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The glycocalyx: a diagnostic and therapeutic target in cardiometabolic diseases

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THE GLYCOCALYX

a diagnostic and therapeutic target in cardiometabolic diseases

Anouk Ilse Maria van der Velden

The glycocalyx; a diagnostic and therapeutic target in cardiometabolic diseases

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The glycocalyx; a diagnostic and therapeutic target in cardiometabolic diseases

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CHAPTER 1

General Introduction

General Introduction

Diabetes mellitus is a chronic metabolic disorder with a complex pathogenesis. Patients with diabetes are characterized by hyperglycemia, obesity, hypertension, hypercholesterolemia and endothelial dysfunction. In the long term, type 2 diabetes mellitus (T2DM) can lead to various microvascular and macrovascular complications that are responsible for the high morbidity and mortality associated with this disease. Intensive management of glucose and lipid concentrations as well as blood pressure control are used to minimize the risk of developing vascular complications. However, current therapies are not sufficient enough to stop vascular disease progression.

Micro- and macrovascular complications in diabetes have been pathophysiologically linked with endothelial dysfunction [1] and one of the first signs of vascular damage results from endothelial dysfunction. The metabolic milieu of T2DM, including hyperglycemia, hypercholesterolemia and low grade inflammation affects endothelial cells and their glycocalyx. This glycocalyx is a negatively charged dynamic mesh of glycoproteins, glycolipids, glycosaminoglycans (GAG) and proteoglycans surrounding the cells and is the first barrier between the vascular wall and flowing blood. The endothelial glycocalyx exerts various important functions to maintain vascular homeostasis; It acts as a mechanosensor for shear stress induced release of nitric oxide [2], plays an important role in regulation of the inflammatory- and thrombotic response of the endothelium and acts as a permeability barrier for circulating blood components. The main glycosaminoglycans (GAGs) represented in the endothelial glycocalyx are heparan sulphate (HS) and hyaluronan, accounting for up to 90% of the total GAGs. Other less abundant GAGs are chondroitin sulphate, keratan sulphate and dermatan sulphate. The glycocalyx is highly interactive with circulating cytokines, proteins, growth hormones and other blood components. Under normal conditions, there is a dynamic balance between biosynthesis and shedding of glycocalyx constituents. However, the endothelial glycocalyx is very susceptible for stressors in pathophysiological conditions such as diabetes [3, 4].

Metabolic alterations in the diabetic environment, with production of toxic metabolites (advanced glycation end products, reactive oxygen species, free fatty acids and inflammatory cytokines), together with hemodynamic changes, provides a milieu for sustained activation of the endothelium. Such activation represents the switch from a quiescent phenotype towards a pro-inflammatory pro-thrombotic phenotype, which together with expression of chemokines and adhesion molecules, results in increased interactions with leukocytes and platelets [5]. Furthermore, upregulation of glycocalyx degrading enzymes such as heparanase-1 (HPSE-1), hyaluronidase-1 (HYAL-1), monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinases (MMP) impairs the endothelial glycocalyx layer. These glycocalyx degrading enzymes play an important role in the pathophysiol-

ogy of diabetic vascular complications, as genetic deletion of these enzymes in diabetic mouse models prevented the development of endothelial dysfunction and albuminuria [6, 7]. Hyaluronidase-1 (HYAL-1) cleaves hyaluronan, a major glycosaminoglycan of the glycocalyx and increased plasma hyaluronan levels and HYAL-1 activity have been found in patients with diabetes [3, 4]. MCP-1 is a potent chemokine that regulates the renal influx of macrophages and activation of tissue resident macrophages. HPSE-1 is a beta-glucuronidase capable of cleaving heparan sulphate in the endothelial glycocalyx. It is secreted as the inactive precursor pro-heparanase and activation requires cleavage of its pro enzyme by proteases such as cathepsin-L [8, 9]. The heparan sulphate fractions cleaved by HPSE-1 can in turn serve as toll-like receptor ligands inducing inflammatory cytokines secretion by macrophages [10]. In patients with diabetes, HPSE-1 and MCP-1 have been found in renal tissue and urine, correlating with the degree of albuminuria and kidney function [11-17]. Prolonged exposure to the diabetic environment results in chronic endothelial activation, endothelial dysfunction and eventually structural vascular damage affecting the brain, retina, peripheral nerves and kidneys.

The renal glomerulus contains a specialized microvascular bed, as it excretes waste products from the plasma toward the urinary tract collection system via the glomerular filtration barrier (GFB). The glomerular filtration barrier is composed of the fenestrated endothelium which are covered with a dense glycocalyx layer [18], the glomerular basement membrane and podocyte foot processes with their slit diaphragms. The glomerular glycocalyx contributes significantly to the perm-selective filtration properties of the GFB [19, 20]. Altering the composition of the glomerular glycocalyx via enzymatic removal of the glycocalyx or indirect alterations results in albuminuria in several experimental models [18]. Albuminuria has therefore been implicated as indicator of glomerular glycocalyx damage in the context of direct glomerular injury or glomerular involvement in generalized vascular dysfunction [21, 22]. In diabetic nephropathy, albuminuria is considered the first hallmark of diabetic kidney disease. However, the glycocalyx is present throughout all capillary beds, preventing protein filtration across the endothelium. Loss of the glycocalyx not only drives the development of albuminuria but also contributes to cardiovascular diseases. In addition, albuminuria may not only be a sign of kidney disease, but is an expression of systemic dysfunction of the vascular endothelium. This is seen in various studies were there is a strong association between albuminuria and vascular disease [22-24]. The mechanism was first proposed as the Steno hypothesis in 1989, which states that excessive urinary albumin loss is the result of widespread peripheral vascular damage or endothelial dysfunction [25]. This resulted in the acceptance of albuminuria and endothelial function as therapeutic target in the prevention of cardiovascular disease [26, 27].

Ethnic disparities in vascular complications in patients with T2DM

The incidence and prevalence of T2DM is higher among ethnic minorities. Compared with Caucasians, individuals from Black African, African Caribbean and South-Asian ethnic origin suffer disproportionately from type 2 diabetes and its long-term vascular complications. Diabetic nephropathy is one of the major complications of T2DM, as it is the main cause of end-stage renal disease (ESRD) worldwide. Among the different ethnic groups and across various regions of the world there is a striking difference in prevalence and progression of diabetic nephropathy [28-30].

Patients with T2DM from South-Asian or African descent seem to be more prone to develop albuminuria [31-34], show a faster decline of kidney function [32, 35] and have a 40 times higher risk at developing ESRD compared to patients with diabetes from European origin [36, 37]. The cause for these ethnic differences in disease phenotype have not been fully elucidated.

Proposed underlying mechanisms for the high rate of vascular complications in South-Asian individuals with diabetes are increased visceral adipose tissue, systemic inflammation and endothelial dysfunction, in addition to the classic diabetic risk factors [38, 39]. Interestingly, signs of endothelial activation have already been found in South-Asian neonates, compared to European neonates [40]. Other studies have shown that even healthy South-Asians are characterized by endothelial dysfunction compared to Europeans [41, 42]. As we know, signs of endothelial dysfunction can be evident before the occurrence of clinically detectable vascular complications [43-45]. Sustained endothelial dysfunction in individuals of South-Asian descent may explain their predisposition to develop vascular complications. As this ethnic group suffers from an excessive rate of vascular complications, there is a need for therapeutic interventions aimed at slowing down vascular disease progression. Therefore, South-Asian patients with T2DM may benefit from therapeutic interventions aimed at improving endothelial function.

In the Netherlands, the biggest South-Asian group are the immigrated South-Asians from Suriname. In this thesis, this group is mostly studied and referred to as South-Asian Surinamese. These South-Asian immigrants originally descent from the Indian Subcontinent (India, Pakistan, Bangladesh). They migrated to Suriname due to the economic situation in North India and worked mostly on the plantation upon arrival to Suriname. Around 1975 and 1980, two political migration waves caused the South-Asian population to migrate to the Netherlands where they mainly settled in The Hague, Rotterdam and Amsterdam.

The glycocalyx as a therapeutic target in diabetes

Increased understanding of the role that the endothelial glycocalyx plays in the development of vascular complications has led to novel interventions and therapeutic treatments

aimed at the improvement of the endothelial glycocalyx. Inhibiting glycocalyx degrading enzymes and supplementing glycocalyx mimetics are strategies used to preserve and restore the endothelial glycocalyx. Heparan sulphate mimetics aimed at inhibiting HPSE-1 activity are mainly developed and investigated in the field of cancer research but may also be of interest in the diabetic field. So far, HPSE-1 inhibitors which also have been studied in experimental diabetes are PI-88 [46], PG545 [47] and SST0001 [48]. PI-88 is a mixture of highly sulfated oligosaccharides derived from yeast. PI-88 has been investigated in autoimmune type 1 diabetes, where it preserved HS content in the pancreatic islet [49]. PG545 is a sulfated tetra-saccharide with the addition of a lipophilic moiety which has been studied in experimental diabetic retinopathy and ischemic reperfusion during acute kidney injury (I/R AKI), were it was able to inhibit the inflammatory response and upregulation of HPSE-1 [50, 51]. SST0001, a non-anticoagulant *N*-acetylated glycol split heparin, showed to reduce renal damage and albuminuria in experimental diabetes [7]. However, none of these HPSE-1 inhibitors have been approved for clinical application.

One of the most extensively studied heparan sulphate mimetic in the diabetes field is sulodexide, a purified mixture of low-molecular-weight heparin and dermatan sulfate. In vitro, sulodexide showed to be a potent inhibitor of HPSE-1 and MMP [52, 53]. Broekhuizen et al. showed that in type 2 diabetes patients both sublingual as retinal glycocalyx dimensions increased after 2 months of sulodexide administration, whereas plasma hyaluronidase decreased [4]. In a larger trail with both type 1 and type 2 diabetic patients, sulodexide improved albuminuria in a dose dependent manner, indicating the effect of this GAG supplementation on the glomerular glycocalyx layer and filtration barrier [54]. However, phase II studies with sulodexide in overt diabetic nephropathy failed to demonstrate beneficial effects and were therefore discontinued [55]. An explanation for the disagreement in clinical trials may be due to different sources of the drug components [56]. It may also be that stabilization and preservation of the glycocalyx is only effective in early diabetes, before the occurrence of irreversible morphological changes in the kidney. Currently, sulfated polysaccharides derived from marine algae are under investigation, as they resemble biological properties of especially heparan sulfate glycosaminoglycans.

Assessment of the endothelial glycocalyx in vivo

Despite the important role of the endothelial glycocalyx in vascular homeostasis, it has been challenging to study the endothelial glycocalyx *in vivo*. The microvasculature covers more than 95% of the total vascular surface area, therefore most glycocalyx volume resides in the microvasculature. The development of non-invasive methods that are able to assess the endothelial glycocalyx *in vivo* has become of major importance and new techniques have been developed over the years.

Sidestream dark field (SDF) imaging is a non-invasive intravital microscopy imaging technique that allows visualization of the sublingual microcirculation in a clinical setting at bedside. The SDF imaging camera can be used in various vascular beds, however, not every microvascular bed can be easily visualized at bedside. The sublingual microcirculation is one of the most easily accessible surface in human and has proved to be a clinically relevant location, as alterations in the microvascular bed have been associated with several clinical parameters and outcomes in different patient groups [57-61]. The SDF camera uses green light-emitting stroboscopic diodes to detect the hemoglobin of passing red blood cells (RBCs). Over the years, several visual scoring systems and automated analysis software have been developed to standardize and improve acquisition and analysis of the microcirculation. The Glycocheck^{TM} software automatically detects, records and analysis the microvessels with a diameter between 4 and 25 µm. Several videos per individual are recorded and subjected to predefined quality criteria. Around 3000 valid vascular segments are collected and analyzed in one measurement. The software automatically generates several microvascular parameters based on these valid vascular segments, such as the vascular density, red blood cell velocity, blood volume and the perfused boundary region (PBR, an inverse estimation of the endothelial glycocalyx).

Damage to the glycocalyx allows RBCs to penetrate deeper towards the endothelial surface, which is expressed by an increased perfused boundary region (PBR). In previous studies, the PBR was found to be increased in dialysis patients [57], sepsis patients [62], patients with SARS-CoV-2 [63] and patients with type 2 diabetes [64]. In addition, the PBR has proven to be a valuable additive predictor for adverse cardiovascular events [65]. However, the variability of the PBR has been an issue of concern. Due to its dynamic nature, substantial differences in measured PBR have been reported, especially in healthy individuals. Average PBR in healthy volunteers ranged from 3.3 to 1.8 µm in two different studies [57, 64]. Only in patients with severe disease states, such as sepsis patients, the PBR revealed to have an acceptable intra- and interobserver variability, with comparable PBR values across different studies [66, 67]. To improve reliability of the PBR, the average of more than one measurement per individual used to be estimated [66, 68]. Recently, it was hypothesized that PBR dimensions are dependent on the velocity of passing RBCs in the vessels. Therefore, to minimize this flow dependent variability in PBR estimation, the slope of measured PBR values (called PBR_{static}) with corresponding red blood cell velocity (V_{RBC}) is used to estimate the PBR in the absence of RBC velocity (V_{RBC} =0 µm/s). This V_{RBC} independent PBR is called the $PBR_{dynamic}$ and is expected to have a much lower variation [59]. In the current thesis, we use SDF-imaging with the new Glycocheck software to assess the microvascular health in patients at risk for developing vascular complications and to monitor the effect of dietary interventions on the microvascular health.

Thesis objectives and outline

The objectives of this thesis are first to investigate if microvascular changes can be detected and used as a diagnostic marker in individuals at risk of developing cardiovascular disease. Secondly, we investigated if urinary HPSE-1 and MCP-1 can serve as biomarkers in individuals with type 2 diabetes. Lastly, we explored two dietary interventions aimed at stabilizing and preserving the endothelial glycocalyx in diabetes. The effect on the glomerular glycocalyx will be investigated in an experimental mouse model next to the effect on the sublingual microvasculature in South-Asian Surinamese patients with type 2 diabetes.

We first investigated in **chapter 2** whether changes in the microcirculation already could be detected in individuals with increased cardiovascular risk with the newly developed Glycocheck^{TM} software. Therefore, we assessed SDF-imaging parameters in individuals of the Netherlands Epidemiology of Obesity (NEO) Study stratified by risk groups according to the Framingham risk score, which is used to assess the risk of developing cardiovascular disease within 10 years.

Because individuals of South-Asian descent have a predisposition to develop diabetes and vascular complications, we wanted to investigate inflammatory markers involved in degradation of the endothelial glycocalyx in a multi-ethnic cohort of patients with type 2 diabetes in **chapter 3.** We determined ethnic differences in MCP-1 and HPSE-1 activity in participants of the HELIUS study. In addition, we investigated associations between these markers and the degree of albuminuria per ethnic group.

Next, we focus on two dietary interventions aimed at stabilizing or preservation of the endothelial glycocalyx and investigate the effect of these interventions on the diabetic kidney.

In **chapter 4,** we conducted a randomized controlled trial to investigate the effect of supplementation of glycocalyx mimetics or a repeated fasting mimicking diet on microvascular health in South-Asian Surinamese patients with type 2 diabetes. In addition, the effect on metabolic markers, inflammatory markers and albuminuria are investigated.

If supplementation with glycocalyx mimetics could preserve the glomerular endothelial glycocalyx in an experimental diabetic nephropathy mouse model was studied in **chapter 5**.

In **chapter 6**, the fasting mimicking diet intervention was studied in an experimental diabetic nephropathy mouse model. The effect on weight loss, inflammatory markers, albuminuria and the glomerular glycocalyx was investigated.

Finally, this thesis is summarized in **chapter 7** where the relevance of the results are discussed and future perspectives are proposed.

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CHAPTER 2

Microvascular differences in individuals with obesity at risk of developing cardiovascular diseases

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Abstract

Objective

To investigate microvascular differences in individuals with obesity at risk for developing cardiovascular diseases.

Methods

In this cross-sectional Netherlands Epidemiology of Obesity study, the sublingual microcirculation was assessed with a new developed GlycoCheck™ software, which integrates red blood cell (RBC) velocity within the smallest capillaries (4-7 μm) and feed vessels (>10 μm). Framingham risk score was used to calculate 10-year cardiovascular risk, divided into low-, intermediate- and high-risk groups. Analysis of Variance was used to evaluate microvascular differences between the groups.

Results

A total of 813 participants were included. The high-risk group (n=168) was characterized by differences in the microvasculature compared to the low-risk group (n=392), with a 49% reduction in the number of smallest capillaries and a 9.1 μm/sec (95% CI:5.2–12.9) higher red blood cell velocity in the feed vessels. No differences in velocity corrected perfused boundary regions were found.

Conclusions

We observed that with adding RBC velocity to the software, SDF imaging is able to detect microcirculatory differences in a cohort of individuals with obesity at risk for developing cardiovascular diseases.

Introduction

Obesity is a well-established risk factor for developing cardiovascular disease (CVD), the leading cause of mortality worldwide. One of the earliest changes in CVD pathogenesis is microvascular endothelial dysfunction [1, 2]. Recently, we showed that early in diabetes the endothelial glycocalyx is perturbed, which resulted in reduced tissue perfusion and decreased perfused capillary density [3, 4]. Detecting early microvascular changes, long before the onset of clinical symptoms of CVD, and monitoring the response of therapeutic interventions may improve cardiovascular outcome. However, techniques to easily assess the dynamic microcirculation in humans are limited [5, 6].

Subsequent to our previous sidestream dark field (SDF) imaging analysis in a sub-population of the Netherlands Epidemiology of Obesity (NEO) study [7], we present a newly developed software application which facilitates automatic analysis of red blood cell velocity, allowing to include flow changes between feed vessels and capillaries to be coupled to the perfused boundary region and perfused capillary density measurements. In the current study we re-analyzed our previous SDF measurements and divided the cohort into cardiovascular risk groups according to their Framingham Risk Score (FRS). The Framingham Risk Score is a sex-specific algorithm that is widely used to assess the risk of cardiovascular events (coronary, cerebrovascular, and peripheral artery disease and heart failure) within 10 years.

As microvascular dysfunction is one of the first signs of CVD, we aimed to investigate whether individuals with obesity and a high risk for developing CVD could be characterized by microvascular changes measured with SDF-imaging.

Methods

Study design and population

The population-based prospective cohort NEO study, designed to investigate pathways leading to obesity-related diseases, started in 2008 and included 6671 individuals aged 45–65 years, with an oversampling of overweight individuals with a body mass index (BMI) of 27 kg/m^2 or higher. Detailed information about the NEO study design and data collection are described elsewhere [7]. The Medical Ethical Committee of the Leiden University Medical Center approved the design of the study. All participants gave their written informed consent.

In the present study, 918 participants in which SDF-imaging was performed between January and October 2012 as part of the baseline visit at the LUMC NEO study center were included.

Framingham Risk Score

The Framingham risk score was used to calculate the risk of general CVD by using the risk factors gender, age, total and high-density lipoprotein cholesterol, systolic blood pressure, treatment for hypertension, current smoking, and diabetes status. The Framingham Risk Score was reported as absolute risk percentage classified as low (<10%), intermediate (10% - 20%), or high (>20%) 10-year predicted risk of CVD [8].

SDF microcirculation imaging

Intravital microscopy was performed earlier using a SDF camera (MicroVision Medical Inc., Wallingford, PA) and acquired using Glycocheck™ software (Microvascular Health Solutions Inc., Salt Lake City, UT, USA) as described elsewhere [5].

The new software includes red blood cell velocity as a new parameter. After re-analyzing the following parameters were obtained: perfused capillary density; capillary blood volume; RBC velocity; static and dynamic perfused boundary region. Detailed information about the new software used in the NEO study is described elsewhere [5, 9]

Statistical analysis

The Framingham risk score was calculated by using the Stata module of A. Linden, installed with the syntax 'ssc install framingham'. The resulting participants were divided into absolute risk percentage groups classified as low (<10%), intermediate (10% to 20%), or high (>20%) risk group. Data is presented as mean (standard deviation, SD), median (25^{th}-75^{th} percentile), or as percentage. Differences in microvascular parameters between the risk groups were analyzed by Analysis of Variance (ANOVA). Capillary blood volume and perfused capillary density were transformed into the natural logarithm. The percentage change of capillary density compared to the low risk group (reference) was calculated within the capillary diameters class. The above-mentioned analyses were performed with STATA Statistical Software (StataCorp, College Station, TX, version 14.1).

Results

Differences in microvascular parameters between Framingham risk groups

For stratifying the participants (n=918) by the Framingham risk score, participants with pre-existing cardiovascular disease (n=60) were excluded for this analysis, as were participants with missing data on diabetes status (n=5), systolic blood pressure (n=2), $PBR_{4\text{-}25\mu m}$ (n=30) and RBC velocity measurements (n=8). This resulted in a total of 813 participants (382 men and 431 women), included in the present analysis. For each participant, the Framingham risk score was calculated, and individuals were divided into risk groups reported as low-, intermediate-, or high- 10-year predicted risk of CVD. Study characteristics and microvascular parameters derived from SDF imaging stratified by Framingham risk groups and in the total cohort are shown in table 1 and figure 1.

For the statistical analysis, capillary blood volume and capillary density were log transformed. After log transformation, capillary blood volume was 0.085 (95% CI 0.003 – 0.166, fig 1A) lower in the high-risk group compared to the low-risk group and the capillary density was 0.063 (95% CI 0.006 – 0.121, fig 1B) lower in the high-risk group compared to the low-risk group. This reduced number of perfused capillary density in the high-risk group was accompanied by increased RBC velocity in the feed vessels and capillaries. RBC velocity in the feed vessels was, compared to the low-risk group, higher in the intermediate group (difference 7.0 µm/sec with 95% CI 3.7 – 10.4, fig 1C) and the high-risk group (difference 9.1 µm/sec with 95% CI 5.2 – 12.9, fig 1C). This higher RBC velocity was also observed within capillaries, with an increase of 6.1 µm/sec (95% CI 2.4 – 9.8) in the intermediate risk group-, and an increase of 8.1 µm/sec (95% CI 3.8 – 6.6) in the high-risk group compared to the low-risk group (fig 1D). The PBR static was lower in the intermediate group compared to low-risk group (difference -0.06 µm with 95% CI -0.10 – -0.01, fig 1E), and high-risk group (difference of -0.06 µm with 95% CI -0.12 – -0.01 compared to the low-risk group, fig 1E). However, velocity corrected PBR (PBR dynamic), based on per group analysis [9], did not differ across the Framingham risk groups (fig 1F).

An in-depth analysis of perfused capillary density loss is shown in figure 2. Capillaries were categorized according to their diameter and percentage change in capillary density in intermediate and high-risk groups compared to low-risk group was calculated. The number of capillaries with a diameter of 4 µm was 49% lower in the high-risk group, and 29% lower in the intermediate-risk group. Similarly, densities of 5 µm capillaries were 23% and 10% lower in the high- and the intermediate-risk group, respectively.

Table 1. Characteristics and SDF derived parameters of the study population stratified by Framingham risk group and total cohort.

	Low risk (N=392)	Intermediate risk (N=253)	High risk (N=168)	Total cohort (N=813)
Demographics				
Age (years)	54 (6)	57 (6)	60 (5)	56 (6)
Women (%)	81	37	12	53
Post-menopausal in women (% yes)	52	77	95	60
Ethnicity (% Caucasian)	94	94	98	95
Tobacco smoking (% current)	5	12	32	12
Prevalent diabetes[a] (%)	1	6	23	7
Treatment for hypertension (% yes)	17	30	50	28
Anthropometrics				
Systolic blood pressure (mmHg)	123 (13)	136 (14)	143 (16)	131 (16)
Diastolic blood pressure (mmHg)	80 (8)	87 (10)	88 (9)	84 (10)
BMI (kg/m^2), M/W	27.0 (3.6)/ 27.8 (5.1)	28.2 (3.5)/ 29.0 (4.1)	29.1 (4.1)/ 31.8 (6.3)	28.3 (3.8)/ 28.3 (5.2)
Waist circumference (cm), M/W	96.9 (11.5)/ 90.5 (13.1)	99.9 (9.2)/ 94.7 (12.2)	103.5 (12.2)/ 100.8 (13.8)	100.7 (11.2)/ 91.9 (13.2)
Waist-to-hip ratio, M/W	0.92 (0.07)/ 0.84 (0.07)	0.95 (0.06)/ 0.88 (0.07)	0.97 (0.07)/ 0.90 (0.07)	0.95 (0.07)/ 0.85 (0.07)
Total body fat (%), M/W	24 (7)/39 (7)	26 (5)/41 (6)	29 (6)/42 (6)	27 (6)/40 (7)
Laboratory markers				
Fasting glucose (mmol/l)	5.2 (4.9-5.6)	5.5 (5.2-5.9)	5.7 (5.3-6.6)	5.4 (5.0-5.9)
Fasting insulin (IU/l)	8.5 (5.8-12.3)	9.9 (6.3-14.4)	12.4 (9.0-18.2)	9.6 (6.2-14.3)
Hba1c (%)	5.31 (0.33)	5.42 (0.50)	5.69 (0.87)	5.42 (0.55)
Total cholesterol (mmol/L)	5.64 (1.00)	5.92 (1.12)	6.00 (1.07)	5.80 (1.06)
Triglycerides (mmol/L)	0.84 (0.63-1.21)	1.20 (0.82-1.65)	1.36 (0.98-1.98)	1.05 (0.73-1.48)
HDL-cholesterol (mmol/L)	1.67 (0.44)	1.42 (0.38)	1.25 (0.31)	1.51 (0.43)
hsCRP (mg/L)	1.32 (0.7-3.10)	1.31 (0.73-2.92)	1.66 (0.88-3.55)	1.37 (0.73-3.06)
eGFR CKD-EPI (ml/min/1.73m^2)	87 (12)	86 (12)	84 (12)	86 (12)
Albumin/creatinine ratio (mg/mmol)	0.43 (0.26-0.69)	0.41 (0.26-0.60)	0.43 (0.30-0.71)	0.42 (0.27-0.68)
Microvascular parameters				
Capillary blood volume (pL/mm^2)	2.74 (1.41-4.87)	2.41 (1.43-4.24)	2.21 (1.36-3.52)	2.52 (1.40-4.31)
Capillary density (µm/mm²)	40 (25-59)	36 (25-57)	33 (24-47)	37 (25-55)
RBC velocity feed vessels (µm/sec)	53 (18)	60 (19)	62 (17)	57 (18)
RBC velocity capillaries (µm/sec)	54 (20)	60 (21)	62 (18)	57 (20)
PBR_{static} (µm)	2.37 (0.24)	2.32 (0.23)	2.31 (0.23)	2.34 (0.24)
$PBR_{dynamic}$ (µm)	2.54 (0.24)	2.57 (0.22)	2.53 (0.22)	2.55 (0.23)

Abbreviations: *BMI* body mass index, *M* men, *W* women, *HDL* High-density-lipoprotein, *HsCRP* high sensitivity c-reactive protein, *PBR* perfused boundary region, *RBC* red blood cell

Data are presented as mean (SD), median (25th -75th percentile) or percentage.

Framingham risk groups: low risk: <10%, intermediate risk: 10-20%, high risk: >20%

[a] Self-reported DM I or II, medication use or fasting plasma glucose >7.0 mmol/L

Missing: 12 Hba1c, 3 eGFR CKD-EPI, 3 Albumin/creatinine ratio

Abbreviations: *PBR* perfused boundary region, *RBC* red blood cell

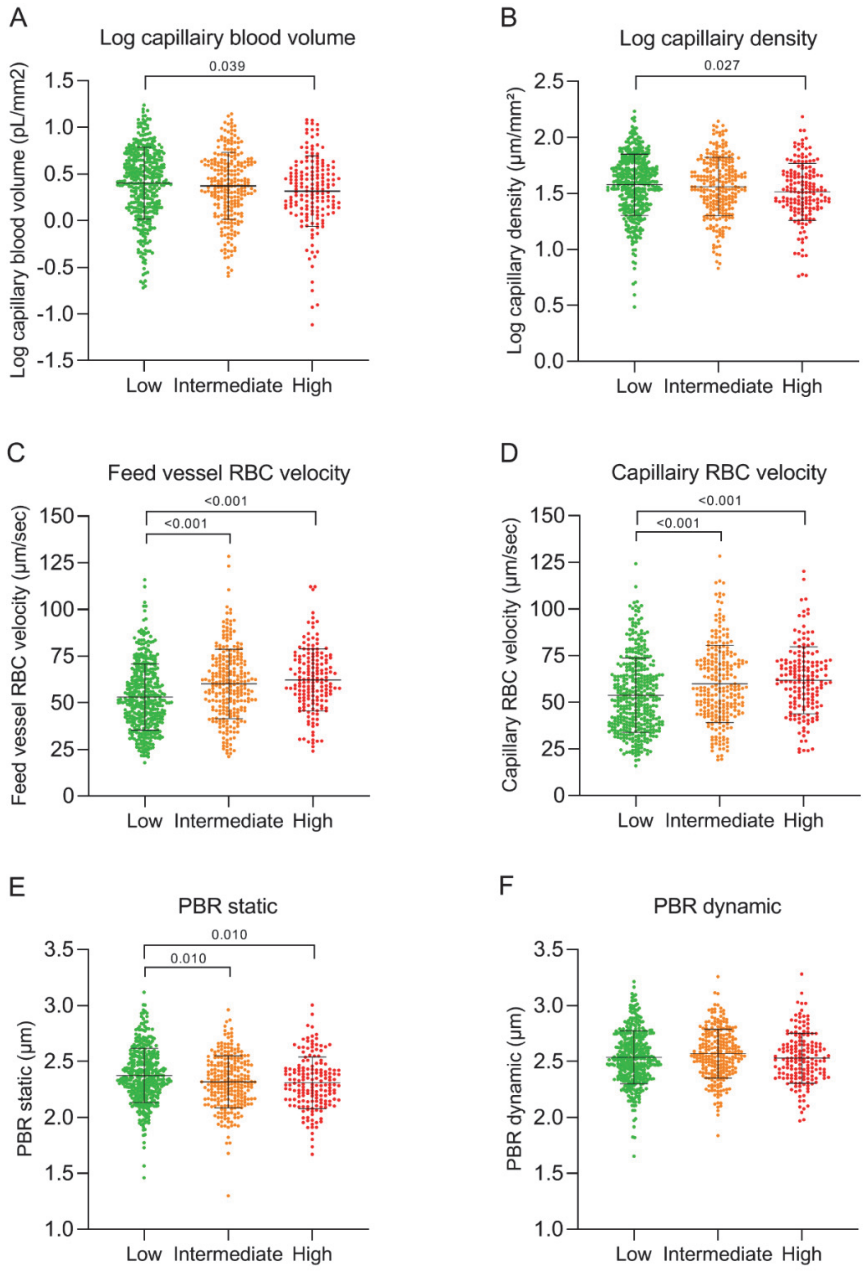


Figure 1. Sidestream dark field imaging derived parameters of the study population stratified by Framingham risk group. Log transformed capillary blood volume (A), capillary density (B), feed vessels red blood cell velocity (C), capillary red blood cell velocity (D), static perfused boundary region (PBR) (E) and dynamic perfused boundary region (F) in the low-, intermediate and high Framingham risk groups. Differences in microvascular parameters between the risk groups were analyzed by analysis of variance.

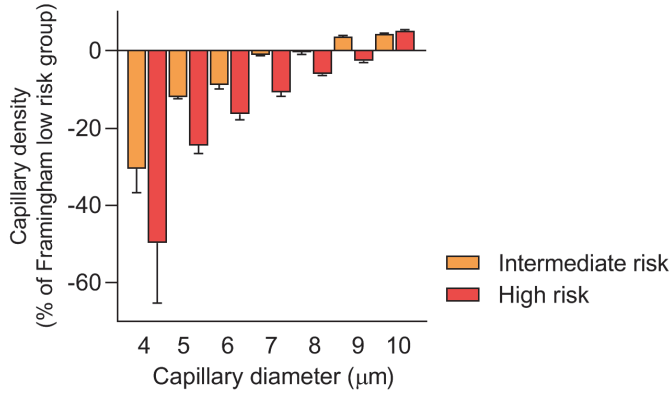


Figure 2. Capillary density of capillaries with different diameter in the intermediate and high-risk group compared to the low-risk group. Percentage difference in the number of perfused capillaries (capillary density) per capillary diameter group in the intermediate- and high Framingham risk groups compared to the low-risk group (reference).

Discussion

In the current study, we observed moderate microvascular differences detected with SDF-imaging in individuals with obesity at risk of developing cardiovascular disease. The number of the smallest functional perfused capillaries (4-6 μm) in individuals with a high risk for developing CVD was reduced coinciding with moderately lower perfused capillary density and capillary blood volume in the intermediate- and high-risk group. RBC velocity at intermediate- and high-risk was higher in both feed vessels (>10 μm) and capillaries, possibly due to higher metabolic demand in tissues [10]. The loss of functional capillaries has been a consistent observation over the years in hypertensive or diabetic patients [11-13]. However, in our current study, we cannot distinguish between capillary rarefaction or reduced NO production due to endothelial dysfunction that could lead to impaired vasodilatation and perfusion.

While the estimated PBR (4-25 μm) seem to differ between the risk groups, the difference was abolished when PBR was corrected for RBC velocity. In previous studies PBR was shown to discriminate between specific patient groups and controls [14, 15]. Interestingly, there is an inconsistency in the range of the measured PBR values in healthy individuals across these studies and our current study. This intra-variability across various studies possibly reflects the inter-individual variability due to the different flow stages within one person at the time of the SDF measurement, especially in healthy persons. By correcting the PBR for these velocity changes the newly PBR (dynamic) will represent a better estimate of changes in the endothelial glycocalyx layer, as also observed between sepsis patients and healthy controls [9].

A limitation of the current study is that only one SDF measurement per individual was performed. To capture different flow states of the feed vessels and capillaries, new recording and analysis strategies have to be developed to calculate microvascular changes on a per-patient basis. Another limitation is the cross-sectional design of the study. In the current study, minor differences between the cardiovascular risk groups could be detected, with a considerable overlap between the groups. It would be interesting to investigate whether high-risk individuals with microvascular changes develop cardiovascular disease in the future. A strength of the current study is the large number of participants in the cohort.

In conclusion, we observed, that by adding red blood cell velocity to the software tool, SDF imaging was able to detect differences within the microvasculature in a cohort of individuals with obesity stratified by cardiovascular risk profile.

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CHAPTER 3

Ethnic differences in urinary monocyte chemoattractant protein-1 and heparanase-1 levels in individuals with type 2 diabetes, the HELIUS study

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Abstract

Introduction: We aimed to investigate ethnic differences in two urinary inflammatory markers in participants with type 2 diabetes mellitus (T2DM).

Research design and methods: We included 55 Dutch, 127 South-Asian Surinamese, 92 African Surinamese, 62 Ghanaian, 74 Turkish and 88 Moroccan origin participants with T2DM from the HEalthy LIfe in an Urban Setting study. Using linear regression analyses we investigated differences in urinary monocyte chemoattractant protein-1 (MCP-1) and heparanase-1 (HPSE-1) levels across ethnic minorities compared to Dutch individuals. Associations between the urinary markers and albuminuria (ACR) was investigated per ethnicity.

Results: Urinary MCP-1 levels were higher in the Moroccan participants (0.15 log ng/mmol, 95% CI 0.05–0.26) compared to Dutch after multiple adjustments. Urinary HPSE-1 levels were lower in the African Surinamese and Ghanaian participants compared to the Dutch, with a difference of -0.16 log mU/mmol (95% CI -0.29– -0.02) in African Surinamese and -0.16 log mU/mmol (95% CI -0.31– -0.00) in Ghanaian after multiple adjustments. In all ethnic groups except the Dutch an Ghanaian participants, MCP-1 was associated with ACR. This association remained strongest after multiple adjustment in South-Asian and African Surinamese participants, with an increase in log ACR of 1.03% (95% CI 0.58–1.47) and 1.23% (95% CI 0.52–1.94) if log MCP-1 increased 1%. Only in the Dutch participants, an association between HPSE-1 and ACR was found, with increase in log ACR of 0.40% (95% CI 0.04–0.76) if log HPSE-1 increased 1%.

Conclusions: We found ethnic differences in urinary MCP-1 and HPSE-1 levels, in a multi-ethnic cohort of T2DM participants. In addition, we found ethnic differences in the association of MCP-1 and HPSE-1 levels with albuminuria. These findings suggest differences in renal inflammation across ethnic groups.

Introduction

Over the years, our knowledge of underlying processes involved in the pathophysiology of diabetic nephropathy (DN) has evolved tremendously. Growing evidence indicates that inflammatory pathways play a central role in the development and progression of the disease [1]. Elucidating important molecular and cellular pathways in the pathophysiology of DN is highly important, as this diabetic complication is one of the leading cause of end stage renal disease (ESRD) worldwide [2].

There are striking differences in the prevalence and progression of DN across various regions of the world and among different ethnic groups [3-5]. Interestingly, these disease disparities still remain even after migration to other continents. In ethnic minority groups of non-European descent, DN is more prevalent and progresses faster to ESRD [6, 7]. Patients with type 2 diabetes mellitus (T2DM) originally from South-Asian or African descent seem to be more prone to develop albuminuria [8-11], show a faster decline of kidney function [9, 12] and have a higher risk at developing ESRD compared to Caucasian patients with diabetes [13, 14]. These ethnic differences in disease phenotype have not been fully elucidated. However, variances in the degree of inflammation in the diabetic kidney may give insight in possible mechanisms to explain ethnic differences in DN phenotype.

The role of chronic low-grade inflammation in the multifactorial pathogenesis of DN has been widely accepted [1]. Metabolic alterations, with the production of toxic metabolites (advanced glycation end products, free fatty acids and inflammatory cytokines), together with hemodynamic changes, provide a chronic low inflammatory environment in patients with T2DM. This inflammatory environment induces the activation of glomerular cells (endothelial cells, podocytes) and macrophages to express adhesion molecules and to produce pro-inflammatory metabolites, such as monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and heparanase-1 (HPSE-1). Chronic exposure to the diabetic stressors and inflammatory metabolites results in structural glomerular and tubular damage leading to albuminuria and eventually kidney function decline [1].

Both MCP-1 and HPSE-1 are known as key players in the development and progression of the disease [15-18]. Heparanase-1 is a beta-glucuronidase that is capable of cleaving heparan sulphate in the glomerular endothelial glycocalyx. The glycocalyx is an important carbohydrate rich layer that covers the luminal and abluminal side of fenestrated endothelium, where it acts as the first barrier in the glomerular filtration barrier. MCP-1 is a potent chemokine that regulates the renal influx of macrophages and activation of tissue resident macrophages. Several studies found that MCP-1 and HPSE-1 are released in renal tissue and urine of patients with DN, correlating with the degree of albuminuria

and kidney function [19-25]. These urinary markers may give insight in the degree of inflammation in the diabetic kidney.

We sought to investigate differences in urinary levels of MCP-1 and HPSE-1 in a multi-ethnic cohort of T2DM patients living in Amsterdam, the Netherlands. In addition, the association between urinary inflammatory markers and the ACR may differ across populations of various ethnicity, for instance due to differences in systemic low-grade inflammation [26]. Therefore, we investigated the association between these urinary markers and the degree of albuminuria per ethnicity.

Research Design and Methods

Participants

For this study, baseline data and morning urine samples from participants from the HELIUS (HEalthy LIfe in an Urban Setting) study were used. The HELIUS study is a large multi-ethnic prospective cohort study conducted in Amsterdam, the Netherlands. Between 2011 and 2015, the study included people of Turkish, Moroccan, South-Asian-Surinamese, African Surinamese, Ghanaian and Dutch origin between 18 and 70 years old. In- and exclusion criteria for the HELIUS study together with detailed study design and methods is described elsewhere [27]. The study protocols were approved by the Academic Medical Center Ethical Review Board (protocol ID NL32251.018.10, approval number 10/100# 10.17.1729), and all participants provided written informed consent.

Participants were asked to bring a morning urine sample the day of the physical examination on the baseline visit. Creatinine and albumin were determined directly at the Clinical Chemistry Laboratory of the AMC. Remainder urine samples were stored at -80°C.

The country of birth of the participants as well as that of his/her parents, both obtained from the municipal registry, was defining for the participant's ethnicity. If the participant was born abroad and has at least one parent born abroad (first generation), or was born in the Netherlands but both parents were born abroad (second generation), the participant was considered of non-Dutch ethnic origin. After data collection, participants from Surinamese ethnic origin were classified into 'African Surinamese' or 'South-Asian Surinamese' according to self-reported ethnic origin. The Dutch participants had to be born in the Netherlands and their parents also had to be born in the Netherlands to be considered as of Dutch ethnic origin.

For the present study, data from a sub-sample of participants with T2DM within the HELIUS study were included. Participants were considered to have diabetes if the measured

fasting blood glucose level was ≥7.0 mmol/L and/or a measured HbA_{1c} level of ≥ 6.5%(48 mmol/mol), and/or antidiabetic medication was used, and/or a self-reported physician diagnosis of diabetes mellitus. Within this selection, the participants with diabetes were divided into normo-albuminuria (albumin/creatinine ratio <3 mg/mmol), micro-albuminuria (albumin/creatinine ratio 3-30 mg/mmol) and macro-albuminuria (albumin/creatinine ratio >30 mg/mmol) groups based on the albumin/creatinine ratio (ACR) in the morning urine sample, with a maximum of 50 participants with normo-albuminuria per group. This maximum was set to prevent a too large sample size of patients with normo-albuminuria. Exclusion criteria were the use of low-molecular weight heparin, as this can interfere with heparanase production [28]. Eventually, 127 South-Asian-Surinamese, 92 African Surinamese, 55 Dutch, 62 Ghanaian, 74 Turkish and 88 Moroccan participants with T2DM were included in this study.

Urinary MCP-1 concentration and HPSE-1 activity

Stored morning urine samples of the participants were obtained to determine MCP-1 and HPSE-1 activity in the LUMC. Urinary MCP-1 concentration was measured with a commercially available immunoassay (R&D Systems Europe, Ltd., Abingdon, UK). Urinary samples were diluted 1:10 with Calibrator Diluent, added to a polystyrene microplate coated with a monoclonal antibody specific for human MCP-1 and incubated for 2 hours at room temperature. The wells were washed with washing buffer consisting of phosphate-buffered saline (PBS) containing 0.1% Tween 20. Then, the human MCP-1 conjugate was added to incubate for 1 hour at room temperature. After washing the plate, a mix of hydrogen peroxide and tetramethylbenzidine was added to start the peroxidase reaction. The reaction was stopped after 20 minutes by adding 2N sulfuric acid and the absorbance was measured at 450 nm with a microplate reader. MCP-1 activity was determined according to a recombinant human MCP-1 standard and corrected for urinary creatinine concentration.

Urinary HPSE-1 activity was measured with a commercially available ELISA assay (Takara Bio Inc., Shiga, Japan). Urine samples were first run through Zeba™ Spin Desalting Columns (ThermoFisher Inc., Waltham, Massachusetts) for removal of salts and other small molecules (<1000 MW). Then, urine samples were diluted 1:1 with reaction buffer and added to 55 µL of dissolved biotinylated HS. After 120 min incubation at 37°C, the reactants were transferred to a well coated with CBD-FGF, an HS-binding protein. During the 15 min incubation at 37°C, non-degraded HS substrates bound to the CBD-FGF in the wells. The plate was washed with washing buffer consisting of phosphate-buffered saline (PBS) containing 0.1% Tween 20. After that, Avidin phenacetin O-de-ethylase (POD) conjugate was added to each well and incubated for 45 min at 37°C. The plate was washed again and the POD substrate was added to start the peroxidase reaction at room temperature. The reaction was stopped by adding 2 M sulfuric acid and the absorbance was measured

at 450 nm with a microplate reader. Heparanase activity was determined according to the provided HS standard and corrected for urinary creatinine concentration.

Additional variables

A history of cardiovascular disease (CVD) was determined with the questions if the person ever had a heart attack, stroke or a catheter intervention on heart or legs. Prevalent CVD was defined as having experienced one of the above conditions. Educational level was based on the highest qualification gained either in the Netherlands or in the country of origin; 1. Never been to school or elementary schooling (non or elementary), 2. lower vocational schooling or lower secondary schooling (lower secondary), 3. intermediate vocational schooling or intermediate/higher secondary education schooling (higher secondary), 4. higher vocational schooling or university (tertiary education). Current smoking was self-reported. Medication use was identified using the ACT classification system. Estimated glomerular filtration rate (eGFR) was calculated using the 2021 CKD-EPI creatinine equation, without race coefficient [29]. Blood pressure was measured twice after five minutes of rest with a validated automated sphygmomanometer, the mean of the two measurements was used. Hypertension was defined as a systolic blood pressure ≥140mmHg or a diastolic blood pressure ≥90mmHg. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Fasting blood samples were drawn at the morning of the study visit.

Statistical analysis

The study population was stratified by ethnicity. Continuous variables with normal distribution are presented as mean with standard deviation (SD) and non-parametric variables by median with interquartile range (IQR). Categorical data are expressed as proportions. Descriptive statistical analyses were carried out on the characteristics of the participants. Normal distributions of the variables were tested with histograms. For comparison of means across the ethnicities, the parametric Welch's ANOVA for unequal variances with Games Howell post-hoc test was used. The Welch's tests were used as the sample sizes of the groups were unequal. Differences were considered statistically significant if $P<0.05$. For comparison of skewed distributed variables across the ethnicities, the non-parametric Kruskal Wallis test was used followed by the Mann-Whitney U test, the significance level was corrected according to the Bonferroni correction (α/number of test). Categorical data was compared using the chi-squared test with post-hoc test with Bonferroni correction.

To investigate the association of ethnicity with urinary MCP-1 levels and HPSE-1 activity, linear regression analyses was used. Because of skewed distributions, urinary MCP-1 and HPSE-1 activity levels were log-transformed before the analysis to achieve normal distribution (of residuals). Due to the occurrence of HPSE-1 levels of 0 mU/mmol, a constant of 0.001 was added prior to transformation, therefore, no back-transformation of HPSE-1

could be performed. Ethnicity was used as independent variable with the Dutch ethnicity as reference group. Multivariable models were constructed. Model 1 was adjusted for age and sex. Model 2 was used to adjust for variables that affect inflammation which additionally included duration of diabetes in years, BMI, hypertension, current smoking, Hba1c and total cholesterol levels. In model 3, the eGFR (2021 CKD-EPI) and ACR were included as continuous variables to investigate if the differences were mediated by these variables. Model 4 additionally included the use of RAAS inhibitors, as this can influence MCP-1 and HPSE-1 levels in the urine [30-32].

Subsequently, we examined the association of the urinary markers MCP-1 levels and HPSE-1 activity with ACR, stratified by ethnicity. To achieve normal distribution (of residuals), ACR was log-transformed before the analysis. We adjusted for known determinants for the ACR such as age, sex, BMI, hypertension, smoking, Hba1c, cholesterol, kidney function and the use of RAAS inhibitors. We also tested formally for interaction between MCP-1 or HPSE-1 and ethnicity by adding a multiplicative interaction term to the fully adjusted model.

Associations were considered statistically significant if $P<0.05$. Statistical analysis were performed using SPSS statistical software version 25 (SPSS Inc., Chicago, IL) and GraphPad Prism version 8 (Graphpad Inc., La Jolla, CA).

Results

Characteristics of the study population

Table 1 shows the baseline characteristics of the study population stratified by ethnicity. In total, 55 Dutch, 127 South-Asian Surinamese, 92 African Surinamese, 62 Ghanaian, 74 Turkish and 88 Moroccan participants with T2DM were included in this study. In concordance with earlier HELIUS publications, baseline differences between the ethnic groups could be detected. The Dutch participants were significantly older compared to the other ethnic minorities, but the age of diabetes onset did not differ across the ethnic groups. Ghanaian and Moroccan participants were less likely to be smokers as compared to the other ethnic groups. The South-Asian Surinamese group had more participants with prevalent CVD compared to the Moroccan participants (29.1% compared to 10.2%). In addition, Moroccan participants were less likely to have hypertension, 35% in the Moroccan group compared to >50% of the participants in the other ethnic groups. Average BMI was significantly higher in the Ghanaian participants compared to the Dutch and South-Asian Surinamese groups, with respectively an average of 33.2 kg/m^2 in the Ghanaian group compared to 29.7 kg/m^2 and 30.1 kg/m^2. Hba1c levels appeared to be lowest in the Dutch group, with an average of 6.9% (51.9 mmol/mol). Urinary creatinine levels did not

Table 1. Characteristics of the participants with type 2 diabetes mellitus stratified by ethnicity.

	Dutch (N=55)	South-Asian Surinamese (N=127)	African Surinamese (N=92)	Ghanaian (N=62)	Turkish (N=74)	Moroccan (n=88)	p-value*
Age, mean (SD)	62 (6)	58 (7) [a]	59 (7) [a]	53 (8) [abc]	54 (8) [abc]	55 (9) [ac]	<0.001
Female, n (%)	26 (47.3)	56 (44.1)	48 (52.2)	34 (54.8)	39 (52.7)	50 (56.8)	0.491
Education level							
- Non or elementary, n (%)	9 (16)	32 (25) [ef]	9 (10)	21 (34) [cef]	50 (68) [abcd]	62 (71) [abcd]	<0.001
- Lower secondary, n (%)	22 (40)	62 (50) [ef]	39 (42)	22 (36)	16 (22) [b]	9 (10) [ab]	<0.001
- Higher secondary, n (%)	11 (20)	17 (13)	26 (28) [e]	16 (26)	6 (8) [c]	12 (14)	<0.001
- Tertiary education, n (%)	12 (22)	16 (13)	16 (17) [e]	3 (5)	1 (1) [ac]	4 (5) [a]	<0.001
Current smoking, n (%)	13 (24)	42 (33) [df]	29 (32) [df]	3 (5) [abce]	18 (23) [d]	8 (9) [bc]	<0.001
Age of diabetes onset, mean (SD)	46 (17)	45 (11)	41 (16)	45 (11)	42 (11)	41 (15)	0.206
Duration of diabetes (years), mean (SD)	17 (18)	13 (9) [d]	17 (17) [d]	8 (7) [abc]	12 (11)	13 (13)	<0.001
Prevalent CVD, n (%)	15 (27)	37 (29) [f]	14 (15)	7 (11)	17 (23)	9 (10) [b]	0.003
Use of RAAS inhibitors, n (%)	35 (64)	83 (65) [d]	43 (47)	26 (42) [b]	33 (45)	40 (46)	0.002
Use of antilipidemic drugs, n (%)	43 (78)	93 (73) [cd]	44 (48) [ab]	29 (47) [ab]	51 (69)	57 (65)	<0.001
Hypertension, n (%) #	28 (51)	70 (55)	53 (58) [f]	39 (63) [f]	40 (54)	31 (35) [cd]	0.012
BMI (kg/m^2), mean (SD)	29.7 (5.3)	30.1 (5.36) [e]	30.7 (5.6)	30.7 (7.0)	33.2 (7.1) [ab]	30.9 (5.4)	0.024
Hba1c (mmol/mol), mean (SD)	51.9 (9.5)	63.1 (15.6) [a]	59.9 (5.6) [a]	58.6 (19.4)	62.0 (15.2) [a]	57.7 (15.2)	<0.001
Hba1c (%), mean (SD)	6.9 (0.9)	7.9 (1.4) [a]	7.6 (1.9) [a]	7.5 (1.8)	7.8 (1.4) [a]	7.4 (1.4)	<0.001
Total cholesterol (mmol/L), mean (SD)	4.50 (1.06)	4.29 (1.08)	4.52 (1.06)	4.44 (1.04)	4.30 (1.04)	4.10 (1.01)	0.082
CKD-EPI eGFR 2021 (mL/min/1.73 m^2), mean (SD)	91 (16)	86 (24) [ef]	85 (22) [ef]	90 (15) [ef]	104 (15) [abcd]	104 (16) [abcd]	<0.001
Urinary creatinine (mmol/L), median (IQR)	7.5 (5.4-10.2)	7.5 (4.8-12.8)	7.6 (5.0-12.0)	9.2 (6.0-12.6)	7.1 (4.8-11.1) [f]	9.4 (6.9-13.5) [e]	0.019
Albumin creatinine ratio (mg/mmol), median (IQR)	0.38 (0.23-1.25)	4.33 (0.44-21.25) [adef]	0.99 (0.35-7.68) [ad]	0.35 (0.17-1.44) [bcf]	0.59 (0.23-5.85) [b]	2.03 (0.46-5.00) [abd]	<0.001

Table 1. Characteristics of the participants with type 2 diabetes mellitus stratified by ethnicity. *(continued)*

	Dutch (N=55)	South-Asian Surinamese (N=127)	African Surinamese (N=92)	Ghanaian (N=62)	Turkish (N=74)	Moroccan (n=88)	p-value*
Normo-albuminuria, n (%)	50 (90.9)	50 (39.4)	50 (54.3)	50 (80.6)	50 (67.6)	50 (56.8)	<0.001
Micro-albuminuria, n (%)	5 (9.1)	50 (39.4) [a]	31 (33.7) [a]	12 (19.4) [b f]	24 (32.4) [a b]	38 (43.2) [a d]	<0.001
Macro-albuminuria, n (%)	0	27 (21.3)	11 (12.0)	0	0	0	<0.001

Abbreviations: *SD* standard deviation *CVD* cardiovascular disease *RAAS* renin-angiotensin-aldosterone system *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *IQR* interquartile range

defined as systolic BP ≥140 mmHg *or* diastolic BP ≥90 mmHg.

* Welch ANOVA, Kruskall Wallis or chi squared test, P<0.05 is considered statistically significant. Post-hoc test: Games Howell test, Mann-Withney U test with Bonferroni correction or chi squared with Bonferroni correction

[a] Significant difference compared to Dutch

[b] Significant difference compared to South-Asian Surinamese

[c] Significant difference compared to African

[d] Significant difference compared to Ghanaian

[e] Significant difference compared to Turkish

[f] Significant difference compared to Moroccan

significantly differ across the ethnic groups except for a higher median urinary creatinine level in the Moroccan group (9.4 mmol/L IQR 6.9–13.5) compared to the Ghanaian group (7.1 mmol/L IQR 4.8–11.1). The number of participants with normo-, micro- and macro-albuminuria was not equally distributed among the ethnicities. This is in concordance with literature as South-Asian Surinamese and African Surinamese individuals with T2DM had a higher prevalence of albuminuria [10, 11]. As a consequence, median albuminuria levels were highest in the South-Asian Surinamese group compared to the other ethnic groups. In addition, the South-Asian- and African Surinamese had a lower eGFR compared to the other ethnic groups, suggesting a more advanced stage of DN in these participants.

Ethnic differences in urinary MCP-1 levels and HPSE-1 activity

Urinary MCP-1 concentrations and HPSE activity levels were corrected for urinary creatinine levels and log transformed due to their skewed distributions. Urinary MCP-1 could be detected in every participant. Comparing urinary MCP-1 levels in the ethnic groups with the Dutch participants revealed that in South-Asian Surinamese and Moroccan participants, MCP-1 levels were significantly higher with a difference of 0.11 log ng/mmol in the South-Asian Surinamese (95% CI 0.02–0.20) and 0.12 log ng/mmol (95% CI 0.03–0.22) in the Moroccan group, respectively, compared to the Dutch group (Figure 1A and table 2). Without an effect of adjusting for age and sex (model 1, table 2) only after adjusting for age, sex, duration of diabetes, BMI, Hba1c, total cholesterol, smoking and hypertension

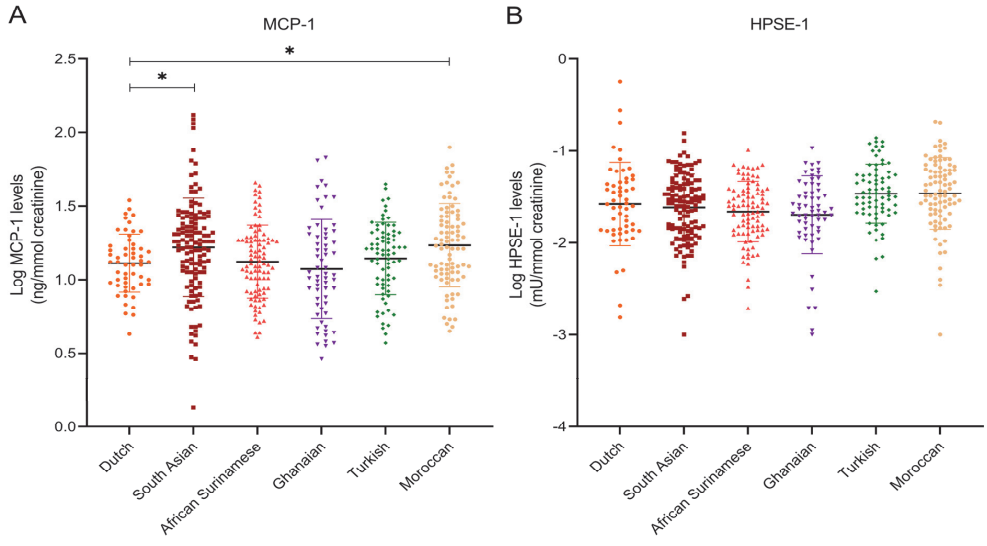


Figure 1. Ethnic differences in urinary MCP-1 and HPSE-1 levels.

A. Log urinary MCP-1 (monocyte chemoattractant protein-1) levels per ethnic group. B. Log urinary HPSE-1 (heparanase-1) levels per ethnic group. Linear regression with Dutch as the reference group, * P<0.05.

Table 2. Ethnic differences in urinary MCP-1 and HPSE-1 levels.

	Unadjusted Difference (95% CI)	Model 1 Difference (95% CI)	Model 2 Difference (95% CI)	Model 3 Difference (95% CI)	Model 4 Difference (95% CI)
Log MCP-1 (ng/mmol)					
Dutch (reference)					
South-Asian Surinamese	**0.11 (0.02–0.20)**	**0.12 (0.02–0.21)**	**0.11 (0.01–0.21)**	0.04 (-0.06–0.14)	0.04 (-0.06–0.14)
African Surinamese	0.01 (-0.09–0.11)	0.02 (-0.08–0.12)	0.01 (-0.10–0.11)	-0.01 (-0.12–0.08)	-0.01 (-0.11–0.09)
Ghanaian	-0.04 (-0.14–0.06)	-0.02 (-0.13–0.09)	-0.01 (-0.13–0.11)	-0.02 (-0.13–0.09)	-0.01 (-0.13–0.10)
Turkish	0.03 (-0.07–0.13)	0.05 (-0.06–0.15)	-0.004 (-0.12–0.11)	-0.01 (-0.13–0.10)	-0.01 (-0.12–0.10)
Moroccan	**0.12 (0.03–0.22)**	**0.13 (0.04–0.24)**	**0.16 (0.05–0.27)**	**0.15 (0.05–0.26)**	**0.15 (0.05–0.26)**
Log HPSE-1 (mU/mmol)					
Dutch (reference)					
South-Asian Surinamese	-0.04 (-0.16–0.08)	-0.03 (-0.15–0.09)	-0.10 (-0.23–0.03)	-0.11 (-0.24–0.02)	-0.11 (-0.24–0.02)
African Surinamese	-0.08 (-0.21–0.04)	-0.08 (-0.21–0.05)	**-0.18 (-0.31– -0.04)**	**-0.16 (-0.29– -0.02)**	**-0.16 (-0.29– -0.02)**
Ghanaian	-0.12 (-0.25–0.02)	-0.11 (-0.25–0.03)	**-0.19 (-0.34– -0.04)**	**-0.16 (-0.31– -0.00)**	**-0.16 (-0.31– -0.00)**
Turkish	0.11 (-0.02–0.24)	0.12 (-0.02–0.26)	0.06 (-0.09–0.20)	0.05 (-0.09–0.19)	0.05 (-0.09–0.20)
Moroccan	0.11 (-0.01–0.24)	0.12 (-0.01–0.25)	0.13 (-0.00–0.27)	0.12 (-0.02–0.25)	0.12 (-0.02–0.26)

Abbreviations: *MCP-1* monocyte chemoattractant protein-1 *HPSE-1* heparanase-1 *CI* confidence interval *BMI* body mass index, *ACR* albumin-creatinine ratio, *RAAS* renin-angiotensin-aldosterone system

Linear regression analyses with Dutch as reference group, P<0.05 is considered statistically significant

model 1: age and sex

model 2: +duration of diabetes, BMI, current smoking, hypertension, Hba1c and total cholesterol

model 3: +eGFR and ACR

Model 4: +use of RAAS inhibitors

(model 2, table 2), differences in MCP-1 levels compared to Dutch participants slightly increased in the Moroccan group (difference of 0.16 log ng/mmol 95% CI 0.05–0.27) with minimal effects in the South-Asian Surinamese group (difference of 0.11 log ng/mmol 95% CI 0.01–0.21). However, after additional adjusting for kidney function (2021 CKD-EPI) and ACR (model 3, table 2), only Moroccan participants with diabetes still revealed a significant higher MCP-1 level compared to Dutch participants, with a difference of 0.15 log ng/mmol (95% CI 0.05–0.26). Instead, adjusting for eGFR and ACR normalized urinary MCP-1 levels in the South-Asian Surinamese group (difference of 0.04 log ng/mmol 95% CI

-0.06–0.14). The differences compared to the Dutch participants remained similar when additionally adjusting for the use of RAAS inhibitors in model 4 (table 2).

In all participants, low urinary HPSE-1 activity was observed, of which in several patients, no HPSE-1 activity could be detected at all. Heparanase-1 levels revealed to be non-significant elevated in the Turkish- and Moroccan participants compared to the Dutch, and significantly lower in the, African Surinamese and Ghanaian participants. After adjusting for age, sex, duration of diabetes, BMI, smoking, Hba1c, total cholesterol, hypertension, eGFR, ACR and the use of RAAS inhibitors (model 1-4, table 2), HPSE-1 levels were 0.16 mU/mmol (95% CI 0.02–0.29) lower in the African Surinamese participants and 0.16 mU/mmol (95% CI 0.00–0.31) lower in the Ghanian participants compared to Dutch participants.

Association of urinary MCP-1 levels and HPSE-1 activity with albuminuria

Table 3 shows the association of urinary MCP-1 concentration and HPSE-1 activity with the degree of albuminuria stratified by ethnicity. Linear regression analyses revealed that in the South-Asian- and African Surinamese participants the association between urinary MCP-1 levels and ACR was stronger compared to the other ethnic groups and Dutch participants. For every 1% increase in log MCP-1, log ACR increased with about 1.40% in the South-Asian Surinamese participants (95% CI 0.94–1.86) in the unadjusted model. In Moroccan participants, every 1% increase in log MCP-1 is accompanied by a 0.70% increase in log ACR (95% CI 0.26–1.15). In contrast, no association between log MCP-1 and log ACR was seen in the Dutch and Ghanian participants. After adjusting for known risk factors for albuminuria, eGFR and the use of RAAS inhibitors (model 4), weaker but still significant associations between log MCP-1 and log ACR were seen in all ethnic groups except for the Dutch and Ghanaian.

Notably, only in the Dutch participants, an association with log HPSE-1 activity and log ACR levels was found, with around 0.42% increase in log ACR for every 1% increase in log HPSE-1 (95% CI 0.10–0.74) in the unadjusted model. This association became less strong (but still significant) after adjustment for age, sex, known risk factors for albuminuria, eGFR and the use of RAAS inhibitor (model 4). In the other ethnic groups, no significant association between log HPSE-1 activity and log ACR levels were observed.

However, the formal test for interaction provided no evidence for a differential association for both urinary markers across ethnic groups.

Table 3. Associations between urinary MCP-1 or HPSE-1 levels and albumin-creatinine ratio stratified by ethnicity.

	Unadjusted Difference (95% CI)	Model 1 Difference (95% CI)	Model 2 Difference (95% CI)	Model 3 Difference (95% CI)	Model 4 Difference (95% CI)
Log MCP-1 (ng/mmol)					
Dutch	0.73 (-0.02–1.47)	0.73 (-0.02–1.48)	0.70 (-0.12–1.53)	0.77 (-0.04–1.58)	0.77 (-0.05–1.59)
South-Asian Surinamese	**1.40 (0.94–1.86)**	**1.42 (0.94–1.86)**	**1.35 (0.86–1.83)**	**1.02 (0.54–1.50)**	**1.03 (0.58–1.47)**
African Surinamese	**1.11 (0.45–1.78)**	**1.14 (0.95–1.88)**	**1.19 (0.34–2.03)**	**1.25 (0.55–1.95)**	**1.23 (0.52–1.94)**
Ghanaian	**0.67 (0.20–1.14)**	**0.67 (0.22–1.11)**	0.22 (-0.24–0.69)	0.24 (-0.23–0.70)	0.22 (-0.24–0.68)
Turkish	**0.91 (0.23–1.59)**	**0.88 (0.19–1.56)**	**0.81 (0.14–1.49)**	**0.87 (0.21–1.53)**	**0.87 (0.27–1.47)**
Moroccan	**0.70 (0.26–1.15)**	**0.64 (0.18–1.10)**	**0.64 (0.17–1.11)**	**0.63 (0.18–1.08)**	**0.64 (0.22–1.07)**
Log HPSE-1 (mU/mmol)					
Dutch	**0.42 (0.10–0.74)**	**0.43 (0.10–0.74)**	**0.42 (0.03–0.77)**	**0.40 (0.05–0.76)**	**0.40 (0.04–0.76)**
South-Asian Surinamese	0.20 (-0.28–0.69)	0.18 (-0.30–0.67)	0.31 (-0.20–0.83)	0.30 (-0.16–0.75)	0.30 (-0.13–0.73)
African Surinamese	-0.04 (-0.57–0.50)	-0.03 (-0.58–0.51)	0.08 (-0.63–0.78)	0.54 (-0.07–1.16)	0.54 (-0.08–1.15)
Ghanaian	0.30 (-0.09–0.69)	0.22 (-0.16–0.60)	0.08 (-0.27–0.43)	0.08 (-0.27–0.43)	0.10 (-0.25–0.44)
Turkish	0.13 (-0.42–0.68)	0.11 (-0.44–0.66)	-0.06 (-0.68–0.56)	0.10 (-0.56–0.75)	-0.25 (-0.88–0.38)
Moroccan	0.15 (-0.19–0.50)	0.12 (-0.22–0.47)	0.18(-0.21–0.57)	0.21 (-0.16–0.58)	0.23 (-0.13–0.58)

Abbreviations: *MCP-1* monocyte chemoattractant protein-1 *HPSE-1* heparanase *CI* confidence interval *BMI* body mass index *ACR* albumin-creatinine ratio *RAAS* renin-angiotensin-aldosterone system

Linear regression analyses, P<0.05 is considered statistically significant

Model 1: age and sex

Model 2: + duration of diabetes, BMI, current smoking, hypertension, Hba1c and total cholesterol

Model 3: +eGFR

Model 4: +use of RAAS inhibitors

Discussion

In this multi-ethnic cohort study of participants with type 2 diabetes mellitus, we found ethnic differences in urinary MCP-1 and HPSE-1 levels. The Moroccan participants had significantly higher urinary MCP-1 levels compared to the Dutch participants. This difference could not be explained by differences in age, sex, factors that may influence inflammation (i.e., duration of diabetes, BMI, smoking, hypertension, Hba1c and total cholesterol levels) or kidney function, degree of albuminuria or RAAS inhibition. In South-Asian Surinamese participants, significant higher urinary MCP-1 levels compared to the Dutch group were seen after correcting for confounders that influence inflammation (i.e., age, sex, BMI, smoking, hypertension, Hba1c and total cholesterol levels). However, adjusting for eGFR and ACR attenuated this difference, suggesting that MCP-1 levels are partly elevated due to a higher degree of albuminuria and decreased renal function between these groups. In contrast, lower urinary HPSE-1 activity were found in the African Surinamese and Ghanian participants compared to the Dutch participants. These differences could not be explained by differences in age, sex, factors that may influence inflammation (i.e., duration of diabetes, BMI, smoking, hypertension, Hba1c and total cholesterol levels), kidney function, degree of albuminuria or the use of RAAS inhibition.

Several studies report that higher levels of certain systemic and urinary inflammatory biomarkers such as MCP-1, TNF-a, VCAM-1 or urinary ICAM-1 are associated with kidney function or albuminuria in patients with diabetes and a specific ethnic background, such as African-Americans [33, 34] or Asians [35]. We found that in all ethnic minority groups studied, a higher urinary MCP-1 level was associated with a higher level of albuminuria. These associations were still apparent after adjustment for factors that may influence albuminuria (i.e. age, sex, BMI, smoking, hypertension, Hba1c, total cholesterol levels, kidney function and the use of RAAS inhibitors). To this, we found strongest associations in African- and South-Asian Surinamese, and Turkish participants which could suggest that renal inflammation may contribute to the development of albuminuria in these ethnic groups. Similar to our results, several studies showed positive correlations with urinary MCP-1 activity and degree of albuminuria in patients with diabetes [20, 22, 23]. Using a larger participant cohort in a recent post-hoc analysis of the ROADMAP study revealed that even despite very high variations in outcome, serum and urinary MCP-1 levels were significant predictors of the onset of albuminuria in patients with diabetes [36].

Only in the Dutch participants, a positive association was found between urinary HPSE-1 activity and albuminuria levels. Several studies found increased HPSE-1 activity levels in patients with diabetes [24, 25] but only one study found a correlation between urinary HPSE-1 activity and the degree of albuminuria [19]. Urinary HPSE-1 activity appeared to be low in all participants. It is known that active heparanase has an important role in the

cleavage of heparan sulfate in the glomerulus, contributing to the development of albuminuria [19, 37]. However, it is not known how much of this activity is still detectable in the urine. It is possible that heparanase is bound to heparan sulphates shed by other renal or bladder cells, as every cell in the urinary tract expresses heparan sulphates. This may give an underestimation of the amount of active heparanase in urine with the currently available ELISA kits that uses an indirect method to detect HPSE-1 activity. Therefore, a new test to detect active HPSE-1 and inactive proHPSE-1 directly in urine is needed in future research [38, 39].

We corrected urinary MCP-1 and HPSE-1 levels for urinary creatinine levels to correct for variations in urine concentration and flow rate. However, it is known that urinary creatinine levels may vary across ethnic groups due to differences in muscle mass. Therefore, major differences in urinary creatinine levels may influence the levels MCP-1 and HPSE-1. We, however, found no major differences in urinary creatinine levels across the ethnic groups.

The attenuation of the difference in urinary MCP-1 levels in South-Asian Surinamese compared to the Dutch after adjusting for eGFR and ACR could be related to the possible negative correlation between eGFR and urinary MCP-1 levels in South-Asian individuals with type 2 diabetes mellitus. This negative correlation with kidney function has already been found for serum MCP-1 levels [34, 40], attenuating the positive association between ethnicity and MCP-1.

The strength of our study is the representation of six large ethnic groups based in Amsterdam with participants between 18 and 70 years of age making it possible to early on detect possible disease related risk factors. Our study also has several limitations. Due to the cross sectional nature of the study, no conclusions on causal relations between urinary MCP-1 or HPSE-1 levels and progression of albuminuria can be drawn. As no renal biopsy material was collected in the HELIUS study, no correlations with local glomerular expression of the inflammatory markers could be investigated. Due to the low number of patients with micro- and macro-albuminuria in the Dutch and Ghanaian groups, those groups may be underpowered to show an effect or association. In addition, high variability of the urinary MCP-1 (as also shown earlier [36]) within the ethnic groups prevents for further stratification in quartiles to assess a more robust association with the occurrence of micro- and macroalbuminuria. The ACR was used based on a single urine sample. A 24-hour urine collection may give a more accurate estimation of the ACR then a single urine sample. However, several studies showed that in patients with diabetes, a morning urine sample correlates well with the 24-hour urine collection [41, 42].

It can be discussed that the urinary levels of MCP-1 and HPSE-1 activity may not reflect local inflammation in the kidney. Although the nature of the HELIUS study ruled out collection of renal biopsy material, it has been confirmed previously that urinary MCP-1 levels were elevated in accordance with the progression of diabetic glomerular lesions [43, 44]. For HPSE-1 activity, increased local glomerular and tubular HPSE-1 expression has been found in T2DM patients, however, without a direct correlation between histological findings and urinary HPSE-1 activity levels [19, 37]. It therefore needs to be established whether urinary HPSE-1 activity levels will reflect renal perturbation in early diabetes related disease progression. An interesting approach to study the ethnic differences in developing DN would be in dissecting possible genetic predispositions and epigenetic modifications, albeit a recent study within the HELIUS cohort revealed the specific admixed genetic variability within the three mayor Surinamese ethnic groups (besides the Africans and South Asians also the Java Surinamese) increasing the complexity of determining such modifications at a global ethnic level [45].

To our knowledge, no prior studies have studied the difference in these urinary markers between ethnic groups to give more insight in differences in the degree of inflammation in the diabetic kidney. It is known that the pathogenesis of DN is a multifactorial pathway where inflammation appears to be a relevant contributor to its development. Ethnic differences in urinary inflammatory markers can be of interest and may explain the heterogeneity in DN across ethnic groups. In addition, it could indicate that some ethnic groups may respond well to targeted anti-inflammatory therapy whereas other ethnic groups may experience less therapeutic response. MCP-1 and HPSE-1 are known as in important promotors of inflammation, however, the meaning of differences in urinary MCP-1 and HPSE-1 levels found in our study has yet to be determined. It would be interesting to investigate other inflammatory markers across ethnic groups and relate these findings to DN markers.

In the present study, we found ethnic differences in urinary MCP-1 and HPSE-1 levels, in a multi-ethnic cohort of T2DM participants. In addition, we found ethnic differences in the association of urinary MCP-1 and HPSE-1 levels with the degree of albuminuria. This suggests that the contribution of renal inflammation in the pathogenesis of diabetic nephropathy may vary between ethnic groups.

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CHAPTER 4

Role of dietary interventions on microvascular health in South-Asian Surinamese people with type 2 diabetes in the Netherlands; a randomized controlled trial

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Abstract

Background: We investigated whether dietary interventions, i.e. a fasting mimicking diet (FMD, Prolon®) or glycocalyx mimetic supplementation (Endocalyx™) could stabilize microvascular function in Surinamese South-Asian patients with type 2 diabetes (SA-T2DM) in the Netherlands, a patient population more prone to develop vascular complications.

Methods: A randomized, placebo controlled, 3-arm intervention study was conducted in 56 SA-T2DM patients between 18 and 75 years old, for 3 consecutive months, with one additional follow up measurement 3 months after the last intervention. Sublingual microcirculation was assessed with SDF-imaging coupled to the GlycoCheck™ software, detecting red blood cell velocity, capillary density, static and dynamic perfused boundary region (PBR), and the overall microvascular health score (MVHS). Linear mixed models and interaction analysis were used to investigate the effects the interventions had on microvascular function.

Results: Despite a temporal improvement in BMI and HbA1c after FMD the major treatment effect on microvascular health was worsening for RBC-velocity independent $PBR_{dynamic}$, especially at follow-up. Glycocalyx supplementation, however, reduced urinary MCP-1 presence and improved both $PBR_{dynamic}$ and $MVHS_{dynamic}$, which persisted at follow-up.

Conclusions: We showed that despite temporal beneficial changes in BMI and HbA1c after FMD, this intervention is not able to preserve microvascular endothelial health in Dutch South-Asian patients with T2DM. In contrast, glycocalyx mimetics preserves the microvascular endothelial health and reduces the inflammatory cytokine MCP-1.

Introduction

Diabetic vascular complications account for an enormous health burden worldwide with various ethnic groups that are more prone to develop such vascular complications [1, 2]. In the Netherlands, people from South-Asian Surinamese descent are characterized by such high vascular vulnerability, resulting in increased prevalence of micro- and macrovascular complications in diabetes [3-5]. At time of diagnosis these higher rates of complications are already found [3, 6, 7] and progression is also much faster compared to other ethnic groups, translating into a 40 times higher risk for end stage renal disease [8] and a 50% higher age-adjusted mortality rate from coronary heart disease [9].

One of the first hallmarks of vascular damage is endothelial dysfunction, which can progress to structural microvascular changes, and eventually result in irreversible vascular damage [10-14]. Upregulation of glycocalyx degrading enzymes and inflammatory cytokines such as heparanase-1 (HPSE-1) and monocyte chemoatracctant-1 (MCP-1) augments endothelial dysfunction by impairing the endothelial glycocalyx (EG), a mesh of glycosaminoglycans (GAGs), proteoglycans, glycoproteins and glycolipids on the apical side of endothelial cells [15]. Next to conventional therapeutic options, preserving endothelial function in South Asian patients with T2DM (SA-T2DM) could prevent or reduce the fast progression of vascular complications through inhibiting glycocalyx degrading enzymes or reducing metabolic risk factors that contribute to glycocalyx degradation.

Intermitting fasting or fasting mimicking diets (FMD) have been shown to be able to induce cellular changes that affect inflammation and cellular protection [16-21]. A study of T2DM patients with micro-albuminuria showed that FMD could be conducted safely and showed beneficial effects on albuminuria [22]. Recently, we revealed in an experimental diabetic study that repeated FMD partially preserved the glomerular endothelial glycocalyx coverage, however, perturbing glomerular metabolic responses [23]. Also a new promising dietary intervention through supplementation with GAGs mimetics such as fucoidan, a marine organism-derived fucosylated and sulfated polysaccharide and major constituent of Endocalyx™, has been shown to preserve the EG layer [24, 25].

Here, we investigated the effects of these two dietary interventions, repeated FMD cycles or Endocalyx™ supplementation in SA-T2DM in a multi-arm randomized controlled study. We hypothesized that these dietary interventions are able to preserve endothelial stability and in turn preserve microvascular health.

For this, microvascular health was assessed on the sublingual microvasculature with the non-invasive sidestream dark field (SDF) imaging method with newly developed and validated Glycocheck™ software [26, 27]. In addition, besides demographics, medication

use, blood pressure and laboratory markers for diabetes and renal function testing, we measured the HPSE-1 and hyaluronidase 1 (HYAL-1) activity, and HYAL-4 activity and concentration, together with the endothelial activation markers angiopoietin-2 (ANG2), soluble thrombomodulin (sTM) and MCP-1.

Research design and methods

Clinical study design and patient recruitment

We conducted a multi-arm parallel-group randomized study in SA-T2DM of Surinamese descent in The Hague area of the Netherlands between May 2018 and September 2020, recruited at general practitioners' offices. In this study 3 parallel arms were selected: 1) receiving a diet regime of FMD boxes (Prolon®, L-Nutra Inc., Los Angeles, CA, USA); 2) receiving the dietary supplement Endocalyx™ (MicroVascular Health Solutions LLC, Alpine, UT, USA); or 3) receiving placebo capsules (figure 1A). For sample size estimation the primary outcome was determined as improvement of the Microvascular Health Score ($MVHS_{dynamic}$) according to a pilot study with 13 healthy volunteers receiving the food supplement Endocalyx™ for 3 consecutive months (supplemental figure 1). Eligible patients, with inclusion criteria of age between 18 and 75 years old, self-identified as from South-Asian Surinamese descent, treatment with hypoglycemic drugs for type 2 diabetes and proven albuminuria with an albumin to creatinine ratio (ACR) between 0.3 and 30 mg/mmol in the last 12 months. Moreover, eGFR had to be above 45 mL/min/1.73 m^2 (CKD-EPI formula [28]). The study (NCT03889236) was conducted in accordance with the Declaration of Helsinki (October 2013) and approved by the Ethics Committee of the Leiden University Medical Center (LUMC) in agreement with the Dutch law for medical research involving human subjects.

Intervention, randomization and blinding

Eligible patients were randomized via randomization envelopes made by the Pharmacy department of the LUMC into the diet-, supplement-, or placebo-arm, after given informed consent (figure 1). In agreement with the CONSORT statement, randomisation was performed to provide blinding of the supplement and placebo arm to the participants, care providers and researchers. The Pharmacy department redistributed, labeled and blinded the capsules.

The FMD (Prolon®) consisted of a 5-day low protein plant-based diet regime that contained energy bars, vegetable-based soups, kale chips, olives, energy drinks, a supplement and natural tea's. Day 1 provided 1,090 kcal (containing 34% carbohydrate, 56% fat and 10% protein), and days 2 to 5 were identical in formulation and provided 725 kcal (47% carbohydrate, 44% fat and 9% protein). As previous studies with this diet showed benefi-

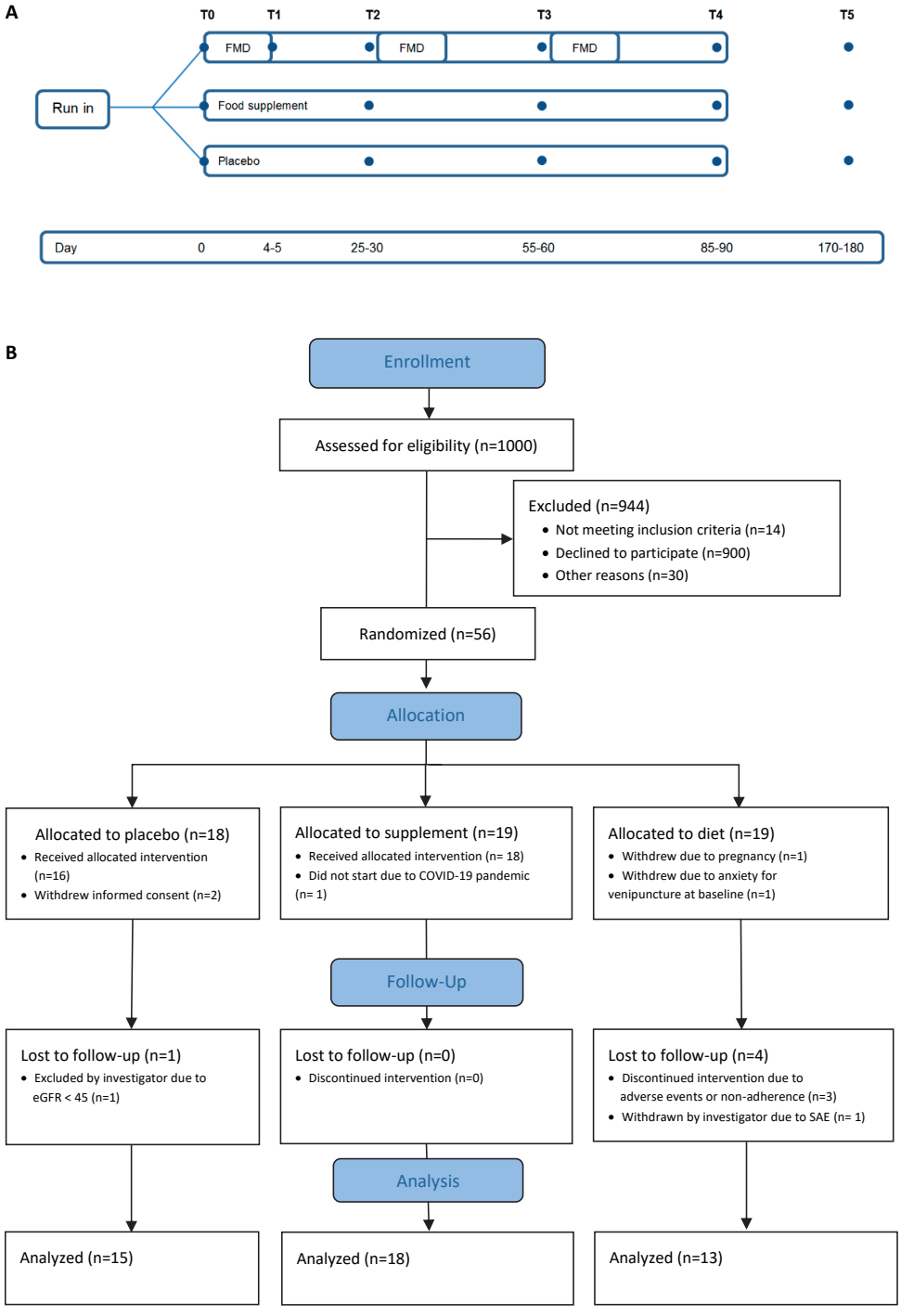


Figure 1. Study design (A) and CONSORT Flow diagram (B).

cial effects after 3 monthly cycles [16], our patients also followed the diet once a month, with a total of 3 cycles in 3 months. Trajectory patients' satisfaction about FMD intervention was reviewed with the Diabetes Treatment Satisfaction Questionnaire (DTSQ) [29]. Endocalyx™ supplement was produced and provided by Microvascular Health Solutions (Alpine, UT, USA). One capsule contained fucoidan extracted from *Laminaria japonica* (106.25 mg), glucosamine sulphate (375.0 mg), hyaluronic acid (17.5 mg), a blend of superoxide dismutase and polyphenols (120.0 mg) and stabilizers/bulking agents. The placebo capsules were manufactured by the Pharmacy department of the LUMC and contained microcrystalline cellulose. Patients were instructed to take 4 capsules a day for 3 consecutive months. Patients received no dietary advice and maintained their normal diet during the study.

Data collection

Study visits were conducted at the general practitioner's office of that specific patient and were executed by the researcher or research assistants. The web based relational database management system Castor Electronic Data Capture (*EDC*) (https://www.castoredc.com) was used for data storage. Patients were instructed to fast overnight and not to smoke before each study visit. In the first three months, patients had a study visit each month. After completing the first 3 months of the intervention, the interventions were discontinued, and patients had one follow-up study visit at month 6 (figure 1A).

At baseline, self-identified ethnicity, age and smoking status were collected. Medical history and medication use was extracted from the personal health records. Microvascular complications were defined as having retinopathy and/or neuropathy. Macrovascular complications were defined as having myocardial infarction, angina pectoris, cerebrovascular accident and/or peripheral artery disease.

Systolic and diastolic BP was measured twice with an automated blood pressure monitor (OMRON, Model M6, Omron Health Care Inc, IL, USA) after patients were sitting calmly for about 5 minutes. BMI was calculated by dividing the weight (measured with indoor clothing but without shoes) in kilograms by the self-reported height in meters squared. Waist circumference was measured with a measuring tape mid-way between the lower costal margin and the iliac crest. Fasting blood glucose levels were measured with a finger prick blood sample (Accu-chek Aviva, Roche, Basel, Swiss).

Blood samples were collected after overnight fast at the morning of the study visit at baseline, 3- and 6 months through vena puncture. Serum levels of C-peptide, Insulin growth factor 1 (IGF-1), creatinine, high sensitivity C-reactive protein (HsCRP), total cholesterol, high-density lipoprotein cholesterol, triglycerides levels and plasma levels of HbA1c were determined in the central clinical chemistry laboratory of the LUMC using standard assays.

Low-density lipoprotein cholesterol was calculated using the Friedewald formula [30]. The CKD-EPI formula was used to estimate the glomerular filtration rate [28].

Plasma HPSE-1 (heparan sulfate, HS degradation) activity (Takara Bio Inc., Shiga, Japan), HYAL-1 [31] (chondroitin sulfate/hyaluronan, CS/HA) and HYAL-4 [32] (CS) activity in-house developed ELISA [33], optimized by use of recombinant active human HYAL-1 or HYAL-4 (7358-GH-020 and #6904-GH-020, Bio-techne, Abingdon, UK). HYAL-4 protein was measured (AMS Biotechnology, Abingdon, UK) according to the manufacturer's instructions. Plasma levels of ANG2 (DANG20, R&D Systems, Abington, UK) and sTM (850.720.096, Diaclone, Besançon, France) were determined as described [32], and measured according to the protocol supplied by the manufacturer.

On the day of study visit, first morning urine was collected to determine albumin and creatinine (CCL, LUMC), albumin-creatinine ratio (ACR) was calculated and for albumin levels lower than 3.0 mg/mL (displayed as <3.0 by CCL), 2.9 was used for ACR calculation. Urinary HPSE-1 activity and MCP-1 concentration were measured (Takara Bio Inc., Shiga, Japan and R&D Systems Europe, Ltd., Abingdon, UK, resp.) according to manufacturer's protocols and corrected for creatinine concentration.

Microvascular imaging

Sublingual microcirculation was assessed with SDF-imaging (CapiScope HVCS, KK Technology, Honiton, UK) coupled to the GlycoCheck^{TM} software (Microvascular Health Solutions Inc., Salt Lake City, UT, USA). Image acquisition was automatically mediated through the Glycocheck™ software as described elsewhere [26, 27, 34]. The GlycoCheck™ software detects and extracts the following microvascular parameters: red blood cell velocity(V_{RBC}), perfused capillary density, static and dynamic capillary blood volume (CBV), static and dynamic perfused boundary region (PBR), and the overall microvascular health score (MVHS), validated and described earlier [27, 35].

Glucose monitoring and diet compliance

To minimize the occurrence of hypoglycemia during the diet cycles, dosages of hypoglycemic medications were temporarily altered during the 5 day diet cycle. Sulfonylurea derivatives and short acting insulin were discontinued, long acting insulin reduced by 50%, with fasting glucose monitoring on days 6, 7 and 8. Metformin, DPP-4 inhibitors, SGLT-2 inhibitors or GLP-1 agonists were continued during the diet cycle. Compliance was checked on the morning of day 5 of the first FMD cycle by measuring fasting ketone body concentration in blood (CareSens Dual, Zkope Healthcare, Sittard, Netherlands) and with ketone sticks in a fresh morning urine sample (Ketostix, Bayer, Leverkusen, Germany). During the cycles patients were contacted by the investigator to check glucose monitoring and compliance with the diet.

Clinical data and resource availability

Most of the data generated or analyzed during this study are included in the published article (and its online supplementary files). The remainder clinical data generated during and/or analyzed during the current study are not publicly available due to hospital privacy restrictions but can be made available as anonymized data from the corresponding author upon reasonable request.

Statistical analysis

The primary endpoint was improvement of microvascular function within 3 months as determined with SDF-imaging (capillary density, CBV, PBR and MVHS). Secondary endpoints were improvement in clinical parameters (BP, BMI, waist-to-hip ratio), laboratory markers (ACR, fasting glucose, HbA1c, C-peptide, IGF-1, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, hsCRP, HPSE-1, HYAL-1, HYAL-4, ANG2, sTM and MCP-1). Potential legacy effects were determined using the microvascular and clinical parameters, 3 months after discontinuation (at month 6).

Continuous variables with normal distribution were presented as mean with standard deviation (SD) and variables with skewed distribution as median with 25-75 percentile. Categorical data were expressed as proportions.

Treatment effects within and between groups (diet vs. placebo and supplement vs. placebo, respectively) were investigated with intention to treat analysis by linear mixed models for repeated measurements with Bonferroni post hoc test (values expressed as estimated marginal means (SE) or estimated mean differences with 95% CI). The models were adjusted for age, gender, microvascular and macrovascular history at baseline and hypertension at baseline as this can influence microvascular function. For the per protocol analysis, delta changes within 3 months intervention were compared between the intervention groups with an unpaired t-test.

Results from the DTSQ questionnaire (see supplemental data) were compared between FMD and placebo groups with an unpaired t-test.

Statistical analysis was performed using SPSS version 25 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8 (Graphpad Inc., La Jolla, CA, USA). A significance level of 0.05 was considered statistically significant.

Results

Inclusion and drop-outs

A total of 56 patients were included and randomized (see CONSORT diagram in figure 1), with 19 patients in the diet group, 19 in the supplement group and 18 patients in the placebo group. In the diet group, one patient withdrew due to pregnancy at the start of the study and one patient withdrew during the baseline visit due to anxiety for the venipunctures. 3 Patients discontinued due to adverse events or non-adherence during or after the first diet cycle and one patient was withdrawn by the investigator due to an SAE after the first diet cycle. A total of 13 patients completed the 3 diet cycles and the follow-up study visit at month 6 (figure 1). In the supplement group, 19 patients were randomized and completed the baseline measurement. One patient did not start due to the COVID-19 pandemic, which resulted in 18 patients completing the 3-month intervention and follow-up. In the placebo group, 2 patients withdrew informed consent before the baseline visit and one patient was withdrawn by the investigator after the baseline measurements due to an eGFR below the inclusion criteria threshold. A total of 15 patients completed the 3 months placebo intervention and 14 patients completed the follow-up study visit at month 6, as one patient migrated to Surinam. Due to the COVID-19 pandemic all inclusions had to stop, preventing completion of the estimated number of patients per group.

Baseline characteristics of the study population

Baseline characteristics of FMD- (n=18), Endocalyx- (n=19) and placebo group (n=16) and total cohort (n=53) are shown in Table 1. Overall, baseline characteristics were almost similar between both dietary intervention groups and placebo group, except mean age in

Table 1. Baseline characteristics of the study population

	Placebo (n=16)	Diet (n=18)	Supplement (n=19)	Total (n=53)
Demographics				
Age, years (SD)	63 (±7)	61 (±6)	56 (±7)	60 (±7)
Women, n (%)	7 (44)	12 (67)	12 (63)	31 (59)
Current tobacco smoking, n (%)	5 (31)	4 (22)	6 (32)	15 (28)
Medical history				
Duration diabetes mellitus, years (SD)	7 (±5)	11 (±4)	9 (±5)	9 (±5)
Retinopathy, n (%)	4 (25)	10 (56)	5 (26)	19 (36)
Neuropathy, n (%)	1 (6)	2 (11)	4 (21)	7 (13)
Coronary artery disease, n (%)	4 (25)	3 (16)	2 (11)	9 (17)
Angina pectoris, n (%)	4 (25)	2 (11)	1 (5)	7 (13)
CVA/TIA, n (%)	3 (19)	0	4 (21)	7 (13)

Table 1. Baseline characteristics of the study population *(continued)*

	Placebo (n=16)	Diet (n=18)	Supplement (n=19)	Total (n=53)
Medication use				
Metformin, n (%)	16 (100)	17 (94)	19 (100)	52 (98)
DPP-4 inhibitor/GLP-1-RA/SGLT2 antagonist, n (%)	2 (13)	3 (17)	3 (16)	8 (15)
Sulfonylurea derivatives, n (%)	3 (19)	9 (50)	9 (47)	21 (40)
Insulin, n (%)	2 (13)	3 (17)	3 (16)	8 (15)
Anti-hypertensive medication, n (%)	11 (61)	12 (67)	12 (63)	35 (66)
RAAS inhibitors (n)	10 of 11	10 of 12	10 of 12	30 of 35
Statins, n (%)	12 (75)	16 (89)	16 (84)	44 (83)
Blood pressure				
Systolic blood pressure, mmHg (SD)	139 (±16)	148 (±19)	132 (±15)	140 (±18)
Diastolic blood pressure, mmHg (SD)	86 (±9)	87 (±10)	82 (±11)	85 (±10)
Hypertension, n (%)	13 (72)	16 (89)	11 (58)	40 (76)
BMI, kg/m^2 (SD)	27.4 (±4.3)	28.8 (±5.5)	30.1 (±4.7)	28.8 (±4.9)
Laboratory markers				
Fasting glucose, mmol/L (range)	7.3 (±1.6)	7.6 (±1.7)	8.3 (±2.2)	7.8 (±1.9)
HbA1c, % (range) / mmol/mol (range)	6.8 (6.5-8.0) /51 (47-63)	7.2 (7.0-7.7) /56 (50-60)	7.2 (6.8-7.9) /56 (51-62)	7.2 (6.7-7.8)/ 55 (50-61)
Total cholesterol, mmol/L (SD)	3.9 (±0.7)	4.4 (±0.8)	4.4 (±1.1)	4.3 (±0.9)
hsCRP, mg/L (range)	1.6 (0.8-5.2)	2.1 (0.9-5.0)	4.7 (2.9-8.1)	3.2 (1.0-4.7)
eGFR CKD-EPI (ml/min/1.73m^2)	80 (±17)	84 (±19)	85 (±19)	83 (±18)
Urinary markers				
Albumin/creatine ratio (mg/mmol)	1.8 (0.9-3.2)	1.0 (0.6-1.6)	1.0 (0.6-3.9)	1.1 (0.6-2.4)
Normo-albuminuria[b], (n, %)	9 (56)	17 (94)	15 (79)	41 (77)
Micro-albuminuria at baseline[c] (n, %)	7 (44)	0 (0)	3 (16)	10 (19)
Macro-albuminuria at baseline[d] (n, %)	0 (0)	1 (6)	1 (5)	2 (4)

Data is presented as mean (SD), median (25-75 percentile) or number with percentage.
Abbreviations: *CVA/TIA* cerebrovascular event/transient ischemic attack, *DPP-4* dipeptidyl peptidase-4, *GLP-1-RA* glucagon-like peptide-1 receptor agonist, *SGLT2* sodium-glucose cotransporter 2, *RAAS* renin-angiotensin-aldosterone system, *BMI* body mass index, *HbA1c* hemoglobin A1c, *hsCRP* high sensitivity c-reactive protein, *eGFR CKD-EPI* estimated glomerular filtration rate according to chronic kidney disease epidemiology collaboration
[a]According to the AHA guidelines ≥ 130 mmHg systolic or ≥80 mmHg diastolic
[b]Albumin-creatinine ratio between <3.0 mg/mmol [c]Albumin-creatinine ratio between 3.0 – 30 mg/mmol [d]Albumin-creatinine ratio > 30 mg/mmol.

Endocalyx group was younger and mean duration of diabetes mellitus was higher in FMD group compared to placebo group. Several patients in all groups had prevalent micro- or macrovascular diabetes complications. All patients used various combinations anti-diabetes medication. More than half of the patients used antihypertensive medication,

of which a large number used renin-angiotensin-aldosterone system (RAAS) inhibitors. Despite this, percentage of patients with hypertension (according to the AHA guidelines ≥ 130 mmHg systolic or ≥80 mmHg diastolic) was around 89% (FMD), 58% (Endocalyx) and 72% (placebo). Mean BMI was high in all groups, 28.8 ± 5.5kg/m^2 (FMD), 30.1 ± 4.7kg/m^2 (Endocalyx) and 27.4 ± 4.3kg/m^2 (placebo). In general, patients were well regulated as reflected by the average low HbA1c levels. The placebo group had the highest number of patients with albuminuria in the past 12 months (n=7) compared to FMD (n=1) and Endocalyx (n=4).

Microvascular health in placebo group

First, when combining all baseline data (FMD, Endocalyx and placebo) of measured capillary densities, the capillary density in the total cohort was lower when compared to our earlier published Framingham risk groups in the Netherlands Epidemiology of Obesity (NEO) study (supplemental figure 2) [26]. These results reflect the already poor perfused capillary network in South Asian patients with T2DM, compared to the general Dutch population. As the patients in the placebo group did not receive any dietary restrictions, it was used as a control for both the dietary interventions. Using linear mixed model analysis adjusted for age and sex revealed that besides the perturbed anatomic vascular structures, endothelial health (PBR, as the inverse glycocalyx dimension) and overall microvascular health score ($MVHS_{dynamic}$) worsened over time in the placebo group (table 2, supplemental figure 3A and supplemental table 1) [27].

FMD effect on microvascular health

During the monthly 5-day diet cycle, daily glucose measurements (self-measurement) resulted in 2 reported occurrences of hypoglycemia (<4.0 mmol/L) upon continued use of SU-derivates or long acting insulin, of which one patient discontinued due this occurrence of hypoglycemia. Overall, patients experienced less of the time the feeling of hyper- or hypoglycemia after the diet cycles (DTSQ questionnaire, supplemental table 2). On day 5 of the first diet cycle, in only 3 of 15 patients, ketone bodies could be detected in urine and in 5 patients (of 13) capillary ketone levels of ≥ 0.5 mmol/L (ketosis) were detected.

Intention to treat analysis using linear mixed model analysis adjusted for age, sex, microvascular or macrovascular complications at baseline and hypertension revealed that after 3 months of recurrent FMD, 3 weeks after last cycle, only $PBR_{dynamic}$ significantly increased (estimated mean difference 0.32 µm (95% CI 0.18 – 0.50; table 2). Treatment effects between FMD and placebo groups revealed significant differences within 3 months for $CBV_{dynamic}$, PBR_{static}, and $MVHS_{dynamic}$ (table 2). This was also shown with the per protocol analysis showing the delta differences in microvascular parameters between FMD and placebo in figure 2.

Table 2. Differences between baseline and after 3 months in the interventions groups and treatment effects

	Placebo			Diet			Supplement				
	Baseline (n=16)	3 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	3 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	3 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
Microvascular parameters											
Capillary density (4-6) (µm/mm²)	43 (6)	34 (4)	-9 [-23;4]	32 (6)	24 (4)	-7 [-20;6]	32 (5)	35 (3)	3 [-10;15]	0.79	0.11
Capillary blood volume static (pL/mm²) /10³ µm³	12.7 (1.7)	9.9 (1.1)	-2.8 [-6.8;1.3]	10.1 (1.7)	8.1 (1.2)	-2.1 [-6.1;2.0]	10 (1.5)	10.9 (1)	1.0 [-2.8;4.7]	0.76	0.10
Capillary blood volume dynamic (pL/mm²) /10³ µm³	21.6 (2.5)	11.3 (1.4)	**-10.3 [-16.2;-4.3]**	11.8 (2.5)	14 (1.6)	2.2 [-3.7;8.1]	14.7 (2.3)	13.9 (1.3)	-0.8 [-6.2;4.7]	**0.001**	**0.01**
PBR static (µm)	2.08 (0.05)	2.24 (0.05)	**0.16 [0.03;0.28]**	2.26 (0.05)	2.2 (0.06)	-0.05 [-0.19;0.08]	2.21 (0.04)	2.18 (0.05)	-0.04 [-0.16;0.08]	**0.01**	**0.01**
PBR dynamic (µm)	2.19 (0.05)	2.46 (0.05)	**0.28 [0.15;0.40]**	2.35 (0.05)	2.66 (0.06)	**0.32 [0.18;0.50]**	2.74 (0.04)	2.01 (0.05)	**-0.73 [-0.85;-0.61]**	0.59	**<0.001**
MVHS dynamic (µm)	3.9 (0.5)	1.8 (0.3)	**-2.1 [-3.2;-1.1]**	2.1 (0.5)	2.1 (0.3)	0 [-1.0;1.1]	2.2 (0.4)	2.8 (0.2)	0.7 [-0.3;1.7]	**<0.001**	**<0.001**
Clinical parameters											
Systolic blood pressure (mmHg)	132 (4)	136 (4)	4 [-5;13]	141 (4)	134 (4)	-7 [-16;3]	133 (3)	130 (3)	-3 [-11;5]	0.05	0.17
Diastolic blood pressure (mmHg)	82 (2)	82 (2)	1 [-5;6]	81 (2)	80 (2)	-2 [-7;4]	80 (2)	81 (2)	1 [-4;5]	0.43	0.95
BMI (kg/m²)	26.4 (1.3)	26.4 (1.2)	0 [-0.5;0.4]	28.3 (1.3)	27.3 (1.3)	**-1.0 [-1.5;-0.5]**	30.2 (1.1)	30.3 (1.1)	0.1 [-0.3;0.6]	**<0.001**	0.53
Laboratory markers											
Fasting glucose (mmol/L)	7.3 (0.5)	7.1 (0.6)	-0.1 [-1.4;1.1]	7.2 (0.5)	7.1 (0.6)	-0.2 [-1.5;1.1]	8.3 (0.4)	8.1 (0.5)	-0.2 [-1.3;1]	0.97	0.98
Hba1c (mmol/mol)	55.6 (3.3)	55.9 (3)	0.4 [-3.9;4.6]	55.3 (3.4)	50.3 (3.3)	**-5.1 [-9.6;-0.6]**	57.9 (2.9)	60.2 (2.7)	2.3 [-1.7;6.3]	**0.03**	0.41

Table 2. Differences between baseline and after 3 months in the interventions groups and treatment effects *(continued)*

	Placebo			Diet			Supplement				
	Baseline (n=16)	3 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	3 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	3 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
C-peptide (nmol/L)	1.2 (0.1)	1.2 (0.1)	0 [-0.2;0.2]	1.4 (0.1)	1.3 (0.1)	-0.1 [-0.3;0.1]	1.5 (0.1)	1.5 (0.1)	0.1 [-0.1;0.2]	0.47	0.62
eGFR CKD-EPI (ml/min/1.73m^2)	86 (5)	84 (5)	-2 [-8;5]	83 (5)	81 (5)	-2 [-9;5]	80 (4)	83 (4)	3 [-2;9]	0.97	0.16
Total cholesterol (mmol/L)	4.03 (0.23)	4.12 (0.26)	0.09 [-0.33;0.51]	4.34 (0.24)	4.21 (0.28)	-0.13 [-0.58;0.32]	4.35 (0.2)	4.34 (0.23)	0 [-0.39;0.38]	0.38	0.68
HDL-cholesterol (mmol/L)	1.27 (0.08)	1.35 (0.09)	0.08 [-0.01;0.18]	1.22 (0.08)	1.23 (0.09)	0.01 [-0.09;0.11]	1.08 (0.07)	1.07 (0.08)	-0.01 [-0.09;0.08]	0.20	0.10
LDL-cholesterol (mmol/L)	2.34 (0.2)	2.32 (0.2)	-0.02 [-0.33;0.29]	2.4 (0.21)	2.31 (0.21)	-0.09 [-0.42;0.24]	2.41 (0.18)	2.43 (0.18)	0.02 [-0.27;0.31]	0.70	0.80
Triglycerides (mmol/L)	1.11 (0.5)	1.19 (0.33)	0.07 [-0.6;0.75]	1.65 (0.5)	1.77 (0.35)	0.12 [-0.56;0.8]	2.31 (0.45)	2.09 (0.29)	-0.22 [-0.84;0.4]	0.90	0.43
hsCRP (mg/L)	4.0 (1.1)	3.0 (1.0)	-1.1 [-2.8;0.6]	3.6 (1.1)	2.9 (1.1)	-0.7 [-2.4;1.1]	6.0 (1.0)	6.2 (0.9)	0.2 [-1.3;1.8]	0.68	0.16
IGF-1 (nmol/L)	18.3 (1.3)	17.9 (1.2)	-0.4 [-2.6;1.8]	17.9 (1.4)	19.6 (1.3)	1.6 [-0.7;3.9]	17.1 (1.2)	16.5 (1.1)	-0.6 [-2.7;1.4]	0.13	0.82

Abbreviations: *PBR* perfused boundary region, *MVHS* microvascular health score, *BMI* body mass index, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *Hba1c* hemoglobin A1c, *eGFR CKD-EPI* estimated glomerular filtration rate according to chronic kidney disease epidemiology collaboration, *hsCRP* high sensitivity c-reactive protein, *IGF-1* Insulin growth factor 1, *ns* non-significant; Estimated marginal means with standard error

[1]Linear mixed models adjusted for age, sex, microvascular history at baseline, macrovascular history at baseline and hypertension at baseline with Bonferroni post-hoc, baseline compared to 3 months, [2]interaction term of the groups with measurement over time (Baseline-3 months), p<0.05 is considered significant (bold).

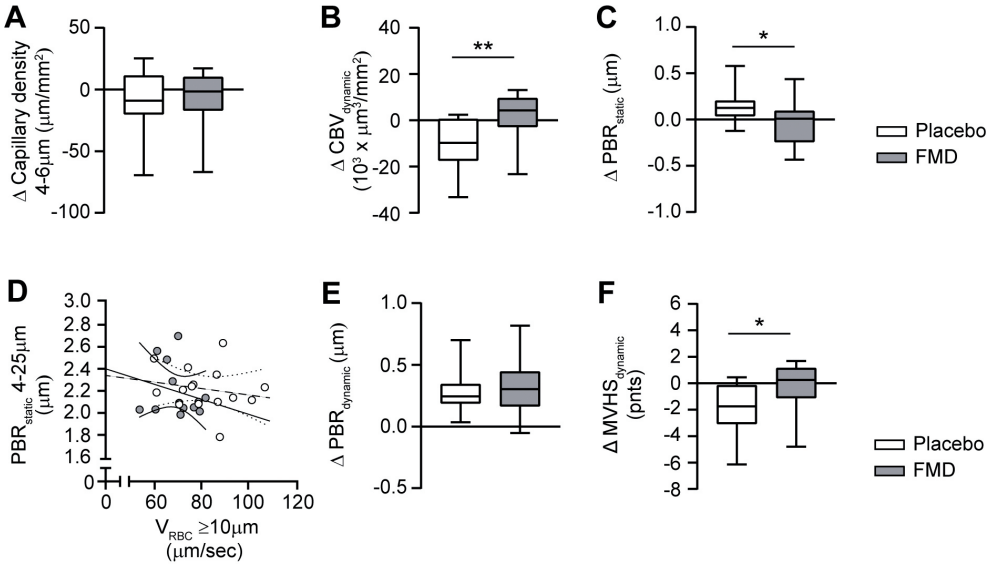


Figure 2. Changes in microvascular parameters upon FMD treatment. Comparison of changes within 3 months intervention between the diet and placebo group of (A) Capillary density, (B) Dynamic capillary blood volume ($CBV_{dynamic}$) and (C) Static perfusion boundary region (PBR_{static}), (D) Scatter dot plots and simple linear regression (slope) with 95% confidence intervals of PBR_{static} plotted against red blood cell velocity (VRBC) in feed vessels. Comparison of changes after 3 months intervention between diet (continuous line) and placebo (dashed line) of (E) dynamic perfusion boundary region ($PBR_{dynamic}$) and (F) dynamic Microvascular Health Score ($MVHS_{dynamic}$). Box plot whiskers indicate 5th and 95th percentiles. Delta changes were compared between the diet and placebo group with an unpaired t-test. *P < 0.05, **P < 0.01

While BP, fasting glucose, hsCRP and cholesterol levels were not affected after the diet cycles, mean BMI and serum HbA1c levels were significantly lower compared to baseline with an estimated difference of -1.0 kg/m^2 (95% CI -1.5 – -0.5) and -5.1 nmol/L (95% CI -9.6 – - 0.6), respectively. Markers related to glycocalyx degradation (HPSE-1) and endothelial and glycocalyx shedding markers (ANG2 and sTM) were not affected (table 3).

The follow-up measurements at month 6 revealed that BMI was still lower compared to baseline, with a difference of -0.6 kg/m^2 (95% CI -1.2 – 0.00). However, the effect on Hba1c was lost after 6 months compared to baseline with a difference of -1.8 mmol/mol (95% CI -6.2 – 2.8) (supplemental table 1). In contrast, $PBR_{dynamic}$ was increased with a positive linear regression slope (supplemental figure 3B) arguing for a continued adverse effect on endothelial function.

Table 3. Differences between baseline and after 3 months in the interventions groups and treatment effects

	Placebo			Diet			Supplement				
	Baseline (n=16)	3 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	3 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	3 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
Glycocalyx plasma markers											
HPSE-1 activity plasma (U/mL)	1.04 (0.16)	1.54 (0.19)	0.50 [-0.01;1.00]	1.33 (0.17)	1.46 (0.21)	0.12 [-0.41;0.65]	1.27 (0.14)	1.54 (0.17)	0.28 [-0.18;0.74]	0.21	0.43
HYAL-1 activity (U/mL)	0.56 (0.02)	0.54 (0.01)	-0.01 [-0.06;0.03]	N/A	N/A	N/A	0.48 (0.02)	0.52 (0.01)	0.04 [-0.01;0.07]	N/A	**0.05**
HYAL-4 activity (U/mL)	25.10 (2.92)	21.47 (2.6)	-3.63 [-8.78;1.52]	N/A	N/A	N/A	17.82 (2.51)	17.55 (2.17)	-0.27 [-4.87;4.33]	N/A	0.23
HYAL-4 protein (ng/mL)	3.45 (0.41)	3.51 (0.39)	0.07 [-0.27;0.41]	N/A	N/A	N/A	2.79 (0.35)	2.61 (0.33)	-0.17 [-0.48;0.13]	N/A	0.19
ANG-2 protein (ng/mL)	2.47 (0.24)	2.40 (0.21)	-0.06 [-0.39;0.27]	2.77 (0.24)	2.59 (0.23)	-0.18 [-0.51;0.16]	2.82 (0.21)	3.04 (0.18)	0.22 [-0.08;0.52]	0.55	0.12
sTM protein (ng/mL)	5.13 (0.60)	5.80 (0.69)	0.67 [-0.16;1.50]	5.37 (0.62)	5.25 (0.72)	-0.12 [-1.00;0.76]	6.57 (0.52)	6.79 (0.60)	0.22 [-0.53;0.98]	0.11	0.33
Urinary markers											
ACR urine (mg/mmol)	3.1 (2.1)	2.6 (0.7)	-0.5 [-5.5;4.4]	2.7 (2.0)	1 (0.8)	-1.7 [-6.4;3.0]	5.0 (1.9)	2.4 (0.6)	-2.6 [-7.2;1.9]	0.68	0.44
HPSE-1 activity urine (U/mL)	0.96 (0.19)	0.81 (0.14)	-0.15 [-0.53;0.24]	1.19 (0.21)	0.89 (0.16)	-0.30 [-0.71;0.12]	0.87 (0.17)	0.83 (0.13)	0.04 [-0.40;0.2]	0.52	0.62
MCP-1 activity urine (ng/mmol)	22.3 (9.7)	39.3 (8.6)	17.1 [-10.2;44.3]	36.6 (9.3)	28.5 (9.4)	-8.10 [-35.7;19.5]	39.3 (8.7)	25.9 (7.8)	-13.4 [-38.3;11.5]	0.11	**0.05**

Abbreviations:, *HPSE-1* heparanase-1*, HYAL-1* hyaluronidase-1*, HYAL-4* hyaluronidase-4*, ANG-2* Angiopotein-2*, sTM* soluble thrombomodulin*, ACR* albumin creatinine ratio, *MCP-1* monocyte chemoattractant protein 1, *ns* non-significant; Estimated marginal means with standard error

[1]Linear mixed models adjusted for age, sex, microvascular complications at baseline, macrovascular complications at baseline and hypertension at baseline with Bonferroni post-hoc, baseline compared to 3 months, [2]interaction term of the groups with measurement over time (Baseline-3 months), p<0.05 is considered significant (bold).

Endocalyx effects on microvascular health

After 3 months of daily supplementation with Endocalyx, a significant improvement in the $PBR_{dynamic}$ (estimated difference of -0.73 µm; 95% CI -0.85 – -0.61) and $MVHS_{dynamic}$ of 0.7 points (95% CI -0.3 – 1.7) was seen, while capillary density, CBV and PBR_{static} did not change significantly (table 2). Treatment effects between Endocalyx and placebo groups for the intention to treat and per protocol analysis showed to be significant for $CBV_{dynamic}$, PBR_{static}, $PBR_{dynamic}$ and $MVHS_{dynamic}$ confirming an overall improvement in vascular health after 3 months of glycocalyx mimetics supplementation (figure 3 and table 2). The follow-up measurements at month 6 revealed a continuation in improved microvascular health (supplemental figure 3C and supplemental table 1).

Markers related to glycocalyx degradation (HPSE-1, HYAL-1 and HYAL-4) and endothelial and glycocalyx shedding markers (ANG2 and sTM), however, were not affected (table 3). Overall, ACR did not significantly change (estimated difference of -2.6 mg/mmol, 95% CI -7.2 – 1.9), although in two patients with the highest baseline ACR (28.8 and 37.7 mg/mmol, respectively) albuminuria decreased to normo-albuminuric levels after 3 months of Endocalyx. Urinary MCP-1 activity did not significantly change within 3 months (estimated

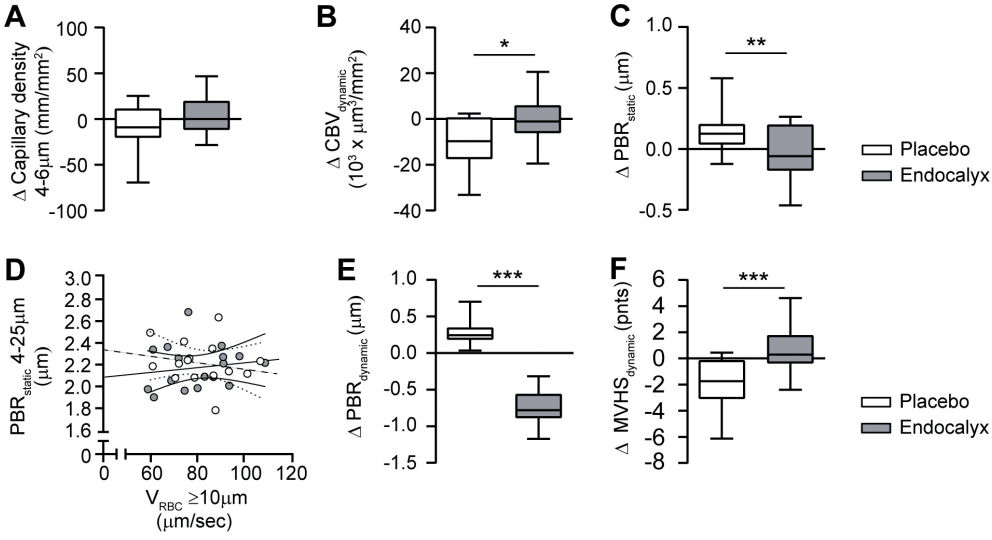


Figure 3. Changes in microvascular parameters upon supplement treatment. Comparison of changes within 3 months intervention between the supplement and placebo of (A) Capillary density, (B) Dynamic capillary blood volume ($CBV_{dynamic}$) and (C) Static perfusion boundary region (PBR_{static}), (D) Scatter dot plots and simple linear regression (slope) with 95% confidence intervals of PBR_{static} plotted against red blood cell velocity (VRBC) in feed vessels. Comparison of changes after 3 months intervention between supplement (continuous line) and placebo (dashed line) of (E) dynamic perfusion boundary region ($PBR_{dynamic}$) and (F) dynamic Microvascular Health Score ($MVHS_{dynamic}$). Box plot whiskers indicate 5th and 95th percentiles. Delta changes were compared between the supplement and placebo group with an unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.0001.

difference of -13.4 ng/mmol with 95% CI -38.3 – 11.5), although compared to placebo urinary MCP-1 activity was significantly lower in the supplement group (p=0.05) (table 3). No significant changes in other clinical or laboratory parameters were observed (table 2).

Discussion

We evaluated the effect of two short-term dietary interventions on microvascular endothelial health in SA-T2DM. Overall, we found that capillary density in this patient population was already lower than expected compared to earlier measurements within the NEO study [26], and seems in line with the observed existing cardiovascular problems these patients are facing [3]. We also observed in the placebo that during the first 3 months microvascular parameters worsened.

After 3 recurrent FMD cycles, $PBR_{dynamic}$ alone increased, which continued to worsen at 3 months after the last diet cycle. We observed a significant reduction in plasma HbA1c levels and BMI in the first 3 months and the effect on BMI was still present after discontinuation of the FMD cycles.

Supplementation with Endocalyx for 3 months, however, showed an improvement in the microvascular parameters $CBV_{dynamic}$, PBR_{static}, $PBR_{dynamic}$ and $MVHS_{dynamic}$ which was also reflected in the linear regression slopes (PBR_{static} vs. V_{RBC}). Even 3 months after the intervention this effect was still present, indicating a possible legacy effect. Interestingly, we found improvement of albuminuria in 2 patients in this group which was accompanied by an improved PBR. The observed significantly reduced urinary MCP-1 activity after 3 months, however, was lost at follow-up. No effect on the other clinical or laboratory markers were observed upon Endocalyx supplementation.

Although in early diabetic nephropathy inhibition of HPSE-1 activity has been shown to protect the endothelial glycocalyx and to prevent development of proteinuria [15, 36], we could not demonstrate this effect in the present study. Following this study, we recently observed ethnic differences in urinary HPSE-1 and MCP-1 activity in individuals with T2DM from the HELIUS study [37]. Interestingly, the South-Asian Surinamese participants showed the lowest urinary HPSE-1 activity. Urinary HPSE-1 activity in that study was only statistically significant in participants of Dutch origin in relation to ACR.

Previous diet intervention studies in patients with type 2 diabetes revealed reduced BMI, blood pressure, fasting glucose, total and low density cholesterol, CRP and IGF-1 levels in a cohort of 100 participants, particularly in those at risk for disease [38, 39, 40]. We recently showed in an experimental diabetic study, that a repeated FMD was able to

partially preserve glomerular endothelial glycocalyx coverage, however, perturbing glomerular metabolic responses resulting in increased oxidative stress and reduced catabolic breakdown products [23]. While capillary loop morphology and endothelial glycocalyx heparan sulfate contents was preserved, hyaluronan surface expression was reduced which coincided with reduced UDP-glucuronic acid, a rate limiting building block in its biosynthesis. Despite the positive effects in the first FMD trial in patients with T2DM [22], showing beneficial effects on albuminuria levels in patients with micro-albuminuria next to beneficial effects on HbA1c levels and BMI, the lack of improvement in inflammatory or glycocalyx degrading markers in our study could be due to the already established cardiovascular problems, such as retinopathy, neuropathy and other cardiovascular disease markers. These cardiovascular comorbidities, however, do not seem to correspond with the observed albuminuria and these observations argue for alternative disease biomarkers in this specific patient population.

The main risk of severe calorie restriction in patients with diabetes is hypoglycemia and several studies on fasting regimes in T2DM patients emphasize the importance of adjusting the dosage of glucose lowering medications during fasting days [38, 41]. In our study, the dosages of the sulfonylurea derivatives and insulins were changed or stopped during the 5-day fasting cycle to minimize the chance of hypoglycemia. This resulted still in 2 reported occurrences of hypoglycemia during the FMD cycles while patients did experience less of the time the feeling of hyper- or hypoglycemia after the diet cycles (see supplemental data). It appears that, when medication dosage is adapted appropriately and patients are frequently monitored, a low caloric diet can be used safely in patients using sulfonylurea derivatives or insulin.

In one patient, the estimated glomerular filtration rate, which was not routinely measured in this study, turned out to be seriously deteriorated after the first diet cycle. This decline of kidney function appeared to be due to dehydration, as the kidney function fully recovered after intravenous fluid therapy (supplemental data). It has been known for a long time that severe calorie restriction is accompanied by loss of sodium and body water via a largely unexplained mechanism [42, 43]. Therefore, people who fast or use a FMD should always be encouraged to drink sufficiently. In our experimental study, we also found that weight loss during the FMD was mainly due to loss of water and lean mass [23]. Currently, a clinical trial with this diet in patients with diabetes is investigating the effect on body composition by collecting MRI data after a diet cycle [44]. For now, it might be advised that FMD should be used with caution in patients with decreased kidney function or CKD and in patients using diuretics, where kidney function should be monitored regularly.

This study has several limitations. Firstly, the low sample size and drop-out rate of the study. We experienced a low response rate in patients that were contacted for the clinical

study. Conducting lifestyle intervention studies in the South-Asian population has been proven to be extremely difficult due to low response rates, high drop-out rates and lack of effect on lifestyle [45, 46]. We experienced a drop-out rate of 30% in the diet group, comparable to other FMD studies [40]. In addition, due to the COVID-19 epidemic, inclusions of the study had to be discontinued. The low sample size may have reduced the power to show significant effects in the intervention groups although still clear primary endpoints were observed. Secondly, in only a few patients after the FMD cycle, capillary or urinary ketone levels were elevated reflecting ketosis. During fasting, when glycogen stores are depleted and glucose is less available, fatty acids are released from adipose tissue to serve as an alternative fuel, and excessive oxidation of fatty acids is accompanied by ketogenesis. These patients may not have been compliant with the dietary regime. Another explanation, however, could be that the switch from carbohydrate to lipid oxidation in response to fasting is impaired in South-Asians (as compared to European) with T2DM, reflecting metabolic inflexibility in South-Asian individuals [47]. As ketones are probably involved in the health effects of fasting [48], this may indicate that the benefits of fasting or fasting mimicking diets are less effective in individuals from South-Asian descent than in individuals of European descent.

A strength of the study is its execution in general practitioner offices with South-Asian Surinamese patients, a patient group that can be difficult to recruit for clinical trials as mentioned above. An intensive 1 and 2 year targeted lifestyle intervention in general practice revealed no significant weight loss or improvement in metabolic profiles in South-Asian Surinamese participants in The Hague [45, 46]. Although we also experienced low response rates and drop-outs, we did see beneficial effects on BMI and HbA1c levels, already at short term which suggest that South-Asian individuals may find it easier to adhere to repeated short term fasting cycles than continuous caloric restriction or continuous lifestyle interventions.

The development of diabetic vascular complications is a multifactorial pathway, therefore, therapies that target different factors in the pathogenesis are strongly recommended for treatment of diabetes [49]. Preserving endothelial function is seen as a valuable pharmacological target for protecting the microvasculature and reducing the incidence of complications in patients with diabetes [50, 51]. In the present study, we showed that supplementation with Endocalyx^{TM} is a potential candidate able to improve microvascular health in SA-T2DM. We failed to demonstrate direct beneficial microvascular effects of FMD but saw temporarily improvement of metabolic risk factors in SA-T2DM. We confirm that FMD can probably be safely used in patients with diabetes glucose lowering drugs if the dose of these medications are adapted appropriately, but it needs to be used with caution in patients with CKD until further studies has been done.

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Supplemental data

Effect of the fasting mimicking diet, continued

In all patients except one, HbA1c levels decreased after the FMD cycles compared to baseline levels. In 2 patients, dosages of anti-diabetic medication were lowered in the 3 month intervention period due to improved fasting glucose levels. In 2 patients (one patient of which HbA1c levels did not decrease), the dosage of anti-diabetic medication was increased by the GP (data not shown). The change in BMI and HbA1c between the FMD and placebo group was significantly different as shown by the treatment effects (table 2).

IGF-1 levels did not change after 3 FMD cycles (table 2). Most likely because the study visit took place 3 weeks after the last FMD cycle at the end of 3 month study, resulting in IGF-1 levels already returned to baseline [1]. Furthermore, plasma levels of angiopoietin-2 (ANG2) and soluble thrombomodulin (sTM), and heparanase-1 (HPSE-1) activity, all markers of endothelial activation and glycocalyx degradation did not change after 3 months of FMD cycles (table 2). Nor did the urinary markers such as the ACR, urinary HPSE-1 and MCP-1 activity levels decrease significantly after 3 FMD cycles (table 2).

FMD treatment satisfaction

The Diabetes Treatment Satisfaction Questionnaire (DTSQ) [2] which was developed to assess the diabetes treatment satisfaction and consists of eight items, each rated on a seven-point scale. The items include satisfaction with current treatment, treatment convenience, flexibility of treatment, understanding of diabetes, continuity of treatment, recommending treatment to others and two additional items about perceived frequency of hyper- and hypoglycemia. The DTSQ static (DTSQs) was conducted at baseline and the items range from 6 (very satisfied) to 0 (very dissatisfied) with the sum of scores ranging from 36 to 0. The perceived frequency of hyper- and hypoglycemia were also scored from 0 (none of the time) to 6 (most of the time). The DTSQ change (DTSQc) was conducted after 3 months of the intervention. DTSQc uses the same eight items as the DTSQs, but assess changes in the treatment satisfaction with their current treatment (intervention) compared to their previous treatment. The items are scored from +3 (much more satisfied now) to -3 (much less satisfied now), with 0 representing no change. The sum of the score thus ranges from 18 to -18. The perceived frequency of hyper- and hypoglycemia are also scored from +3 (much more of the time now) to -3 (much less of the time now).

In both the FMD and placebo group, satisfaction with their current diabetes treatment at the general practitioners office at baseline was high, with a mean DTSQ satisfaction score of 30.3 (± 5.0) in the FMD group and 30.2 (± 4.4) in the placebo group (supplemental table 1). Perceived frequency of hyperglycemia was comparable between both groups as baseline, with a mean score of 2.9 (± 1.7) in the FMD group and 2.9 (± 2.2) in the placebo

group. Perceived frequency of hypoglycemia was also comparable between both groups, with a mean score of 1.8 (± 2.0) in the FMD group and 1.6 (± 2.0) in the placebo group.

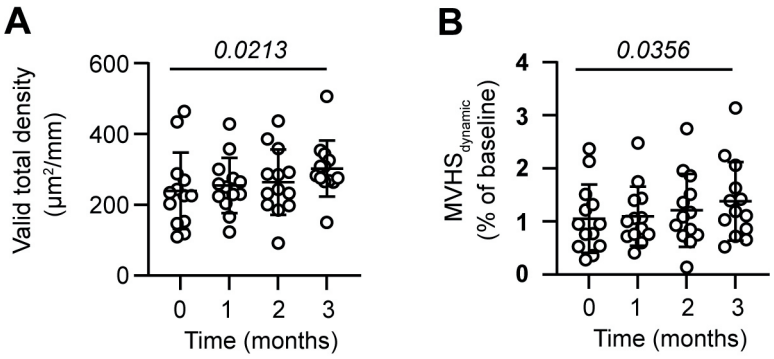
After the 3 FMD cycles, mean treatment satisfaction score with the DTSQc was 11.3 (±4.5), indicating more satisfaction with the intervention compared to the baseline anti-diabetic medication. The placebo group received placebo capsules for 3 months and were also more satisfied with the intervention, with a mean DTSQc treatment satisfaction score of 10.1 (± 7.5). However, perceived hyper- or hypoglycemia did not change in the placebo group with mean scores of 0.1 on both items. After the 3 FMD cycles, mean score of perceived hyperglycemia was -1.0 (± 1.5), indicating that these patients had less of the time the feeling of hyperglycemia after the FMD. These patients also had less of the time perceived hypoglycemia, with a mean score of -0.5 (± 2.0) on this item after 3 FMD cycles.

Adverse events FMD

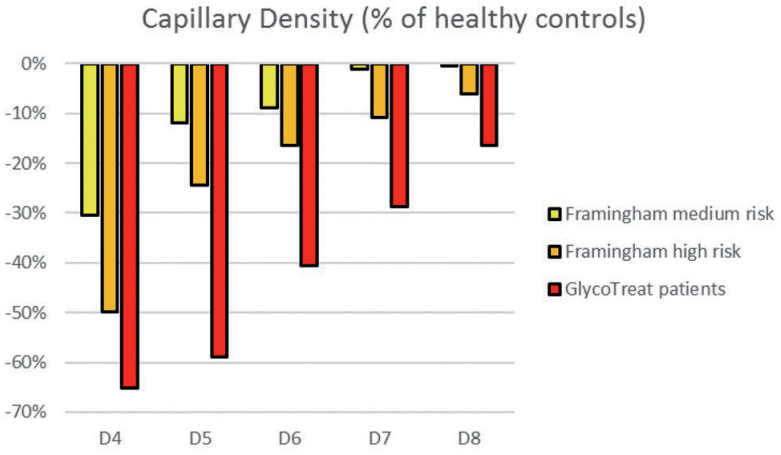
The Common Terminology Criteria for Adverse Events (CTCAE) was used to report adverse event during or after the FMD cycles.The most reported adverse events during the FMD cycles were grade 1 (mild) or grade 2 (moderate) symptoms of headache, fatigue, diarrhea and hypoglycemia. Two patients self-reported the occurrence of hypoglycemia during an FMD cycle. A total of 4 patients dropped out during or after the first cycle due to adverse events. In one patient, a SAE occurred. Kidney function acutely deteriorated at day 5 of the first FMD cycle as compared to previous measurements, which was noticed during a routine check at the hospital. The creatinine level at baseline was 125 µmol/L for this patient, which increased to 169 µmol/L at day 5 of the FMD and increased even further one week later to 202 µmol/L. The patient was hospitalized and rehydrated, after which the creatinine levels normalized to baseline values. This patient was withdrawn from the study by the investigator. No negative effects were seen on kidney function 3 weeks after the third FMD cycle (table 2).

References

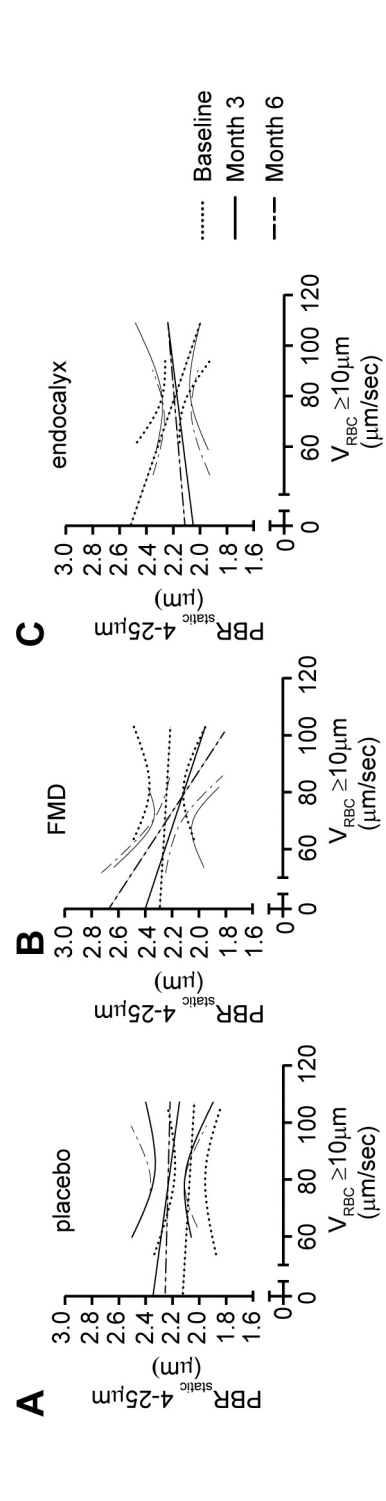
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Supplemental figure 1. Pilot study to determine minimal sample size necessary. Healthy volunteers were given the food supplement Endocalyx™ for 3 consecutive months (n = 13 final measurements) revealing (A) valid total microvascular density, and (B) overall microvascular health score ($MVHS_{dynamic}$). Significance over the three month supplementation is given and was performed using one-way ANOVA (+ Geiser-Greenhouse correction) with mixed effects-model and Dunnet multiple comparison test. After 3 months, a 31% improvement in $MVHS_{dynamic}$ was demonstrated, expressed in a significant increase of from 1.05 at baseline, to 1.38 at 3 months (improvement of 0.33). To achieve per group a 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 group standard deviations of 0.41 and a significance level (alpha) of 0.05 (two-sided two-sample t-test) the estimated group sample size is 23.



Supplemental figure 2. Comparison capillary density loss in South Asian patients with T2DM at baseline. Percentage difference in the number of perfused capillaries (capillary density) per capillary diameter group of total South Asian patients with T2DM in current study at baseline (n = 53) and from intermediate- and high-risk Framingham groups (n = 253 or 168, respectively) from the published NEO study [3] were compared to the low-risk Framingham group (as reference, n = 392).



Supplemental figure 3. Changes in linear regression slopes over time. Comparison of linear regression slopes with 95% confidence intervals of PBR_{static} plotted against red blood cell velocity (V_{RBC}) in feed vessels at each consecutive timepoint of (A) placebo, (B) diet (FMD) and (C) supplement (endocalyx) group. Comparison of changes between baseline, after intervention at 3 months and at month 6 (3 months after last intervention).

Supplemental table 1. Differences between baseline and after 6 months and intervention groups in treatment effects.

	Placebo			Diet			Supplement				
	Baseline (n=16)	6 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	6 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	6 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
Microvascular parameters											
Capillary density (4-6) (µm/mm²)	43 (6)	45 (6)	1 [-16;18]	32 (6)	35 (6)	4 [-14;21]	32 (5)	36 (5)	4 [-12;19]	0.81	0.80
Capillary blood volume static (pL/mm²) /10³ µm³	12.7 (1.7)	11.7 (1.5)	-1.0 [-5.7;3.7]	10.1 (1.7)	10.4 (1.6)	0.3 [-4.5;5.0]	10.0 (1.5)	11.0 (1.3)	1.0 [-3.3;5.2]	0.64	0.45
Capillary blood volume dynamic (pL/mm²) /10³ µm³	21.6 (2.5)	13.1 (1.9)	**-8.5 [-15.2;-1.8]**	11.8 (2.5)	17.3 (2.1)	5.5 [-1.2;12.2]	14.7 (2.3)	14.2 (1.7)	-0.5 [-6.6;5.5]	**0.001**	**0.03**
PBR static (µm)	2.08 (0.05)	2.24 (0.05)	**0.16 [0.01;0.31]**	2.26 (0.05)	2.23 (0.06)	-0.03 [-0.18;0.13]	2.21 (0.04)	2.17 (0.05)	-0.05 [-0.19;0.09]	**0.04**	**0.02**
PBR dynamic (µm)	2.19 (0.05)	2.27 (0.05)	0.08 [-0.07;0.24]	2.35 (0.05)	3.21 (0.05)	**0.86 [0.70;1.00]**	2.74 (0.04)	2.09 (0.04)	**-0.65 [-0.78;-0.51]**	**<0.01**	**<0.01**
MVHS dynamic (µm)	3.9 (0.5)	2.2 (0.3)	**-1.7 [-2.8;-0.5]**	2.1 (0.5)	2.1 (0.3)	0.1 [-1.1;1.2]	2.2 (0.4)	2.7 (0.3)	0.6 [-0.5;1.6]	**0.01**	**0.001**
Clinical parameters											
Systolic blood pressure (mmHg)	132 (4)	135 (3)	3 [-7;14]	141 (4)	131 (3)	-10 [-21;0]	133 (3)	130 (3)	-3 [-13;7]	**0.04**	0.30
Diastolic blood pressure (mmHg)	82 (2)	85 (2)	3 [-3;9]	81 (2)	78 (2)	-3 [-9;3]	80 (2)	80 (2)	-1 [-6;5]	0.06	0.27
BMI (kg/m²)	26.4 (1.3)	26.4 (1.3)	0 [-0.6;0.5]	28.3 (1.3)	27.8 (1.3)	**-0.6 [-1.2;0]**	30.2 (1.1)	30 (1.1)	-0.2 [-0.7;0.3]	0.12	0.61
Laboratory markers											
Fasting glucose (mmol/L)	7.3 (0.5)	7 (0.6)	-0.3 [-1.4;0.8]	7.2 (0.5)	7.6 (0.6)	0.4 [-0.8;1.6]	8.3 (0.4)	8.1 (0.5)	-0.1 [-1.1;0.9]	0.30	0.79

Supplemental table 1. Differences between baseline and after 6 months and intervention groups in treatment effects *(continued)*

	Placebo			Diet			Supplement				
	Baseline (n=16)	6 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	6 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	6 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
HbA1c (%) (mmol/mol)	55.6 (3.3)	56.9 (3.4)	1.3 [-3;5.6]	55.3 (3.4)	53.6 (3.6)	-1.8 [-6.3;2.8]	57.9 (2.9)	59.2 (3)	1.3 [-2.7;5.2]	0.23	0.98
C-peptide (nmol/L)	1.2 (0.1)	1.3 (0.2)	0.1 [-0.1;0.3]	1.4 (0.1)	1.5 (0.2)	0.1 [-0.2;0.3]	1.5 (0.1)	1.6 (0.1)	0.1 [-0.1;0.3]	0.83	0.97
eGFR CKD-EPI (ml/ min/1.73m^2)	86 (5)	84 (4)	-2 [-8;4]	83 (5)	84 (4)	1 [-5;7]	80 (4)	82 (4)	2 [-4;7]	0.42	0.27
Total cholesterol (mmol/L)	4.03 (0.23)	4.09 (0.23)	0.06 [-0.38;0.49]	4.34 (0.24)	4.31 (0.25)	-0.03 [-0.48;0.42]	4.35 (0.2)	4.28 (0.2)	-0.07 [-0.46;0.32]	0.73	0.59
HDL-cholesterol (mmol/L)	1.27 (0.08)	1.31 (0.09)	0.05 [-0.06;0.15]	1.22 (0.08)	1.24 (0.09)	0.02 [-0.09;0.13]	1.08 (0.07)	1.06 (0.07)	-0.02 [-0.11;0.08]	0.71	0.28
LDL-cholesterol (mmol/L)	2.34 (0.2)	2.40 (0.18)	0.06 [-0.31;0.44]	2.4 (0.21)	2.22 (0.20)	-0.18 [-0.57;0.21]	2.41 (0.18)	2.32 (0.16)	-0.09 [-0.44;0.26]	0.27	0.46
Triglycerides (mmol/L)	1.11 (0.5)	1.15 (0.33)	0.04 [-0.65;0.73]	1.65 (0.5)	1.84 (0.35)	0.19 [-0.5;0.88]	2.31 (0.45)	2.17 (0.29)	-0.14 [-0.77;0.49]	0.69	0.64
hsCRP (mg/L)	4.0 (1.1)	1.8 (1.2)	-2.3 [-4.9;0.3]	3.6 (1.1)	3.1 (1.3)	-0.5 [-3.1;2.2]	6.0 (1.0)	6.9 (1.0)	0.9 [-1.4;3.2]	0.23	**0.03**
IGF-1 (nmol/L)	18.3 (1.3)	19.6 (1.4)	1.3 [-1.5;4.2]	17.9 (1.4)	18.7 (1.5)	0.8 [-2.2;3.7]	17.1 (1.2)	17.5 (1.2)	0.4 [-2.2;2.9]	0.72	0.54

Abbreviations: *PBR* perfused boundary region, *MVHS* microvascular health score, *BMI* body mass index, *HbA1c* hemoglobin A1c, *eGFR CKD-EPI* estimated glomerular filtration rate according to chronic kidney disease epidemiology collaboration, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *hsCRP* high sensitivity c-reactive protein, *IGF-1* Insulin growth factor 1, *ns* non-significant; Estimated marginal means with standard error

[1]Linear mixed models adjusted for age, sex, microvascular history at baseline, macrovascular history at baseline and hypertension at baseline with Bonferroni post-hoc, baseline compared to 6 months, [2]interaction term of intervention group with measurement over time (Baseline-6 months), p<0.05 is considered significant (bold).

Supplemental table 2. Diabetes Treatment Satisfaction Questionnaire

	Placebo	Diet	p-value[1]
Baseline – DTSQ static	n=15	n=17	
Treatment satisfaction score	30.2 (4.4)	30.3 (5.0)	ns
Perceived frequency of hyperglycaemia	2.9 (2.2)	2.9 (1.7)	ns
Perceived frequency of hypoglycaemia	1.6 (2.0)	1.8 (2.0)	ns
Month 3 – DTSQ change	n=15	n=13	
Treatment satisfaction score	10.1 (7.5)	11.3 (4.5)	ns
Perceived frequency of hyperglycaemia	0.1 (1.5)	-1.0 (1.5)	ns
Perceived frequency of hypoglycaemia	0.1 (1.8)	-0.5 (2.0)	ns

Abbreviations: *DTSQ* Diabetes Treatment Satisfaction Questionnaire, *ns* non-significant

[1]Unpaired t-test, P<0.05 is considered significant. 1 missing baseline in both diet and placebo group.

Supplemental table 3. Differences between baseline and after 6 months and intervention groups in treatment effects

	Placebo			Diet			Supplement				
	Baseline (n=16)	6 months (n=15)	Difference [95% CI][1]	Baseline (n=18)	6 months (n=13)	Difference [95% CI][1]	Baseline (n=19)	6 months (n=17)	Difference [95% CI][1]	Treatment effect diet[2]	Treatment effect supplement[2]
Glycocalyx plasma markers											
HPSE-1 activity. plasma (U/mL)	1.04 (0.16)	0.85 (0.16)	-0.19 [-0.72;0.34]	1.33 (0.17)	1.09 (0.18)	-0.24 [-0.78;0.30]	1.27 (0.14)	1.23 (0.15)	-0.03 [-0.52;0.45]	0.87	0.58
HYAL-1 activity (U/mL)	0.56 (0.02)	0.54 (0.02)	**-0.02 [-0.06;-0.02]**	N/A	N/A	N/A	0.48 (0.02)	0.47 (0.02)	**-0.01 [-0.05;-0.02]**	N/A	0.65
HYAL-4 activity (U/mL)	25.1 (2.92)	21.33 (2.64)	-3.77 [-8.39;0.85]	N/A	N/A	N/A	17.82 (2.51)	15.86 (2.26)	-1.96 [-6.16;2.24]	N/A	0.47
HYAL-4 protein (ng/mL)	3.45 (0.41)	3.27 (0.39)	-0.18 [-0.46;0.09]	N/A	N/A	N/A	2.79 (0.35)	2.67 (0.33)	-0.11 [-0.36;0.14]	N/A	0.71
ANG-2 protein (ng/mL)	2.47 (0.24)	2.57 (0.27)	0.11 [-0.34;0.56]	2.77 (0.24)	2.94 (0.28)	0.17 [-0.29;0.64]	2.82 (0.21)	3.19 (0.24)	0.36 [-0.05;0.77]	0.81	0.30
sTM protein (ng/mL)	5.13 (0.60)	4.29 (0.61)	**-0.83 [-1.49;-0.17]**	5.37 (0.62)	4.66 (0.64)	**-0.71 [-1.38;-0.04]**	6.57 (0.52)	5.49 (0.53)	**-1.08 [-1.68;-0.48]**	0.74	0.49
Urinary markers											
ACR. urine (mg/mmol)	3.1 (2.1)	2.8 (0.7)	-0.3 [-5.1;4.6]	2.7 (2.0)	1 (0.8)	-1.7 [-6.3;2.9]	5.0 (1.9)	2.1 (0.6)	-2.9 [-7.4;1.5]	0.59	0.31
HPSE-1 activity. urine (U/mL)	0.96 (0.19)	0.55 (0.11)	**-0.41 [-0.83;-0.01]**	1.19 (0.21)	0.67 (0.13)	**-0.52 [-095;-0.07]**	0.87 (0.17)	0.60 (0.09)	-0.28 [-0.65;0.09]	0.68	0.54
MCP-1 activity. urine (ng/mmol)	22.3 (9.7)	20.6 (4.7)	-1.7 [-23.7;20.4]	36.6 (9.3)	28.2 (5.3)	-8.5 [-29.6;12.7]	39.3 (8.7)	25.9 (4.1)	-13.4 [-33.4;6.7]	0.59	0.34

Abbreviations: *HPSE-1* heparanase-1, *HYAL-1* hyaluronidase-*1, HYAL-4* hyaluronidase-4, *ANG-2* Angiopotein-2, *sTM* soluble thrombomodulin, *ACR* albumin creatinine ratio, *MCP-1* monocyte chemoattractant protein 1, *N/A* not applicable, *ns* non-significant; Estimated marginal means with standard error

[1]Linear mixed models adjusted for age, sex, microvascular history at baseline, macrovascular history at baseline and hypertension at baseline with Bonferroni post-hoc, baseline compared to 6 months, [2]interaction term of intervention group with measurement over time (Baseline-6 months), p<0.05 is considered significant (bold).

CHAPTER 5

Supplementation with Endocalyx preserves glomerular endothelial glycocalyx and capillary stability in experimental diabetes

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Abstract

Damage to the glomerular endothelial glycocalyx is one of the various mechanisms in diabetic nephropathy. Over the years, restoring the glomerular glycocalyx has become an interesting therapeutic target, as current treatments are insufficient to slow down progression to end-stage renal disease. In the current study we investigated whether dietary Endocalyx™ suplementation (fucoidan, other glycocalyx constituents and antioxidants) is able to preserve the glomerular endothelial glycocalyx, reduce heparanase-1 (HPSE-1) activity, and affect renal myeloid cells in 8-week-old ApoE-KO mice rendered diabetic through repeated intraperitoneal streptozotocin (60mg/kg) injections with free access to cholesterol-enriched (0.15%) chow. To avoid major metabolic changes, blood glucose was kept between 15-20mmol/L. From week 12 on, mice received supplementation with Endocalyx™. Ten weeks of dietary supplementation with Endocalyx™ in diabetic mice prevented glomerular capillary damage and preserved endothelial glycocalyx coverage of both heparan sulfates and hyaluronan. While a direct HPSE-1 inactivation effect was found *in vitro*, we only observed the heparan sulfate preservation *in vivo*. Preservation was accompanied with a reduced % of CD11b positive renal cortical macrophages and dendritic cells without an effect on the major resident myeloid cell population, macrophage-like dendritic cells.

Introduction

Endothelial cell dysfunction has been implicated as a major contributor in the pathophysiology of diabetic nephropathy (DN) [1, 2]. Chronic exposure to the inflammatory diabetic environment due to circulating advanced glycation end products, reactive oxygen species (ROS) and inflammatory cytokines alters the composition and integrity of the glomerular endothelial glycocalyx. This complex network of glycoproteins, plasma proteins, proteoglycans and glycosaminoglycans maintains an important negatively charged molecular sieve as part of the glomerular filtration barrier [3]. The glycocalyx composition is highly dynamic, with a balance between biosynthesis and shedding of its constituents [4]. However, in a number of pathological conditions such as sepsis or diabetes, the composition of the endothelial glycocalyx can be altered and loses its complex structure and functional properties [5-7]. Several novel treatment strategies for DN have been developed to inhibit the influx of macrophages or glycocalyx degrading enzymes. First, by inhibiting monocyte chemoattractant protein-1 (MCP-1) activity, second by reducing heparanase-1 (HPSE-1) activity, or third by supplementing glycocalyx substituents [8-11]. Therefore, restoring the structure of the glomerular glycocalyx has become an attractive therapeutic target to improve vascular health and prevent disease progression in DN.

In the present study we investigated whether dietary supplementation with glycocalyx mimetics combined in Endocalyx™, containing fucoidan extracted from *Laminaria japonica* as the main compound with added glucosamine sulphate, hyaluronic acid, a blend of superoxide dismutase, catalase and polyphenols is able to restore the glomerular glycocalyx and reduce HPSE-1 activity in a diabetic mouse model. While glucosamine sulphate could stimulate glycocalyx restoration, by increasing N-acetyl glucosamine (GlcNAc)-driven GAG synthesis [12], it is believed that the sulphated polysaccharides in low molecular weight fucoidan with chemical structural properties analogous to HS and CS will act as a heparan sulphate mimetic [13-15]. The distinct nature of this complex sulfated polysaccharide with a backbone of primarily (1 → 3)-linked α-L-fucopyranose residues and branches of 3-linked α-L-fucopyranose residues by β-D-galactopyranose and 3-linked α-L-fucopyranose residues by non-reducing terminal fucose units [16] was further investigated *in vitro* on endothelial glycocalyx restoration [14] and reducing HPSE-1 activity and an *in vivo* experimental glomerulonephritis study [15].

Materials and Methods

Cell culture and treatment

Murine glomerular endothelial cells (mGEnCs) were cultured as described previously [17]. Human Renal Glomerular Endothelial Cells (HRGECs, ScienCell, Uden, The Netherlands)

were cultured on fibronectin (Promocell, Heidelberg, Germany) coated flasks (COSTAR, Amsterdam, The Netherlands) between passage 2-5 with Endothelial Cell Medium (1001, ScienCell) supplemented with 5% FBS (0025, ScienCell), 1% Endothelial Cell Growth Supplement (1052, ScienCell) and 1% Penicillin/Streptomycin (0503, ScienCell) in 5% CO_2 at 37^{o}C [18]. Where indicated, cells were treated with 1 µg/mL LPS O111:B4 (Sigma, Houten, The Netherlands, Cat# L2630) and/or 1-2 µg/mL *L. japonica* fucoidan (≥90%, gift from MicroVascular Health Solutions) for 12-24 hours.

HS release competition ELISA and competition ELISAs to detect Fucoidan

Nunc MaxiSorp™ 96- well ELISA plates (Thermo Scientific) were coated with either 10 µg/mL heparan sulphate from bovine kidney (Sigma) or chondroitin sulphate-A (CS-A) (Iduron) in coating buffer overnight at room temperature in a humidified chamber. Plates were washed 5x with PBS/0.05% Tween20 (PBST) and blocked with 1% bovine serum albumin (Sigma) for 2 hours. 0-4 µg/mL fucoidan (gift from MicroVascular Health Solutions LLC, Alpine, UT, USA) was pre-incubated with glycosaminoglycan antibodies: anti-HS (F58-10E4) (1:100, Amsbio), anti-CS single chain fragment variable (ScFv) I03H10 (1:100), anti-HS (JM403) (1:5000, Amsbio) for 1 hour. Fucoidan/antibody mixtures were then incubated with glycosaminoglycan-coated ELISA plates for 1 hour. Plates were washed 5x PBST and incubated with secondary antibodies: goat anti-mouse IgM-HRP (1:5000, Southern Biotech), mouse anti-VSV-G-peroxidase (1:2000, Sigma). Plates were washed 5x PBST and colour was developed with 1x tetramethylbenzidine ELISA substrate solution (Invitrogen). Absorption was measured at 450 nm.

Immunocytochemistry

Cells were cultured in Nunc™ Lab-Tek™ Flask on Slide chambers (170920, Thermo Scientific, Landsmeer, The Netherlands) until confluent, then treated with LPS for 24 hours. Selected cultures were also subjected to fucoidan treatment for the final 12 hours of LPS stimulation. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 mins at RT. Fixed slides were washed five times with PBS and then blocked with 0.2% BSA for 1 hour at RT. Appropriate sections were incubated with primary mouse anti-rat IgM HS antibody JM403 (1.3 µg/mL) diluted in 0.2% BSA, followed by Alexa Fluor IgM-488κ (1:500, Life Technologies, Bleiswijk, The Netherlands) for 1h each time. Slides were washed between antibodies three times for 10 mins in PBST. Slides were mounted in Vectorshield mounting medium H-1000 with DAPI (Vector Labs Inc., Burlingame, CA, USA) and sealed with a glass coverslip. Images were collected using a Zeiss Axio imager M1 (Zeiss, Breda, The Netherlands) immunofluorescence microscope.

Diabetic apoE KO mouse model

Six-week-old male B6.129P2-*Apoe*^{tm1Unc}/J mice (ApoE-KO; The Jackson Laboratory, Bar Harbor, ME) were rendered diabetic through intraperitoneal injections of 60mg/kg strep-

tozotocin (STZ; Sigma-Aldrich, St. Louis, MO) for 5 consecutive days, as described before [11] (supplemental figure 1A). Control ApoE-KO mice were injected with citrate buffer alone and were used for baseline measurements. All mice had free access to standard rodent diet (NC; Ssniff Spezialdiäten GmbH, Soest, Germany). At week 8, diabetic mice were fed cholesterol enriched (0.15%) chow until the end of the study. At week 11, diabetic mice were randomized into two groups; a diabetic group (diabetes) and a diabetic group that received Endocalyx™ supplementation (US patent # 9943572; MicroVascular Health Solutions LLC, Alpine, UT, USA) starting at week 12. Estimated daily dosage calculations of Endocalyx™ supplement (Endocalyx) per animal (in mg) is: fucoidan sulphate 0.12, hyaluronic acid 0.02, glucosamine-SO_4 0.43 and oxxynea OMD-SOD 0.14 which relates to the recommended daily dose in humans of: 425-, 70-, 1500- and 480mg, respectively. Blood glucose concentrations by tail-tip blood droplets were measured with an Accu-check glucose meter (Roche, Basel, Switzerland). When glucose concentrations exceeded 20 mmol/L, mice were treated with 1–2 units insulin (Lantus, Aventis Pharmaceuticals, Bridgewater, NJ, US) up to three times per week. Mice were sacrificed at week 22 of age. Animal experiments were approved by the Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (permit no. AVD1160020172926). All work with animals was performed in compliance with the Dutch government's guidelines.

Urine collection and analysis

Mice were weighted before and after residing in the metabolic cage (Techniplast S.p.a, Buguggiate, Italy) and water- and food intake, and urine were collected. After acclimatization, 14hrs-urine was collected at week 11, -17 and -21. Urine samples were centrifuged to remove debris and stored at -20°C. Urinary albumin concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories, Inc. Montgomery, TX, USA) and creatinine concentrations were quantified by the Jaffe´ method using 0.13% picric acid and a creatinine standard set (Sigma-Aldrich, Merck Life Science NV, Amsterdam, The Netherlands). Urine MCP-1 activity was measured with an immunoassay according to the manufacturer protocol (R&D Systems Europe, Ltd., Abingdon, UK). Urinary HPSE-1 activity was measured (Takara Bio Inc., Shiga, Japan). Urine samples were run through Zeba™ Spin Desalting Columns (ThermoFisher Inc., Waltham, MA, USA) for removal of salts and other small molecules (<1000 MW) before HPSE-1 activity detection. Excretion of urinary kidney injury molecule-1 (KIM-1) was determined with an ELISA kit (R&D Systems, Minneapolis, MN, USA). Optical densities for albumin, creatinine, MCP-1, HPSE-1 activity and KIM-1 were measured with an ELISA plate reader.

Tissue preparation and histology

Mice were anesthetized by isoflurane inhalation and perfused via the left ventricle with HEPES-buffered salt solution containing 0.5% bovine serum albumin and 5 U/mL heparin to remove blood. After removal of the capsules, one half of the right kidney was fixed in

paraformaldehyde (PFA) solution (4%) for 1 to 2 hours and processed further for endothelial glycocalyx coverage while the other half was frozen in liquid N_2 and stored at -80°C. Both halves of the left kidney were placed in 2% PFA in PBS overnight at 4°C, followed by paraffin embedding for periodic acid-Schiff (PAS), methenamine silver-periodic acid-Schiff (MPAS) or immunofluorescence staining.

A subset of mice (n = 3/group) were anesthetized, the abdominal aorta was exposed and cannulated adjacent to the left renal artery. The right renal artery was ligated at the renal stalk after which the left kidney was perfused with 5mL Hanks-buffered salt solution (HBSS, Gibco) containing 0.5% BSA (Sigma, A7030, essentially globulin free) and 5IU/mL heparin at 2mL/minute to remove blood, followed by 2mL of cationic ferritin (horse spleen, 2.5mg/mL, Electron Microscopy Sciences, Fort Washington, PA) in HBSS at 2mL/minute. The left kidney was excised, its capsule removed, and stored in fixative, 1.5% glutaraldehyde (GA) and 1% PFA (both from Electron Microscopy Sciences, Hatfield, PA) in 0.1M sodium-cacodylate buffered solution (pH 7.4), overnight at 4°C for further processing for transmission electron microscopy (TEM). Of another subset of mice, the left kidney was placed in PBS on ice for single cell isolation for FACScan analysis.

Glomerular endothelial coverage

Glomerular endothelial glycocalyx coverage was determined using fluorescently labelled lectin Lycopersicon esculentum (LEA-FITC) and the N terminus rat neurocan construct of the HA-specific neurocan-dsRed (Ncan-dsRed) construct, as described previously [2, 3]. In short, overnight PFA fixed tissue was subsequently sectioned in 100µm thick slices with a Leica VT1000S vibratome (n = 3/group) and submerged in HBSS (Life Technologies Europe BV, Bleiswijk, The Netherlands) containing 0.5% BSA, 5 mmol/L HEPES, and 0.03 mmol/L EDTA (HBSS-BSA). Slices were incubated with 10µg/mL of fluorescently labeled Lycopersicon esculentum (LEA) or Ncan-dsRed [2] to visualize the glycocalyx, together with 5µg/mL monoclonal mouse anti-mouse CD31 antibody (Ab28364, Abcam, Cambridge, MA) to identify the endothelial cell membrane, overnight at 4°C on a shaker (in dark). After 3 washes with HBSS-BSA slices were incubated for 2 hrs with 10µg/mL Alexa Fluor-568, or AF488–conjugated goat anti-mouse IgG (Molecular Probes, Grand Island, NY) and Hoechst 33528 (Sigma-Aldrich, 1:2000) at 4°C on shaker (in dark). Slices in HBSS-BSA were fixated between glass slide and coverslip in mounting medium and imaged on a LEICA TCS SP8 X WLL microscope (Leica, Rijswijk, The Netherlands) and a 40x objective (HC PL APO CS2 40x/1.30 OIL, Leica). Sequential 16-bit confocal images (xyz dimensions, 0.142 x 0.142 x 0.3 µm) were recorded using LAS-X Image software (Leica). The endothelial glycocalyx was quantified by calculating the distance from the peak of the CD31 signal to the half-width of the intraluminal lectin signal along a line of interest, using intensity profiles (ImageJ).

Cationic ferritin determination with TEM

Cationic ferritin perfused tissue, stored in fixative, 1.5% GA and 1% PFA in 0.1M sodium-cacodylate buffered solution, was subsequently sectioned in 180µm thick slices with a Leica VT1000S vibratome, rinsed 2x with 0.1M sodium cacodylate-buffered solution, and post-fixated for 1hr with 1% osmium tetroxide (Electron Microscopy Sciences) and 1.5% potassium ferrocyanide in demineralized water [2, 3]. Samples were further washed, dehydrated in ethanol, infiltrated with a mixture of epon LX-112 and propylene oxide (1:1) for 1 hr, followed by pure epon for 2hrs, embedded in epon mounted in BEEM capsules (Agar Scientific, Essex, United Kingdom) and polymerized for 48hrs at 60°C. 100nm Thick sections were cut using a diamond knife (Diatome, Biel, Switzerland), collected on single slot copper grids covered with formvar film and carbon layer, and then stained with 7% uranyl acetate in demineralized water for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Data was collected at an acceleration voltage of 120kV on a Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM), equipped with an FEI 4k Eagle CCD camera. Virtual slides were acquired with 18,500x magnification at the detector plane, corresponding to a 1.2nm pixel size at the specimen level. Representative capillary sections of each recorded glomerulus (n = 1/group) from virtual slides were selected for high resolution imaging.

Immunohistochemistry

Deparaffinized kidney sections (4µm thick) were washed in PBS and antigen retrieval was performed in a citrate buffer (Dako S1699, pH 6.0) in an autoclave, blocked with serum-free protein block (Dako X0909) for 1 hour at room temperature. Sections were incubated overnight at 4°C with primary mouse anti-HS antibodies (clone JM403, AMS-Biotechnology, Bioggio-Lugano, Switzerland) or with fluorescently labeled Lycopersicon esculentum (LEA-FITC), in combination with mouse pan-endothelial cell marker (BD Biosciences, San Jose, CA), followed by corresponding fluorescent-labelled secondary antibodies for 1 hour at 4°C, all in blocking buffer. Another set of sections were incubated overnight at 4°C with primary polyclonal rabbit antibodies against HPSE-1 (InSight Biopharmaceuticals, Rehovot, Israel) in combination with rat monoclonal antibodies against mouse F4/80 (ab6640, Abcam, Cambridge, MA) and polyclonal goat antibodies against mouse nephrin (AF3159, R&D Systems), followed by corresponding fluorescent-labelled secondary antibodies for 1 hour at 4°C, all in blocking buffer. Slides were embedded in Prolong™ gold antifade mountant with DAPI (P36931, Thermofisher). Fluorescent images of the slides were recorded using a 3D Histech Pannoramic MIDI Scanner (Sysmex, Etten-Leur, the Netherlands). Annotated glomeruli (± 50/sample) were exported for further analysis in the public domain NIH ImageJ software (http://rsb.info.nih.gov/ij). Fluorescence of the exported 16-bit images were analysed using a macro using the auto threshold function based on the isodata algorithm [19] to generate glomerular surface area, mean fluorescence and % positive

area which are used to calculate total glomerular fluorescence (surface area x (% positive area/100)) x mean fluorescence (mean FL x μm^2).

Single cell isolation for FACscan

Left kidneys placed in PBS on ice for FACScan analysis were cut in small pieces and placed in a 6-well plate containing 1mL of collagenase type 1A (1.6mg/mL) and DNAse (60U/mL) in sterile H_2O and incubated for 30 min at 37°C (5% CO_2 incubator with intermittent agitation). Tissue, including liquid, was transferred on a 100µm cell strainer, placed on a 50 mL tube and was passed through the strainer using the plunger of a 2.5 ml syringe to crush the tissue further and cell strainer is flushed twice with 10mL DPBS at rT. The fall through is transferred to a 40µm cell strainer and passage is repeated, followed by centrifugation for 5 min at 1500rpm (rT). Supernatant was discarded and cells were suspended in 5mL ice-cold shock buffer and incubated for 3-4 min on ice. At least 5mL ice-cold DPBS was added, followed by centrifugation for 5 min at 1500rpm (4°C). Supernatant was discarded cells were suspended in FACS buffer on ice.

Staining protocol for FACScan

Appropriate volumes of adjusted cells (equivalent to 1.0 x 10^6cells) were pipetted into each tube and appropriate volume of the conjugated antibodies directed to the cell surface marker(s) of interest were added. Per sample 4 cocktails were used (supplemental table 1). Each tube was vortexed gently to mix cells with antibodies, and incubated for 15 minutes in the dark at rT. Reagent A (100µL 1% PFA solution) was added and incubated for 15 minutes at rT, washed once in 1mL FACS buffer (FB; 1% BSA 0.01% NaAz in PBS). Centrifuged for 5 minutes at 5000 rpm, supernatant was aspirated and vortexed to fully resuspend the cell pellet. Reagent B (100 µl Permeabilization Medium) was added together with recommended volume of conjugated intracellular antibodies. Vortexed for 1-2 seconds and incubated for 20 minutes, washed once in 1 mL FB. Centrifuged for 5 minutes at 5000 rpm and supernatant was aspirated. Cells were resuspended in 200µL of 0.1% PFA fixative solution for storage at 2-8°C in the dark and analyzed within 24 hours. Samples were measured on a Cytek Aurora 5 flow cytometer (Cytek Biosciences B.V., Amsterdam, The Netherlands), according to manufacturer's guidelines. Characterization of HPSE-1 and cathepsin L positive kidney myeloid cells was based on the antibody cocktails as shown in supplemental table 1. Gating strategy after life/dead discrimination and gating for single cell as shown in supplemental figure 2. In short, first cells positive for CD11b were gated and selected on cell size. Next, MHC-II positive cells were selected (excluding, e.g. naïve monocytes, NK cells, neutrophils and basophils), finishing with selection of the myeloid cell-types based on CD11c and F4/80 expression, and CD370 and Cx3CR1 expression: macrophages (Mϕ; $CD11c^{neg}$/$F4\text{-}80^{hi}$/MHC II^{lo}/$CD11b^{lo}$/$CD370^{lo}$/$CX3CR1^{hi}$), dendritic cells (DC) switched to a macrophage-like phenotype [20] (DC/Mϕ-like; $CD11c^{lo}$/$F4\text{-}80^{hi}$/

MHC II^{hi}/CD11b^{lo}/CD370^{hi}/CX3CR1^{hi}) and DCs (CD11c^{hi}/F4-80^{neg}/MHC II^{hi}/CD11b^{hi}/CD370^{hi}/CX3CR1^{lo}).

Statistical analysis

Data are presented as means with standard deviation (SD). Urine production, albumin-to-creatinine ratio (ACR) and urinary HPSE-1 activity during treatment were analyzed by using linear mixed models with Bonferroni post hoc test. This takes into account that samples over time from the same animal are not independent. Differences per timepoint between groups were analyzed by ANOVA with Tukey's post-hoc test. The ACR and urinary HPSE-1 activity were log-transformed before the ANOVA and linear mixed model analysis.

Differences in other experiments were determined using analysis of variance and post hoc analyses with Tukey's multiple comparison test. Comparison of expression between two different groups was evaluated using a t-test. Statistical analyses were performed using SPSS statistical software version 25 (SPSS Inc., Chicago, IL) and GraphPad Prism version 8 (GraphPad Inc., La Jolla, CA). A significance level of 0.05 was considered statistically significant.

Results

Fucoidan from *Laminaria japonica* inhibits heparanase activity *in vitro*

First, we tested the possible HPSE-1 inhibitory effects of fucoidan, one of the main components of the Endocalyx^{TM} supplement. Previously it has been shown that orally administered fucoidan, through intestinal uptake, in healthy volunteers can be detected in blood with peak levels around 50ng/mL (intake of 1 gram) at 6 hours in its unchanged form, while at 9 hours, fractions can be observed in urine samples [21, 22]. To measure fucoidan levels, we evaluated anti-HS antibodies i.e. F58-10E4 and JM403, respectively [10], and anti-CS antibody via IO3H10 [23] for binding to fucoidan. Anti-HS antibody binding of clone F58-10E4 to fucoidan resulted in a binding ratio of 29.5ng per ng antibody and for the anti-CS antibody, clone IO3H10, of 0.04ng per ng of antibody (supplemental figure 2A-F and supplemental table 1). No binding of the anti-HS antibody clone JM403 to fucoidan was observed (supplemental figure 2G and supplemental table 1). Therefore, we used this antibody to evaluate the inhibitory effect of fucoidan on recHPSE-1 activity. Results revealed a concentration dependent reduction up to about 90% by 10ng/mL fucoidan (figure 1A). In LPS activated murine glomerular endothelial cells (mGEnCs) active HPSE-1 release could be inhibited in the presence of 10ng/mL fucoidan (figure 1B). In addition, fucoidan was also able to protect the endothelial glycocalyx by reducing the release of HS into the extracellular milieu in both murine and human derived glomerular endothelial cells (figure 1C, D).

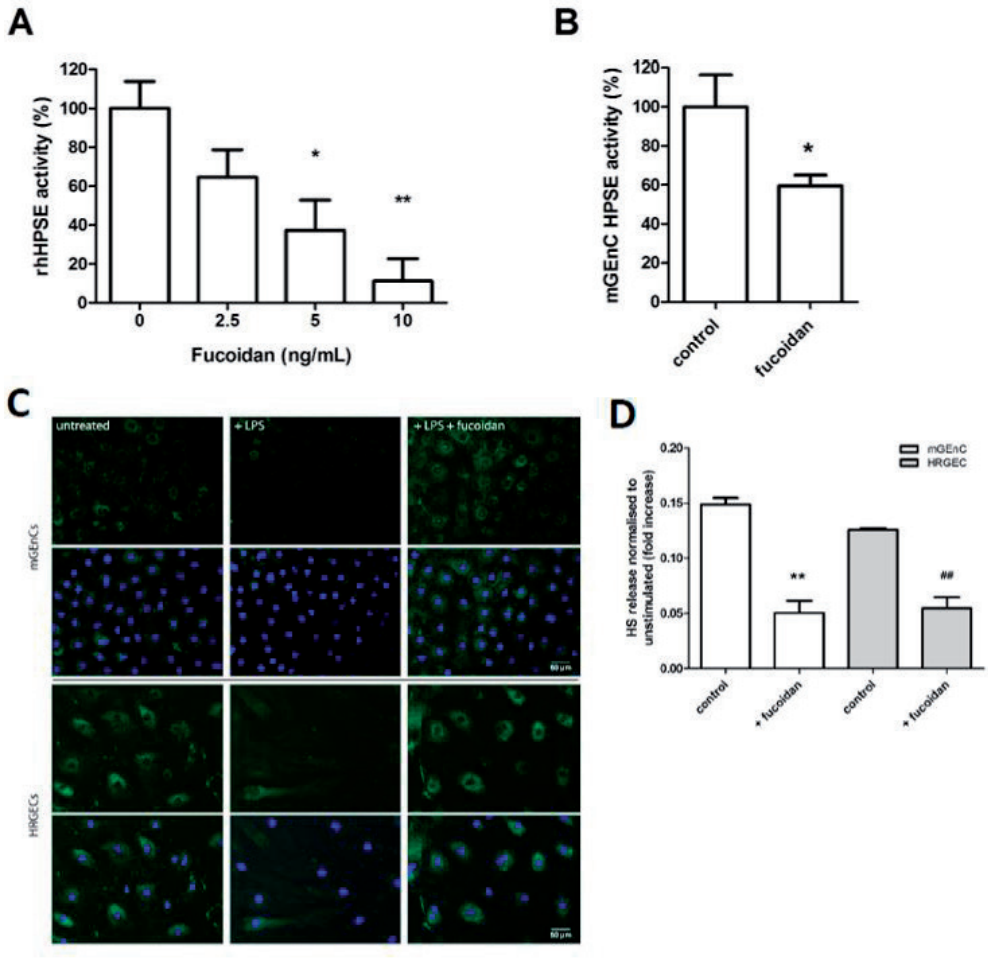


Figure 1. Fucoidan inhibits HPSE-1 activity and protects endothelial surface glycocalyx. (A) Dose-dependent inhibition of recombinant HPSE-1 (rhHPSE-1). rhHPSE activity in presence of 0-10 ng/mL fucoidan was measured by an indirect ELISA. Data significance was analyzed using a one-way ANOVA with Dunnett's post-hoc comparison. *P < 0.05, **P < 0.01 (B) Mouse glomerular endothelial cells (mGEnCs) were treated with LPS to induce the release of active HPSE into the media. Medium HPSE-1 activity was measured in presence and absence of 10 ng/mL fucoidan (n=3). Data significance was analyzed using an unpaired t-test. *P < 0.05 Loss of endothelial heparan sulfate (HS) glycocalyx was measured (C) via immunocytochemistry using anti-HS JM403 and (D) by competition ELISA of HS release into cell media in both mGEnCs and human renal glomerular EC (HRGECs). In the presence of 10 ng/mL fucoidan, endothelial HS glycocalyx was preserved and release of HS was significantly reduced (n= 3). JM403, green; DAPI, blue. Data significance was analyzed using an unpaired t-test. **P < 0.01, LPS-stimulated mGEnCs + fucoidan vs. LPS-stimulated mGEnCs, $^{\#\#}P$ < 0.01, LPS-stimulated HRGECs + fucoidan vs. LPS-stimulated HRGECs.

Diabetes induced ApoE-KO mice

At week 11 (before randomization in diabetes or Endocalyx™ groups), diabetic mice manifested with hyperglycemia, weight loss, polyuria, albuminuria and increased urinary HPSE-1 activity levels (supplemental figure 1B,C). Following the intervention period (week 12-22), high blood glucose levels in diabetic mice were maintained at preferred set glucose levels (15-20 mmol/L) through regular insulin administration to prevent glucotoxicity and a metabolic shift to overt lipid use for energy in these animals (supplemental figure 1D).

Supplementation of glycocalyx mimetics protects the glomerular glycocalyx

We investigated whether 10 weeks of Endocalyx™ supplementation could preserve the glomerular glycocalyx by staining PFA fixed renal sections directly with the fluorescently labelled lectin *Lycopersicon esculentum* (LEA-FITC) (figure 2A). Diabetes reduced the intraluminal endothelial surface lectin thickness, and coverage, to 0.157 µm, compared to 0.272 µm in control mice (difference 0.117 µm 95% CI 0.059 - 0.175) (figure 2B,C). Endocalyx™ supplementation during the 10-week intervention prevented this glycocalyx degradation revealing a 0.227 µm thick layer (difference 0.070 µm compared to diabetes group, 95% CI 0.012 - 0.128). Similarly, staining specifically for hyaluronan, using Ncan-dsRed, revealed protection upon supplementation with Endocalyx™ (figure 2D-F). This endothelial surface layer preservation was further confirmed by visualizing glomerular intraluminal endothelial cationic ferritin coverage (figure 2G). Cationic ferritin was present at the luminal endothelial cell surface, within the fenestrae and underneath the endothelium. In contrast, disruption of the cationic ferritin coverage alongside the luminal endothelial cell surface could be seen in the glomerulus of diabetic mice (figure 2G, middle panel). Total glomerular tuft staining on paraffin embedded sections with LEA-FITC revealed no significant differences in expression between control and both diabetic groups (with or without Endocalyx™), which was corroborated by glomerular tuft heparan sulfate detection using JM403 antibodies (figure 2H,I).

Endocalyx™ supplementation prevents glomerular capillary rarefaction

STZ induction of diabetes combined with a cholesterol-enriched diet (0.15%), resulted in minimal glomerular changes after 16 weeks, as shown by PAS and MPAS stained examples (figure 3A) but a significant reduced number of capillaries per glomerulus in diabetic mice compared to controls (difference of -4.2 95% CI -5.8 - -2.6) was observed (figure 3B). The number of capillaries per glomerulus in diabetic mice supplemented with Endocalyx™ remained equal compared to control (difference of 2.9 95% CI 1.4 - 4.5). No changes were observed in capillary surface- and mesangial surface area (figures 3C,D).

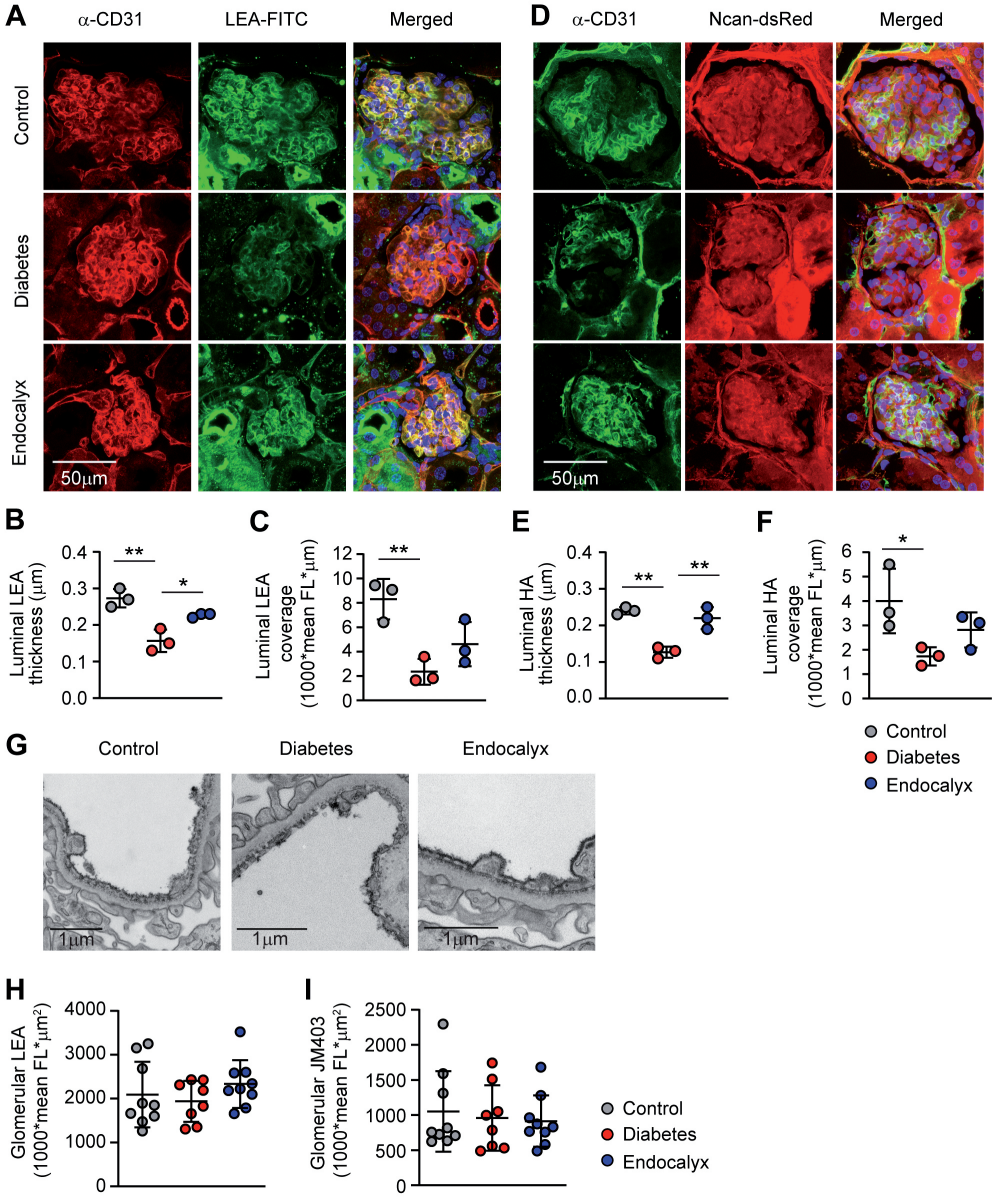

A
α-CD31
LEA-FITC
Merged
Control
Diabetes
Endocalyx
50μm
D
α-CD31
Ncan-dsRed
Merged
50μm
B
Luminal LEA thickness (μm)
C
Luminal LEA coverage (1000*mean FL*μm)
E
Luminal HA thickness (μm)
F
Luminal HA coverage (1000*mean FL*μm)
Control
Diabetes
Endocalyx
G
Control
Diabetes
Endocalyx
1μm
H
Glomerular LEA (1000*mean FL*μm²)
I
Glomerular JM403 (1000*mean FL*μm²)
Control
Diabetes
Endocalyx

Figure 2. Glomerular endothelial glycocalyx coverage (A) Representative images of direct glycocalyx staining using fluorescent labeled lectin *Lycopersicon esculentum* (LEA-FITC) and anti-CD31 antibodies for endothelial cell detection; scale bar 50µm. Reduction of **(B)** the luminal glycocalyx (LEA) thickness and **(C)** luminal glycocalyx (LEA) coverage, assessed in a subset of n = 3 control- (grey), diabetic- (red) and diabetic ApoE-KO mice after Endocalyx intervention (blue). **(D)** Representative images of direct glycocalyx staining using fluorescent labeled neurocan (Ncan-dsRed) and anti-CD31 antibodies for endothelial cell detection; scale bar 50µm. Reduction of **(E)** the luminal glycocalyx (Ncan-dsRed) thickness and **(F)** luminal glycocalyx (Ncan-dsRed) coverage, assessed in a subset of n = 3 control- (grey), diabetic- (red) and diabetic ApoE-KO mice after Endocalyx intervention (blue). **(G)** Representative transmission electron micrographs of cationic ferritin-stained glomerular endothelial surfaces in control-, diabetic- apoE-KO and Endocalyx intervention; scale bars 1µm. Quantification of total glomerular immunofluorescence staining of **(H)** lectin *Lycopersicon esculentum* (LEA-FITC) and **(I)** heparan sulfate (clone JM403). Analysis was performed on ± 50 glomeruli per sample in control- (n = 9), diabetic- (n = 8) and diabetic apoE-KO mice after Endocalyx intervention (n = 9). Values are given as mean with standard deviation. Differences between groups were assessed by ANOVA with Tukey's post-hoc test: $*P < 0.05$, $**P < 0.01$.

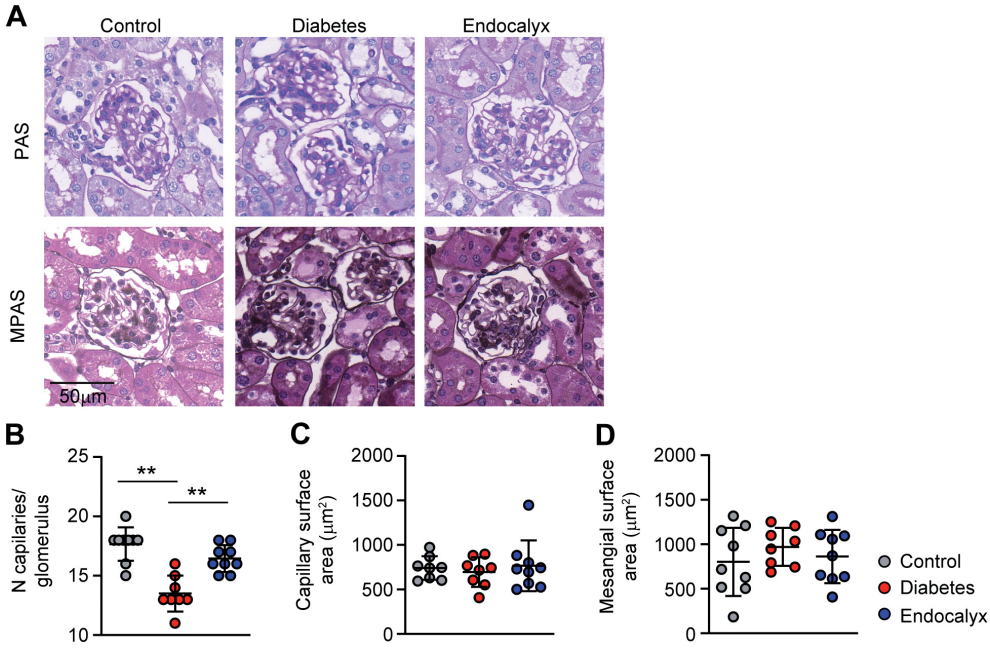


Figure 3. Histological glomerular changes. **(A)** Representative images of periodic acid–Schiff (PAS, top) and (methenamine silver-periodic acid-Schiff (MPAS, bottom) stained glomeruli of control- (left), diabetic- (middle) and diabetic apoE-KO mice with Endocalyx™ supplement (right); scale bar, 50 µm. Quantification of **(B)** number of capillaries per glomerulus, **(C)** glomerular capillary- and **(D)** mesangial surface area. Analysis was performed on ± 50 glomeruli per sample in control- (grey, n = 9), diabetic- (red, n = 8) and Endocalyx intervention (blue, n = 9). Values are given as mean with standard deviation (SD). Differences between groups were assessed by ANOVA with Tukey's post-hoc test: $^{**}P < 0.01$.

Urinary markers and renal HPSE-1 staining after Endocalyx™ supplementation

While at week 11 diabetic mice had elevated albumin-to-creatine ratios (ACR), throughout the rest of the experiments, in the glucose controlled setup, ACR tended to decline over time in parallel to urine production in both diabetic groups (figure 4A). This tendency precluded identifying an isolated effect of glycocalyx restoration on albuminuria. Meanwhile, no differences in urinary HPSE-1 activity were observed during the intervention period, as exemplified by similar trends in both groups between week 11 and 21 (figure 4B). In addition, urinary KIM-1 and MCP-1 levels did not change or remained below threshold levels for detection in both diabetic groups when compared to control non-diabetic mice (data not shown). Total glomerular HPSE-1 (inactive and active) staining on paraffine embedded sections, mostly corresponded with podocyte nephrin-1 staining and was not significantly changed after the intervention period in comparison to the non-diabetic control group (figures 4C,D). Although peritubular F4/80 positive cells were detected, no clear F4/80 positive stained cells were observed within the glomeruli (data not shown).

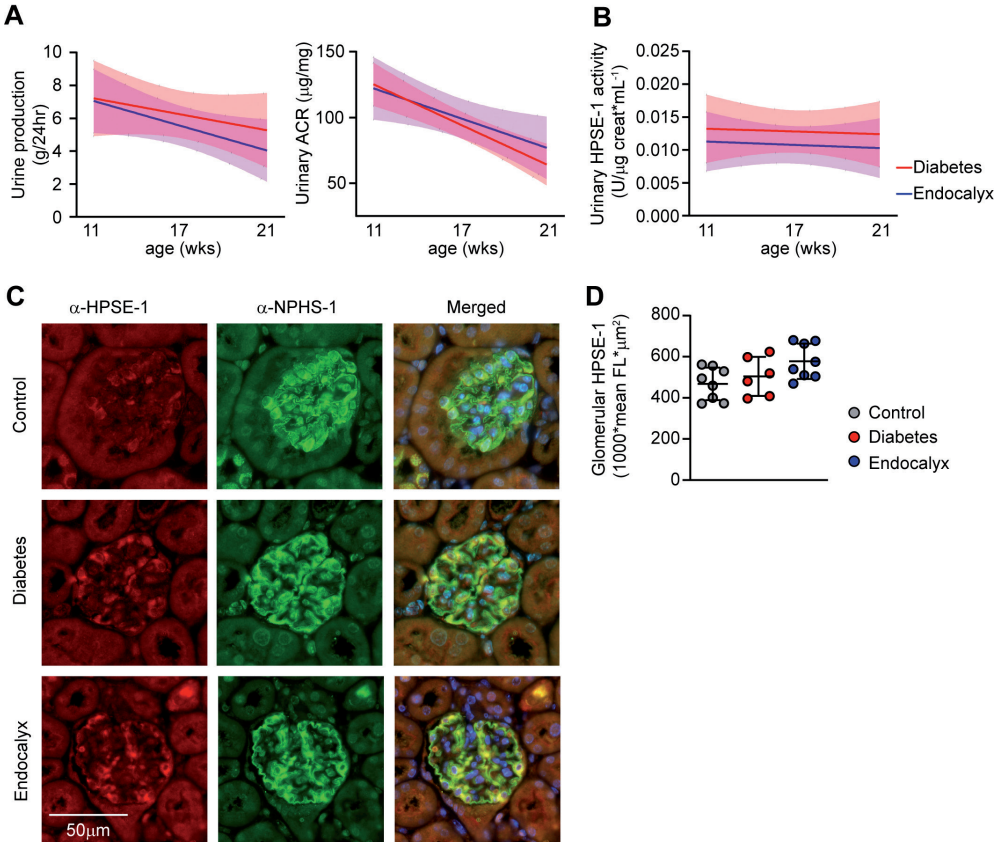


Figure 4. Urinary albumin levels and HPSE-1 activity. Trends in urine production and albumin-to-creatinine ratio (ACR) **(A)** between diabetic- (n = 8) and diabetic apoE-KO mice after Endocalyx intervention (n = 9) over time. Trend in urinary HPSE-1 activity **(B)** between diabetic- (n = 8) and diabetic apoE-KO mice after Endocalyx intervention (n = 9) over time. **(C)** Representative images of glomerular HPSE-1 (red) and nephrin-1 (NPHS-1, green) protein expression in control- (top panels), diabetic- (middle panels) and diabetic apoE-KO mice with Endocalyx™ supplement (bottom panels); scale bar 50 µm.), **(D)** Quantification of total HPSE-1 protein expression. Analysis was performed on ± 50 glomeruli per sample in control- (n = 9), diabetic- (n = 8) and diabetic apoE-KO mice with Endocalyx™ supplement (n = 9). Values are given as mean with standard deviation (D). Differences between groups were assessed by unpaired t-test (D) and simple linear regression (A,B): $*P < 0.05$

Changes in renal myeloid cells in HPSE-1 and cathepsin L expression after supplementation of glycocalyx mimetics

Since pilot immunofluorescence experiments on renal cathepsin L expression revealed very high expression levels in the various epithelial cells, masking possible cathepsin L positivity of myeloid cells (data not shown), we decided to study single myeloid cells with flow cytometry. To investigate renal myeloid cell presence, total kidney single cells were isolated and myeloid cells were determined using a gating strategy shown in supplemental

figure 3A using the antibody cocktails in supplemental table 2, in part according to Cao, *et al.* [24]. The three major myeloid cell types observed were macrophages (Mϕ; CD11c^{neg}/F4-80^{hi}/MHC II^{lo}/CD11b^{lo}/CD370^{lo}/CX3CR1^{hi}), dendritic cells (DC; CD11c^{hi}/F4-80^{neg}/MHC II^{hi}/CD11b^{hi}/CD370^{hi}/CX3CR1^{lo}) and DC switched to a Mϕ -like phenotype [20] (DC/Mϕ-like; CD11c^{lo}/F4-80^{hi}/MHC II^{hi}/CD11b^{lo}/CD370^{hi}/CX3CR1^{hi}), of which the DC/Mϕ-like cell population was found to be about 10 times more present compared to either Mϕ or DCs (figure 5A). Without obvious differences in phenotype differentiation (mean fluorescence intensities) between the diabetes and Endocalyx group (supplemental figure 3B), the percentage of the total CD11b positive population of both Mϕ and DCs were lower in the Endocalyx group compared to the diabetes group (Mϕ: 5 ± 1 vs. 9 ± 2, p = 0.020 and DC: 5 ± 1 vs. 9 ± 3, p = 0.037, respectively; and figure 5B). The DC/ Mϕ-like cell population did not differ between the two groups (77 ± 4 vs. 70 ± 12, Endocalyx and diabetes group, respectively). In neither myeloid cell population tested could a clear cathepsin L expression level be observed (figure 5C), whereas all three cell populations were positive for HPSE-1, however, without differences between the diabetes and Endocalyx group (figure 5D).

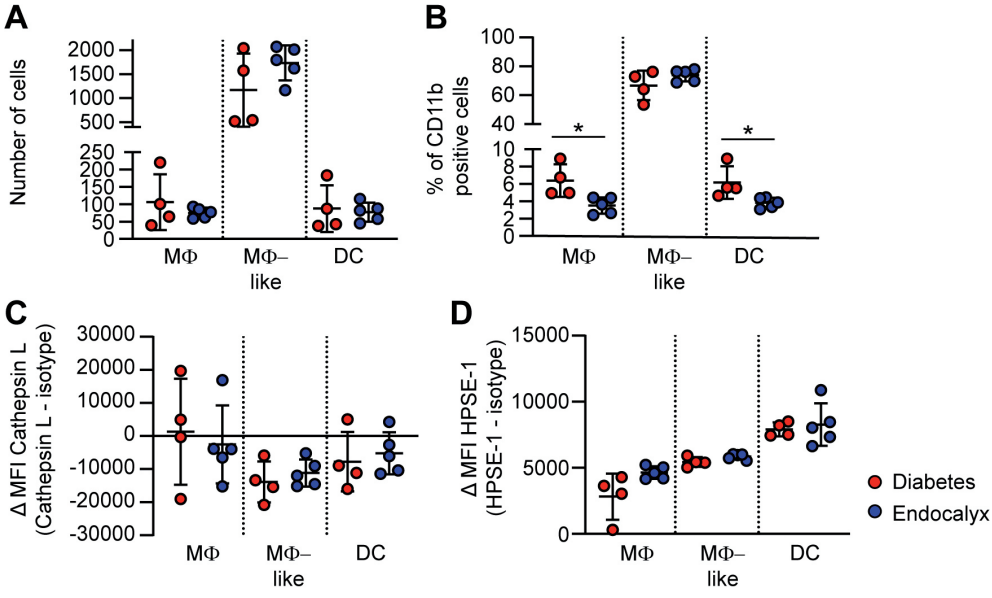


Figure 5. Cytometry of isolated kidney myeloid cells. Isolation from total kidney samples revealed three populations of myeloid cells; macrophages (Mϕ), dendritic cells (DC) switched to Mϕ-like cells and DC in **(A)** number of cells and **(B)** % of CD11b positive cells. Cell type specific expression of **(C)** cathepsin L and **(D)** the heparan sulfate degrading enzyme heparanase (HPSE-1) as function of their respective iso-type controls (ΔMFI), see Table 1. Values are given as mean with standard deviation. Differences between groups were assessed by unpaired t-test: *P < 0.05

Discussion

In the present study, we show that 10 weeks of dietary supplementation with Endocalyx™ preserved the glomerular endothelial glycocalyx coverage in diabetic ApoE-KO mice and prevented glomerular capillary rarefaction. While the main component of Endocalyx™, fucoidan, is able to inhibit HPSE-1 activity and protect the endothelial glycocalyx by reducing the release of heparan sulphate GAGs, no additional effect was found in the glomeruli. Glomerular HPSE-1 expression was predominantly colocalized with podocytes and urinary HPSE-1 activity remained stable throughout the experimental period. While no glomerular macrophages were detected, the percentage of macrophages and dendritic cells within the total CD11b positive cell population isolated from the cortex was found to be lower in the Endocalyx group.

Preventing uncontrolled gluco-toxic effects in the present STZ-induced diabetic mouse model, through regular insulin administration to keep blood glucose levels between 15-20 mmol/L, inadvertently seemed to induce a much milder pathology than we observed previously [10, 11]. This resulted in undetectable urinary kidney damage markers (KIM-1 and MCP-1) and glomerular increased presence of HPSE-1 and cathepsin L. However, glomerular endothelial changes such as loss of the luminal glycocalyx and capillary rarefaction could still be observed [10, 11, 25]. Interestingly, Endocalyx™ supplementation resulted in a shift in the percentage of macrophages and dendritic cell populations, when testing direct isolated single renal myeloid cell populations. Although the major myeloid cell population, macrophage-like dendritic cells did not change, this reduced presence of Mϕ and DCs could reflect a lower renal inflammation status upon Endocalyx™ supplementation.

We did show preservation of the glomerular capillary loops and endothelial glycocalyx coverage after Endocalyx™ supplementation. In line with the specific fucoidan data in the present study, we recently could prove that fucoidan was not only able to block HPSE-1 activity but that surface glycocalyx of primary human lung- and glomerular endothelial cells in the presence of sera from intensive care unit COVID-19 patients was preserved, preventing increased permeability and a hypercoagulable surface [14]. In these experiments fucoidan bound to the endothelial surface, inhibited the inflammatory phenotype and completely restored the endothelial barrier properties and the coagulation disorder. Similar findings were observed on endothelial glycocalyx perturbation using serum of chronic kidney disease patients with elevated uremic toxins [26], relieving arterial stifness in a mouse model of aged animals [27] and in a glomerulonephritis model [15].

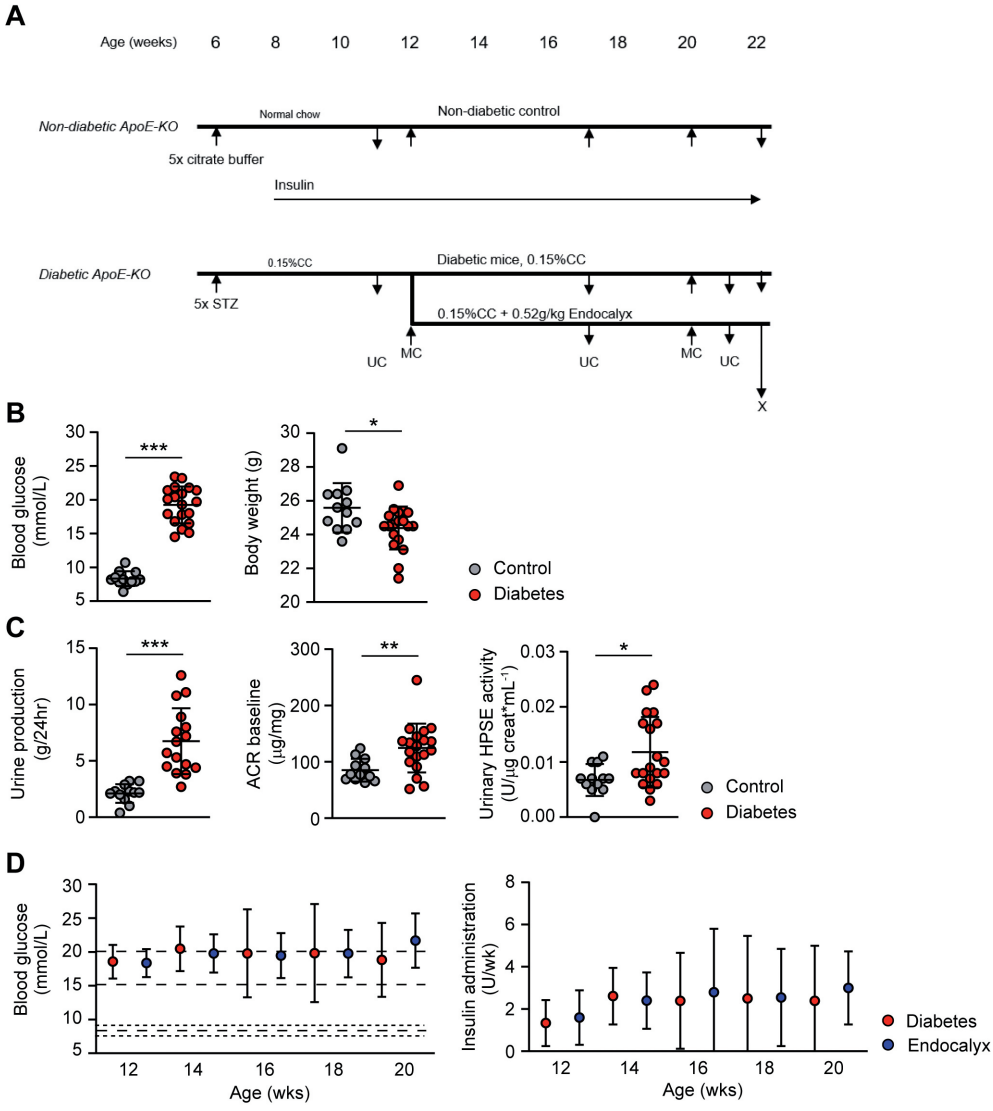
In conclusion, we showed that in the diabetic mouse glomerulus, dietary supplementation with Endocalyx™ was able to prevent capillary damage and glycocalyx degradation.

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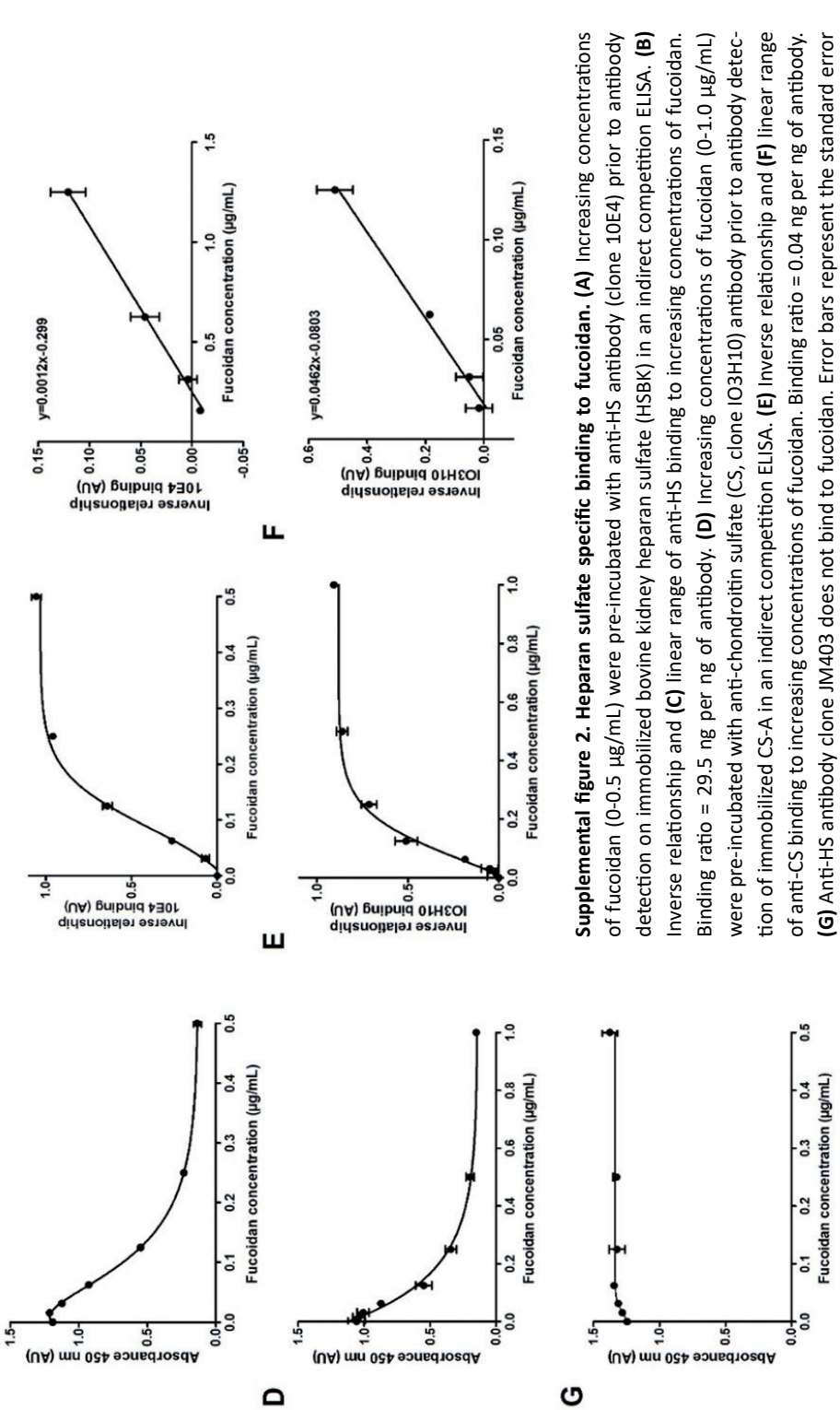
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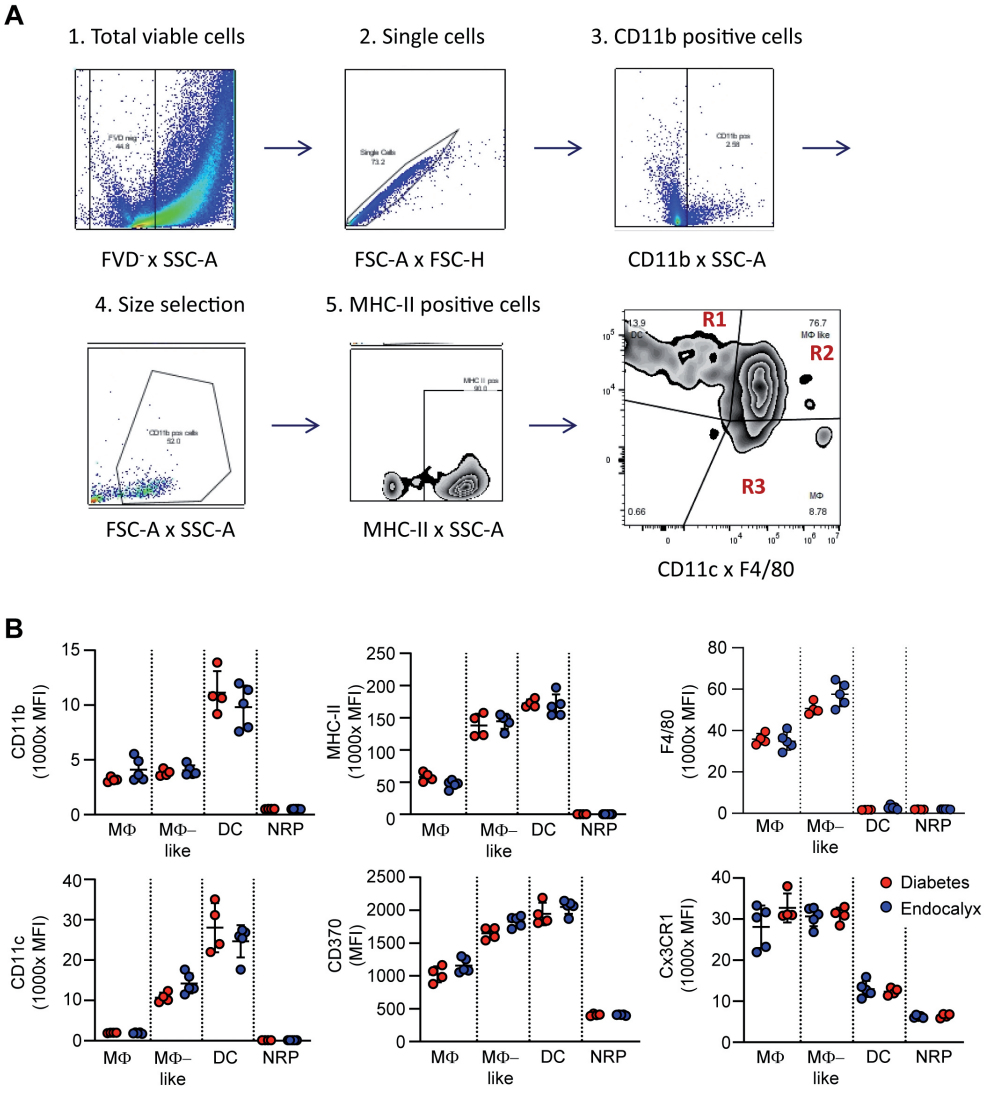
Supplemental



Supplemental figure 1. Design of mouse study and baseline characteristics. (A) Schematic representation of the mouse study. *Abbreviations: STZ = streptozotocin, 0.15%CC = 0.15% cholesterol enriched normal chow, MC = metabolism cage, UC = urine collection. X, endpoint.* Baseline measurements at week 11, before randomization, of **(B)** blood glucose and body weight and **(C)** urine production, albumin-to-creatinine ratio (ACR) and urinary heparanase 1 (HPSE-1) activity of control- and diabetic apoE-KO mice. Averaged weekly **(D)** blood glucose levels and **(E)** insulin administration to maintain preferred blood glucose levels (upper two dashed lines in **D**). **(D)** Lower lines represent mean ± standard deviation blood glucose levels of control apoE-KO mice. Values are given as mean with standard deviation. Differences between groups were assessed by unpaired two-tailed t-test: **P* < 0.05, ***P* < 0.01. ****P* < 0.001.



Supplemental figure 2. Heparan sulfate specific binding to fucoidan. (A) Increasing concentrations of fucoidan (0-0.5 µg/mL) were pre-incubated with anti-HS antibody (clone 10E4) prior to antibody detection on immobilized bovine kidney heparan sulfate (HSBK) in an indirect competition ELISA. **(B)** Inverse relationship and **(C)** linear range of anti-HS binding to increasing concentrations of fucoidan. Binding ratio = 29.5 ng per ng of antibody. **(D)** Increasing concentrations of fucoidan (0-1.0 µg/mL) were pre-incubated with anti-chondroitin sulfate (CS, clone IO3H10) antibody prior to antibody detection of immobilized CS-A in an indirect competition ELISA. **(E)** Inverse relationship and **(F)** linear range of anti-CS binding to increasing concentrations of fucoidan. Binding ratio = 0.04 ng per ng of antibody. **(G)** Anti-HS antibody clone JM403 does not bind to fucoidan. Error bars represent the standard error of the mean.



Supplemental figure 3. Cytometry gating strategy for detection of kidney myeloid cells. To determine and stratify renal myeloid cells from total kidney samples. Single cell suspensions, measured on a Cytek Aurora 5 flow cytometer, were gated after **(A)** life/dead discrimination and single cell selection in further steps using CD11b, MHC-II, CD11c and F4/80 as discrimination expression factors which results in a final gate revealing 3 different myeloid cell populations, i.e., dendritic cells (DC: R1), DC with macrophage-like (Mϕ-like) properties (R2) and Mϕ (R3). **(B)** Mean fluorescent intensities (MFI) of CD11b, MHC-II, F4/80, CD11c, DC marker CD307, and the fractalkine receptor Cx3CR1, with respect to the control non-responsive lymphocyte population (NRP).

Supplemental table 1. Reactivity of antibodies with *L. japonica* fucoidan.

Antibody	Known reactivity/epitope[#]	Fucoidan reactivity	Antibody:fucoidan concentration ratio (ng)	R^2
Anti-HS (F58-10E4)[$]	GlcA-GlcNS-GlcA-GlcNAc	yes	1/28.5	0.998
anti-CS scFv (I03H10)	GlcA2S-GalNAc4S6S	yes	1/0.04	0.99
Anti-HS (JM403)	GlcN	no	-	-

[$]Antibody clone number

[#]GlcA, b-D-glucuronic acid; GlcN, glucosamine; GlcNS, 2-deoxy-2-sulfamido-α-D-glucopyranosyl; GlcNAc, N-acetyl-glucosamine; GlcA2S, 2-sulfated b-D-glucuronic acid; GalNAc4S6S, galactosamine-4,6-disulfate

Supplemental table 2. FACScan antibody cocktails.

Marker	Fluorochrome	Concentration (µg/mL)	Company[#]	Cat no.
Tubes 1-4 (extracellular)				
FVD[$]	eFlour780		eBioscience	65-0865-18
F4/80	SuperBright 436	10	eBioscience	62-4801-82
CX3CR1	BV510	1.3	Biolegend	149025
MHC-II	AF700	5.0	Biolegend	107622
CD11b	PerCP	1.0	Biolegend	101230
CD11c	APC	4.0	BD	550261
CD370 (Clec9a)	AF594	5.0	R&D	FAB67761T
Tube 1 (intracellular), control				
Rabbit IgG	AF488	5.0	eBioscience	53-4616-82
Tube 2 (intracellular)				
Heparanase	AF488	5.0	Bioss Antibodies	BS-1541R-A488
Tube 3 (intracellular), control				
Rabbit IgG	-	20	Dako	X0903
Tube 4 (intracellular)				
Cathepsin L	-			

[$]FVD, fixable viability dye

[#] eBioscience, Thermo Fisher Scientific, Amsterdam, The Netherlands; Biolegend, San Diego, CA, USA; BD Biosciences, Vianen, The Netherlands; R&D Systems, Abingdon, UK; Bioss Antibodies, Woburn, MA, USA; Dako, Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands

CHAPTER 6

Fasting mimicking diet in diabetic mice partially preserves glomerular endothelial glycocalyx coverage, without changing the diabetic metabolic environment

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Abstract

Intermittent fasting has become of interest for its possible metabolic benefits and reduction of inflammation and oxidative damage, all of which play a role in pathophysiology of diabetic nephropathy. We tested in a streptozotocin (60mg/kg) induced diabetic ApoE-KO mouse model whether repeated fasting mimicking diet (FMD) prevents glomerular damage. Diabetic mice received 5 FMD cycles in 10 weeks and during cycles 1 and 5 caloric measurements were performed. After 10 weeks, glomerular endothelial morphology was determined together with albuminuria, urinary heparanase-1 (HPSE-1) activity, and spatial mass spectrometry imaging (MSI) to identify specific glomerular metabolic dysregulation. During FMD cycles, blood glucose levels dropped while a temporal metabolic switch was observed to increased fatty acid oxidation. Overall bodyweight at the end of the study was reduced together with albuminuria, although urine production was dramatically increased without affecting urinary HPSE-1 activity. Weight loss consisted of the loss of lean mass and water, not the loss of fat mass. While capillary loop morphology and endothelial glycocalyx heparan sulfate contents were preserved, hyaluronan surface expression was reduced together with the presence of UDP-glucuronic acid. MSI further revealed reduced protein catabolic breakdown products and increased oxidative stress, not different then diabetic mice. In conclusion, although FMD preserves partially glomerular endothelial glycocalyx, loss of lean mass and increased glomerular oxidative stress argue whether such diet regimes are safe in patients with diabetes.

Introduction

Over the years, intermittent fasting has become a topic of interest in the dietary field, since this type of fasting not only has metabolic benefits, but is also able to induce cellular changes that affect inflammation and oxidative damage [1, 2]. These changes would consequently enhance cellular protection and optimize energy metabolism [2]. The newly introduced periodic fasting-mimicking diet (FMD) regime that induces such beneficial cellular effects, in addition, should be easier to keep up compared to other types of fasting. During the fasting periods, when glucose and glycogen storages are depleted, metabolism switches to the utilization of ketones and fatty acids as the main source of energy. Previous fasting studies showed the beneficial effects of such a diet on endothelial function [3] and reduced body mass index, blood pressure, fasting glucose, total and low-density cholesterol, C-reactive protein and IGF-1 levels in generally healthy participants [4]. Furthermore, FMD in diabetes was effective in experimental type 1 and type 2 mouse models, where diabetic characteristics were ameliorated as blood glucose levels normalized and insulin sensitivity improved, mainly directed by regeneration of the pancreatic beta cells [5, 6].

The effect of FMD specifically on the diabetic kidney has not been studied yet. Endothelial dysfunction is considered the first marker of vascular complications, which can progress to structural microvascular changes and eventually results in irreversible vascular damage [7-9]. Diabetes induced endothelial activation is characterized by a pro-inflammatory and pro-thrombotic phenotype, loss of vascular integrity, and changes in endothelial glycocalyx composition [10]. Prolonged endothelial activation ultimately leads to endothelial dysfunction and upregulation of glycocalyx degrading enzymes such as heparanase-1 (HPSE-1) [11]. As fasting is accompanied by reprogramming of cellular processes towards survival and resilience, we aimed to investigate whether a recurrent 4-day FMD regime has the ability to preserve glomerular capillary morphology and glomerular endothelial surface glycocalyx in a diabetic model. For this, we used a streptozotocin-induced diabetic mice, known to induce impairment of the glomerular glycocalyx [12, 13]. To determine the possible metabolic changes upon FMD, we placed the animals in metabolism cages and at the end of the experiment determined glomerular metabolite changes using spatial mass-spectrometry [14].

Materials and methods

Diabetic ApoE-KO mouse model

Six-week-old male B6.129P2-*Apoe*^{tm1Unc}/J mice (ApoE-KO; The Jackson Laboratory, Bar Harbor, ME) were rendered diabetic through intraperitoneal injections of 60mg/kg

streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) for 5 consecutive days, as described before [12] (figure 1A). ApoE-KO mice were injected with citrate buffer alone (control) and to reduce the overall discomfort level we reduced the amount of handlings during the experimental procedure we only used these non-diabetic mice were used for baseline measurements. All mice had free access to standard rodent diet (NC; Ssniff Spezialdiäten GmbH, Soest, Germany). At week 8, diabetic mice were fed cholesterol enriched (0.15%) chow until the end of the study. At week 11, diabetic mice manifested with hyperglycemia, weight loss, polyuria and albuminuria (figure 2A,B) which were then used in the study. At week 12, six weeks after induction of diabetes, diabetic mice were randomized into the diabetic group (Diabetic) fed cholesterol enriched (0.15%) chow for 10 weeks or into a diabetic group that received a 4 day fasting mimicking diet (FMD) that was repeated 5 times in 10 weeks. In between the diet interventions, FMD mice were fed cholesterol enriched (0.15%) chow. Animal experiments were approved by the Ethical Committee on Animal Care and Experimentation at the Leiden University Medical Center (permit no. AVD1160020172926). All work with animals was performed in compliance with the Dutch government's guidelines.

Blood glucose concentrations were measured with an Accu-check glucose meter (Roche, Basel, Switzerland). When glucose concentrations exceeded 20 mmol/L, mice were treated with 1–2 units insulin (Lantus, Aventis Pharmaceuticals, Bridgewater, NJ, US) up to three times per week (supplemental figure S1D, E). Sixteen weeks after STZ injections, at 22 weeks of age, mice were sacrificed.

Mouse fasting mimicking diet

The FMD used in the study was provided by the Longo group as a gift and consisted of a 4-day regimen similar to the diet used by Brandhorst *et al.* [1]. Day 1 of the diet contained 7.67 kJ/g and consisted of a mix of various low-calorie broth powders, a vegetable medley powder, extra virgin olive oil, and essential fatty acids; day 2–4 diet contained 1.48 kJ/g and consisted of low-calorie broth powders and glycerol. The diet ingredients were thoroughly mixed and then blended together with hydrogel (ClearH_2O, Westbrook, ME, USA).

Caloric measurements

In weeks 12 and 20, control-, diabetic- and FMD mice were placed in metabolism cages (Sable Systems Europe GmbH, Berlin, Germany) for 4 days to measure food intake, oxygen ($V\cdot_{O_2}$) consumption, carbon dioxide ($V\cdot_{CO_2}$) production and ambulatory physical activity (semi quantitative by beam breaks). Followed by Echo-MRI (EchoMRI LLC, Houston, TX, USA) to measure body composition (lean-, fat mass, total- and free water). Based on these values, we calculated the respiratory exchange ratio (RER, the ratio of carbon dioxide produced by the body to oxygen consumed by the body), energy expenditure, carbohydrate and fat oxidation rates to monitor possible metabolic changes. In addition,

from the Echo-MRI data muscle mass and %muscle mass were calculated as followed, since lean mass measurements are the total of muscle mass + (total water- free water), muscle mass was calculated by lean mass - (total water- free water), %muscle mass was calculated as muscle mass/(lean mass + fat mass) x 100.

Urine collection and analysis

Mice were weighted before and after residing in a metabolic cage (Tecniplast S.p.a, Buguggiate, Italy) and water- and food intake and urine were collected. After acclimatization, 14 h-urine was collected at week 11, 17 and 21. Urine samples were centrifuged to remove debris and stored at -20°C. Urinary albumin concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories, Inc. Montgomery, TX, USA) and creatinine concentrations were quantified by the Jaffe´ method using 0.13% picric acid and a creatinine standard set (Sigma-Aldrich, Merck Life Science NV, Amsterdam, The Netherlands). Urinary MCP-1 and KIM-1 (kidney injury molecule-1) were measured with a commercially available immunoassay according to the manufacturer protocol (MJE00B and MKM100, resp.: R&D Systems Europe, Ltd., Abingdon, UK). Urinary heparanase activity was measured with a commercially available ELISA assay (Takara Bio Inc., Shiga, Japan). For this, urine samples were run through Zeba™ Spin Desalting Columns (ThermoFisher Inc., Waltham, MA, USA) for removal of salts and other small molecules (<1000 MW).

Glomerular endothelial glycocalyx coverage

Glycocalyx coverage was determined using fluorescently labelled lectin *Lycopersicon esculentum* (LEA-FITC) and the N terminus rat neurocan construct of the hyaluronan-specific neurocan-dsRed (Ncan-dsRed) construct, as described previously [15, 16]. In short, overnight PFA fixed tissue was subsequently sectioned in 100µm thick slices with a Leica VT1000S vibratome (n = 3/group) and submerged in HBSS (Life Technologies Europe BV, Bleiswijk, The Netherlands) containing 0.5% BSA, 5 mmol/L HEPES, and 0.03 mmol/L EDTA (HBSS-BSA). Slices were incubated with 10 mg/mL LEA-FITC or Ncan-dsRed [16] to visualize the glycocalyx, together with 5 mg/mL monoclonal mouse anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to identify the endothelial cell membrane, overnight at 4°C on a shaker (in dark). After 3 washes with HBSS-BSA slices were incubated for 2 h with 10mg/mL Alexa Fluor-568, or AF488–conjugated goat anti-mouse IgG (Molecular Probes, Grand Island, NY) and Hoechst 33528 (Sigma-Aldrich, 1:1000) at 4°C on a shaker (in dark). Slices in HBSS-BSA were fixated between glass slide and coverslip in mounting medium and imaged on a LEICA TCS SP8 X WLL microscope (Leica, Rijswijk, The Netherlands) and a 40x objective (HC PL APO CS2 40x/1.30 OIL, Leica). Sequential 16-bit confocal images (xyz dimensions, 0.142 x 0.142 x 0.3 µm) were recorded using LAS-X Image software (Leica). The amount of endothelial glycocalyx was quantified in using 5 lines of interest in 3-4 glomeruli per kidney by calculating the distance from the peak of the

CD31 signal to the half-width of the luminal LEA-FITC or Ncan-dsRed signal along a line of interest, using intensity profiles (ImageJ), as described previously [16].

Tissue preparation and histology

Mice were anesthetized by isoflurane inhalation and perfused via the left ventricle with HEPES-buffered salt solution containing 0.5% bovine serum albumin and 5 U/mL heparin to remove blood. After removal of the kidney capsules, the left kidney was placed in 2% PFA in PBS overnight at 4°C, followed by paraffin embedding for periodic acid-Schiff (PAS), methenamine silver-periodic acid-Schiff (MPAS) or immunofluorescence staining. The left kidney of a subset of mice (n = 3/group) was perfused with 5mL Hanks-buffered salt solution (HBSS, Gibco) containing 0.5% BSA (Sigma, A7030, essentially globulin free) and 5U/mL heparin at 2mL/minute to remove blood, followed by 2mL of cationic ferritin (horse spleen, 2.5mg/mL, Electron Microscopy Sciences, Fort Washington, PA) in HBSS at 2mL/minute. The kidney was excised, its capsule removed, and stored in fixative (1.5% glutaraldehyde and 1% paraformaldehyde (PFA) (both from Electron Microscopy Sciences, Hatfield, PA) in 0.1M sodium-cacodylate buffered solution, pH 7.4) overnight at 4°C for further processing for transmission electron microscopy (TEM).

TEM cationic ferritin determination

Cationic ferritin perfused tissue, stored in fixative, 1.5% GA and 1% PFA in 0.1M sodium-cacodylate buffered solution, was subsequently sectioned in 180µm thick slices with a Leica VT1000S vibratome, rinsed 2x with 0.1M sodium cacodylate-buffered solution, and post-fixated for 1hr with 1% osmium tetroxide (Electron Microscopy Sciences) and 1.5% potassium ferrocyanide in demineralized water [15, 16]. Samples were further washed, dehydrated in ethanol, infiltrated with a mixture of epon LX-112 and propylene oxide (1:1) for 1 hr, followed by pure epon for 2hrs, embedded in epon mounted in BEEM capsules (Agar Scientific, Essex, United Kingdom) and polymerized for 48hrs at 60°C. 100nm Thick sections were cut using a diamond knife (Diatome, Biel, Switzerland), collected on single slot copper grids covered with formvar film and carbon layer, and then stained with 7% uranyl acetate in demineralized water for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Data was collected at an acceleration voltage of 120kV on a Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM), equipped with an FEI 4k Eagle CCD camera. Virtual slides were acquired with 18,500x magnification at the detector plane, corresponding to a 1.2nm pixel size at the specimen level. Representative capillary sections of each recorded glomerulus (n = 1/group) from virtual slides were selected for display as a high resolution image.

Glomerular tissue metabolomics

Frozen renal tissue sections (stored at -80°C) of a subset of mice (n= 4/group) were embedded in deionized water (MQ) and sections of 10 µm thickness were cryosectioned

using a Cryostar NX70 cryostat (Thermo Fisher Scientific, MA, USA) at -20 °C. The sections were thaw-mounted onto indium-tin-oxide (ITO)-coated glass slides (VisionTek Systems Ltd., Chester, UK). Mounted sections were placed in a vacuum freeze-dryer for 15 minutes prior to matrix application. After drying, *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) (Sigma-Aldrich, UK) MALDI-matrix solution of 7 mg/mL in methanol/acetonitrile/deionized water (70, 25, 5 %v/v/v) was applied using a SunCollect sprayer (SunChrom GmbH, Friedrichsdorf, Germany). A total of 17 matrix layers were applied with the following flowrates: layer 1-3 at 5 µL/min, layer 4-6 at 10 µL/min, layer 7-9 at 15 µL/min and 10-17 at 20 µL/min (speed *x*, medium 1; speed *y*, medium 1; *z* position, 35). The ITO glass slide containing the slices of all three groups were scanned using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI-TOF-MSI) with the RapifleX MALDI-TOF/TOF system (Bruker Daltonics GmbH, Bremen, Germany). Negative ion-mode mass spectra were acquired at a pixel size of 20 × 20 μm^2 in a mass range from *m/z* 80-1000 in reflectron mode. Prior to analysis the instrument was calibrated using red phosphorus. Spectra were acquired with 200 laser shots per pixel at a laser repetition rate of 10 kHz. Data acquisition was performed using flexControl (Version 4.0, Bruker Daltonics, Germany) and visualizations were obtained from flexImaging 5.0 (Bruker Daltonics). MALDI-FTICR-MSI was performed on a 12T solariX FTICR mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in negative-ion mode, using 30 laser shots and 50 µm pixel size. Prior to analysis the instrument was calibrated using red phosphorus. The spectra were recorded in a *m/z* range of 100-1000 with a 512k data point transient and transient length of 0.1049 seconds. Data acquisition was performed using ftmsControl (Version 2.1.0, Bruker Daltonics), and visualizations were obtained from flexImaging 5.0 (Bruker Daltonics). The *m/z* features present in both MALDI-TOF-MSI and MALDI-FTICR-MSI datasets, and which had similar tissue distributions, were further used for identity assignment of metabolites and lipid species. The *m/z* values from MALDI-FTICR-MSI were imported into the Human Metabolome Database[17] (https://hmdb.ca/) and annotated for metabolites and lipids species with an error < ±10 ppm. For the small molecules detected only in MALDI-TOF, the *m/z* values from MALDI-TOF were imported into the Human Metabolome Database (https://hmdb.ca/) and annotated for metabolites with an error < ±20 ppm. MSI data of the glomerular regions of interest were exported and processed in SCiLS Lab 2016b (SCiLS GmbH). All MALDI-TOF/TOF-MSI data was normalized to the total ion count (TIC). The peaks from the skyline/basepeak spectrum with a signal-to-noise-ratio higher than 3 were selected and all matrix peaks were excluded. A total of 389 *m/z*-features were selected by peak intensities. For specific spatial analysis of the glomerular areas post MALDI-MSI immunofluorescent staining was performed using the endothelial specific lectin from *Bandeiraea simplicifolia* (BS-I-TRITC, 1:200, Sigma, L5264) as described previously [14]. The differential expression of metabolites and lipids between groups were analyzed using both a two-way analysis of variance (ANOVA) test followed with Tukey's post-hoc test and classical univariate ROC curve analysis from the

MetaboAnalyst online version (https://www.metaboanalyst.ca/) [18]. A cutoff (AUC > 0.7 and p < 0.01) was taken for further pathway analysis using Reactome database (https://reactome.org/) [19] and lipid ontology analysis using LION/web (http://www.lipidontology.com/) [20]. Heatmaps were produced using the R pheatmap package based on the average value of metabolites.

Statistical analysis

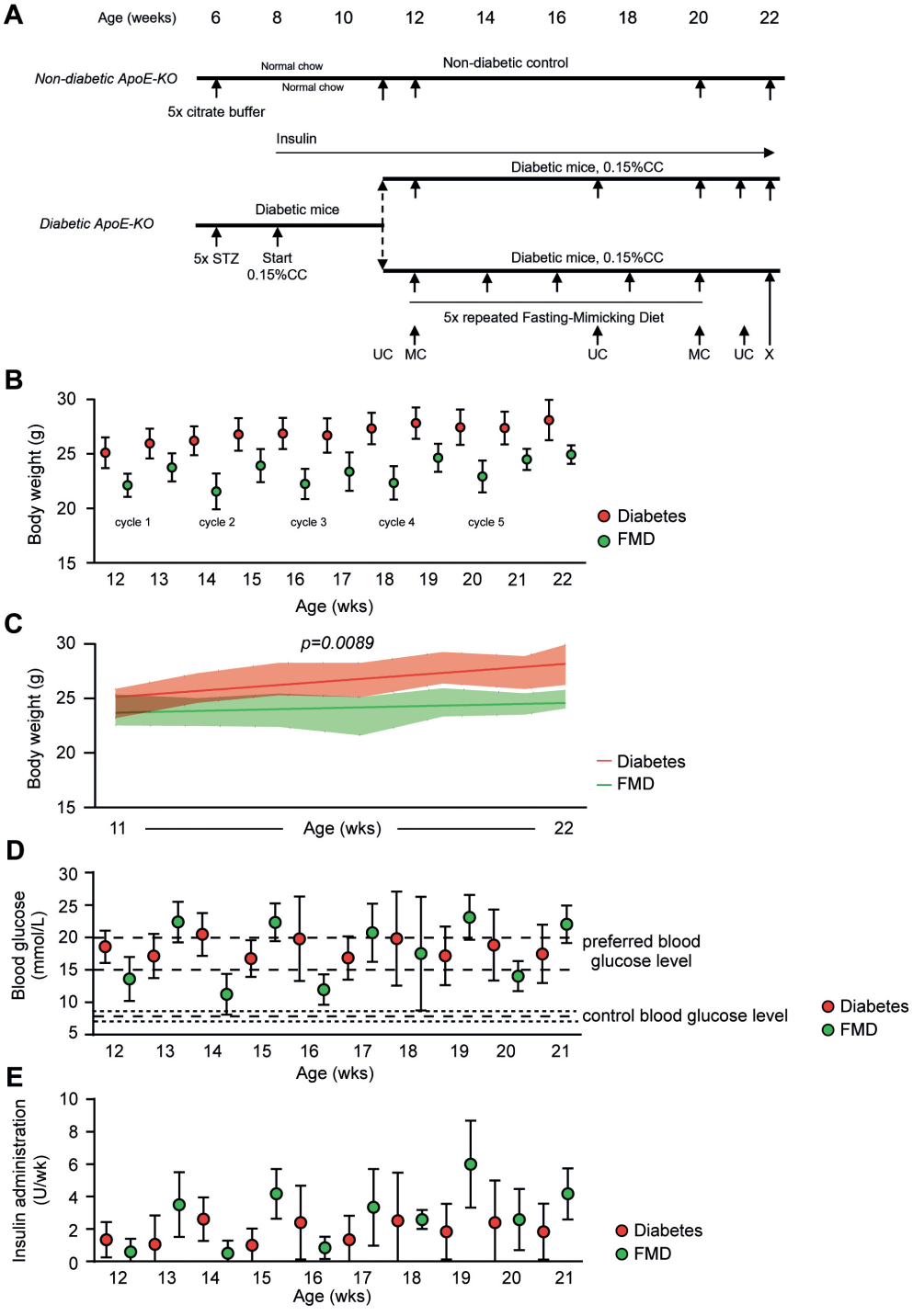
Data are presented as means with standard deviation (SD). Differences in experiments were determined using analysis of variance and post hoc analyses with Tukey's multiple comparison test. Comparison of expression between two different groups was evaluated using a t-test and changes overtime between groups using simple linear regression. Statistical analyses were performed using GraphPad Prism version 8 (Graphpad Inc., La Jolla, CA, USA). A significance level of 0.05 was considered statistically significant.

Results

Study setup diabetic ApoE-KO mice

Throughout the 10-week intervention period, body weight dropped during the FMD cycles but increased again in the refeeding period (figure 1B). Simple linear regression comparison of body weight gain, in weeks excluding FMD cycles, revealed a clear significant (p = 0.0089) change in slopes between diabetic and FMD mice (figure 1C), revealing an overall reduction in total body weight in mice subjected to repeated FMD compared to diabetic mice. Pilot experiments revealed that STZ-induced diabetes in the apoE-KO mouse model resulted in increasing blood glucose levels even above detection which in itself already induced a full body metabolic switch to predominant lipid oxidation were predominantly lipids over carbohydrates are used for energy consumption (comparable to

Figure 1. Design of mouse study and baseline characteristics. (A) Schematic representation of the mouse study. Mice were rendered diabetic using streptozotocin (STZ), controls received citrate buffer, at week 6. From week 8 until end of the experiments diabetic were fed cholesterol enriched chow while the fasting mimicking diet intervention group were replaced on this diet for 5 times which lasted 5 days each cycle. At various intervals urine was collected or mice were housed in metabolism cages for 72hrs each time. *Abbreviations: STZ = streptozotocin, 0.15%CC = 0.15% cholesterol enriched normal chow, MC = metabolism cage, UC = urine collection. X, endpoint.* **(B)** Weekly average body weights during experimental intervention period with fasting mimicking diet (FMD). **(C)** Simple linear regression comparison (slope) in body weights, excluding FMD cycles, between diabetic and FMD mice. Averaged weekly **(D)** blood glucose levels and **(E)** insulin administration to maintain preferred blood glucose levels (upper two dashed lines in **D**). **(D)** Lower lines represent mean ± standard deviation blood glucose levels of control apoE-KO mice. Differences in trend between groups were assessed by simple linear regression.


A
Age (weeks) 6 8 10 12 14 16 18 20 22
Non-diabetic ApoE-KO
Normal chow
Normal chow
Non-diabetic control
5x citrate buffer
Insulin
Diabetic mice, 0.15%CC
Diabetic ApoE-KO
Diabetic mice
5x STZ
Start 0.15%CC
Diabetic mice, 0.15%CC
5x repeated Fasting-Mimicking Diet
UC MC UC MC UC X
B
Body weight (g)
cycle 1
cycle 2
cycle 3
cycle 4
cycle 5
Age (wks)
Diabetes
FMD
C
p=0.0089
Body weight (g)
Age (wks)
Diabetes
FMD
D
Blood glucose (mmol/L)
preferred blood glucose level
control blood glucose level
Age (wks)
Diabetes
FMD
E
Insulin administration (U/wk)
Age (wks)
Diabetes
FMD

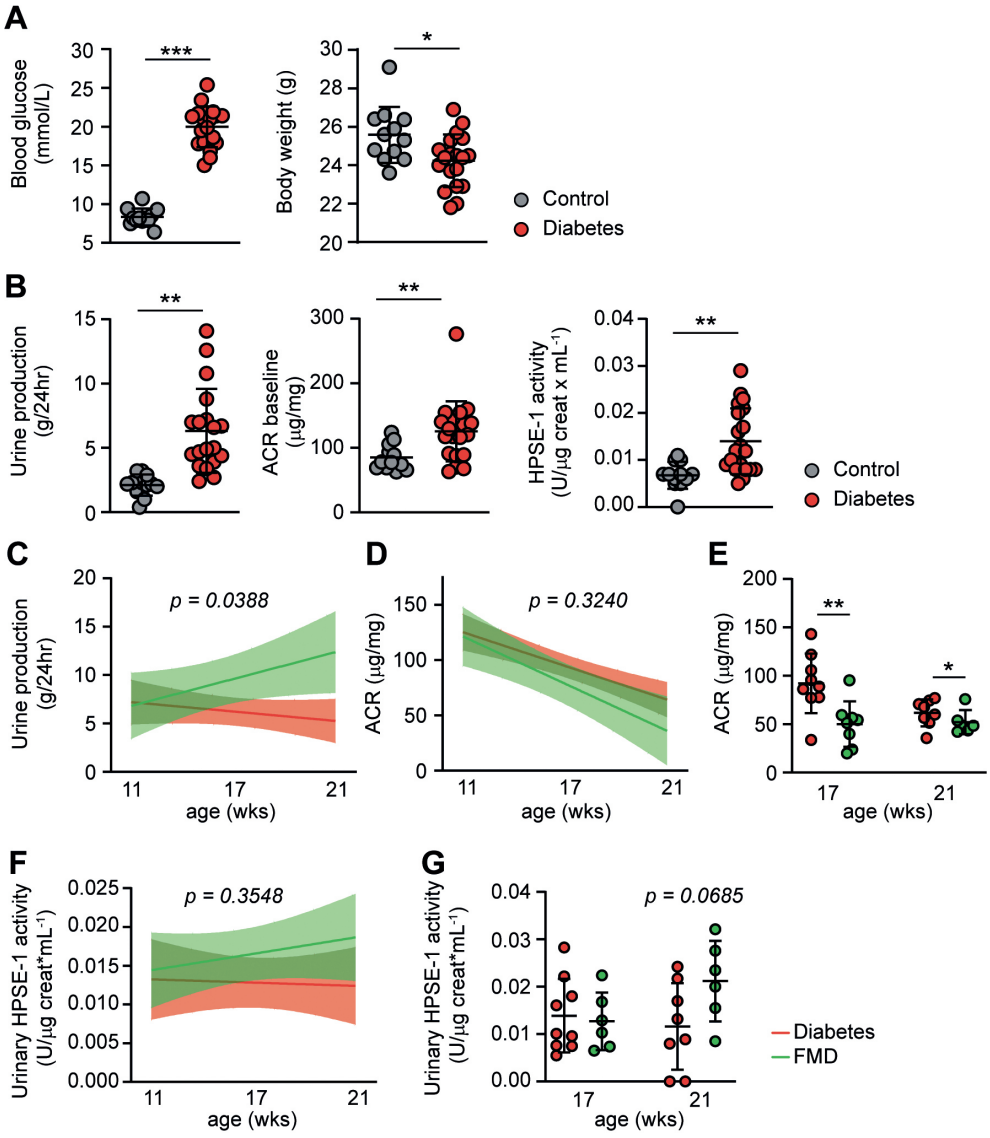


Figure 2. Blood glucose and urine measurements. (A) Blood glucose and body weight, and **(B)** urine production, albumin:creatinine ratio (ACR) and urinary heparanase 1 (HPSE-1) activity of control and diabetic mice at week 11, before randomization of diabetic mice in diabetic and fasting mimicking diet (FMD) groups. **(C)** Trends in urine production, and **(D)** albumin-to-creatinine ratio (ACR) in diabetic- (n = 8-9) and FMD (n = 6-8) mice over time (week 11-, 17- and 21). **(E)** 24-hour urine ACR concentrations at weeks 17 (n = 9 vs. n = 8) and 21 (n = 8 vs. n = 6) in diabetic and FMD mice, respectively. **(F)** Trends in urinary HPSE-1 activity in diabetes and FMD mice over time and **(H)** urinary HPSE-1 activity at week 17 (n = 9 vs. n = 6) and 21 (n = 8 vs. n = 6) in diabetic and FMD mice, respectively. Values are given as mean with standard deviation. Differences between groups were assessed by unpaired two-tailed t-test: **P* < 0.05, ***P* < 0.01. ****P* < 0.001. Differences in trend between groups were assessed by simple linear regression.

FMD group shown below, data not shown). Therefore, we kept the weekly average blood levels around 15-20 mmol/L as much as possible administration of insulin (figure 1D,E), to prevent such a preliminary shift to lipid usage for energy metabolism. Occasional insulin injections have been given throughout the entire experimental procedure with higher amounts in particular in the FMD group (figure 1D).

FMD reduces albuminuria

Before the intervention, at week 11, 5 weeks after STZ induction, diabetic mice manifested with hyperglycemia, weight loss, polyuria, albuminuria and increased urinary HPSE-1 activity levels (figure 1A,B). After these measurements, diabetic mice were randomized into a diabetic group fed cholesterol enriched (0.15%) chow only for 10 weeks or FMD group given a 4 day fasting mimicking diet that was repeated 5 times in 10 weeks with cholesterol-enriched chow ad libitum in between the FMD cycles (Figure 1A). At week 22, one week after the last FMD cycle, urine production was significantly higher in the FMD group compared to the diabetic group (figure 2C). Although during the whole intervention period albumin creatine ratio (ACR) in both diabetes and FMD mice were reduced equally, significantly lower ACR levels in the FMD could be detected at week 17, one week after 3 FMD cycles, and at the end of the experiment at week 21 compared to the diabetic group (figures 2D,E). Urinary levels of the potent heparan sulfate degrading enzyme HPSE-1 were not significantly different when compared to urinary activity in diabetic mice (figures 2F,G). Furthermore, urinary kidney injury molecule-1 (KIM-1) and monocyte chemoattractant protein-1 (MCP-1) levels did not change or remained below threshold levels for detection in both diabetic groups when compared to control mice (data not shown).

FMD prevented glomerular capillary rarefaction

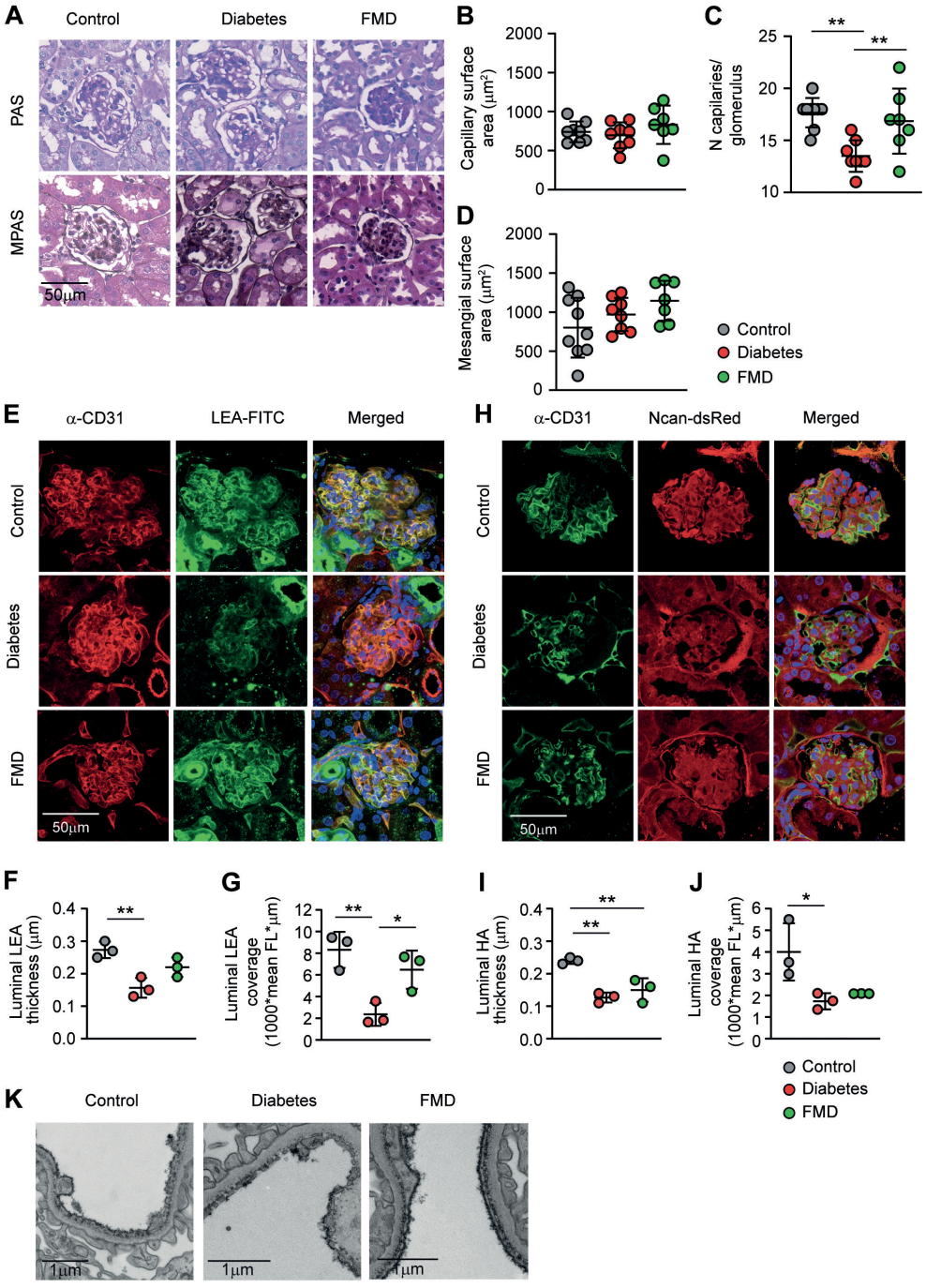
The hyperglycemia controlled diabetic mice, through insulin administration in combination with a cholesterol-enriched diet (0.15%) to induce vascular damage, resulted in minimal glomerular changes at the end of the experiment, as shown by PAS and MPAS stained examples (figure 3A). Although no changes in capillary surface area were observed, the number of capillaries per glomerulus, however, were significantly reduced in diabetic mice compared to controls (difference of -4.2 95% CI -5.8 - -2.6) (figure 3B, C). Diabetic mice after FMD cycles, on the contrary, showed comparable numbers of capillaries per glomerulus compared to control, suggesting that capillary loss and -ballooning were prevented in this group. No changes were observed in glomerular surface area (data not shown) and mesangial surface area (figure 3D).

FMD partially preserved the glomerular glycocalyx

We further investigated the minor glomerular changes through staining PFA fixed renal sections directly with the fluorescently labelled lectin (LEA-FITC) or hyaluronan (HA) specific probe Ncan-dsRed to test the effect of repeated FMD cycles on glomerular glycocalyx

presence (figure 3E and H). Diabetes reduced the glomerular intraluminal lectin thickness on the endothelium to 0.157 µm, compared to 0.272 µm in control mice (difference 0.117 µm 95% CI 0.059 - 0.175) and luminal surface coverage (figures 3E-G). In turn, repeated FMD cycles protected the glomerular endothelial glycocalyx with regard to the lectin stained structures, as the intraluminal thickness was 0.227 µm with a difference of 0.070 µm compared to the diabetes group (95% CI 0.012 - 0.128) and luminal coverage (figure 3E-G). However, staining for HA revealed no protection of surface presence after FMD cycles (figure 3H-J). Visualizing the glomerular endothelial glycocalyx through cationic ferritin coverage using transmission electron microscopy, confirmed protection of at least the charged glycosaminoglycans within the glomerular endothelial glycocalyx by repeated FMD cycles (figure 3K). Cationic ferritin was present at the luminal endothelial cell surface, within the fenestrae and underneath the endothelium of the control mice and FMD group. In contrast, disruption of cationic ferritin coverage alongside the luminal endothelial cell surface could be seen in the diabetic glomerulus (figure 3K, middle panel). These changes were without significant changes in glomerular basement membrane (GBM), podocyte foot process effacement or endothelial fenestration (data not shown).

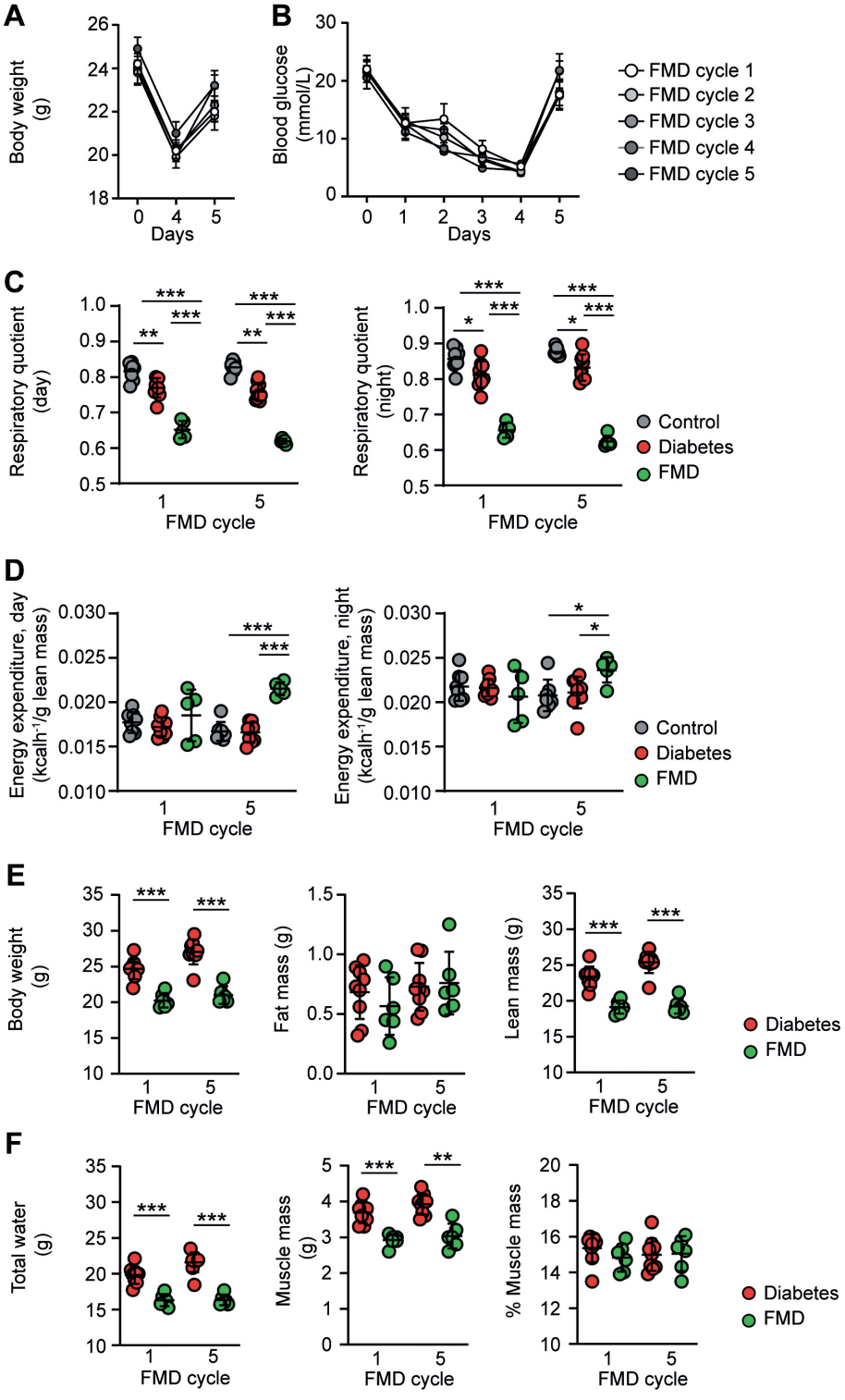
Figure 3. Histological glomerular changes. **(A)** Representative images of periodic acid–Schiff (PAS, top) and (methenamine silver-periodic acid-Schiff (MPAS, bottom) stained glomeruli of control- (left), diabetic- (middle) and diabetic apoE-KO mice after fasting mimicking diet (FMD) interventions (right); scale bar, 50 µm. Quantification of **(B)** glomerular capillary surface area, **(C)** number of capillaries per glomerulus and **(D)** mesangial surface area. Analysis was performed on ± 50 glomeruli per sample in control- (grey, n = 9), diabetic- (red, n = 8) and diabetic apoE-KO mice after FMD interventions (green, n = 9). Values are given as mean with standard deviation (SD). **(E)** Representative images of direct glycocalyx staining using fluorescent labeled lectin *Lycopersicon esculentum* (LEA-FITC) and anti-CD31 antibodies for endothelial cell detection; scale bar 50µm. Quantification of **(F)** luminal glycocalyx (LEA) thickness and **(G)** luminal glycocalyx (LEA) coverage, assessed in a subset of n = 3 control- (grey), diabetic- (red) and diabetic apoE-KO mice after fasting mimicking diet (FMD) interventions (green). **(H)** Representative images of direct glycocalyx staining using fluorescent labeled neurocan (Ncan-dsRed) and anti-CD31 antibodies for endothelial cell detection; scale bar 50µm. Quantification of **(I)** luminal hyaluronan (Ncan-dsRed) thickness and **(J)** luminal hyaluronan (Ncan-dsRed) coverage, assessed in a subset of n = 3 control- (grey), diabetic- (red) and diabetic ApoE-KO mice after FMD intervention (green). **(K)** Representative transmission electron micrographs of cationic ferritin-stained glomerular endothelial surfaces in control-, diabetic- apoE-KO and diabetic apoE-KO after FMD intervention, scale bars 1µm. Values are given as mean with standard deviation. Differences between groups were assessed by ANOVA with Tukey's post-hoc test: $*P < 0.05$, $**P < 0.01$.


A
Control
Diabetes
FMD
PAS
MPAS
50µm
B
Capillary surface area (µm²)
2000
1500
1000
500
0
C
N capillaries/ glomerulus
25
20
15
10
**
**
D
Mesangial surface area (µm²)
2000
1500
1000
500
0
Control
Diabetes
FMD
E
α-CD31
LEA-FITC
Merged
Control
Diabetes
FMD
50µm
H
α-CD31
Ncan-dsRed
Merged
Control
Diabetes
FMD
50µm
F
Luminal LEA thickness (µm)
0.4
0.3
0.2
0.1
0.0
**
G
Luminal LEA coverage (1000*mean FL*µm)
12
10
8
6
4
2
0
**
*
I
Luminal HA thickness (µm)
0.4
0.3
0.2
0.1
0.0
**
**
J
Luminal HA coverage (1000*mean FL*µm)
6
5
4
3
2
1
0
*
Control
Diabetes
FMD
K
Control
Diabetes
FMD
1µm
1µm
1µm

FMD induced metabolic changes in diabetic ApoE-KO mice

During each 4-day FMD cycle, average body weight and glucose levels dropped but raised again as soon as the cycle was finished and animals were given cholesterol enriched food ad libitum (figure 4A, B). As revealed by the respiratory quotient ratio (RQ), defined as the volume of carbon dioxide released over the volume of oxygen absorbed during respiration, the reduced basal metabolic rate in diabetic mice indicated the use of more lipids over carbohydrates for energy consumption (figure 4C). During the FMD cycles, RQ dropped even further, indicating a full body metabolic switch to predominant lipid oxidation within each cycle. In addition, energy expenditure, reflecting animal movement, during the night and especially day cycle was increased during the last FMD cycle (figure 4D). These changes argue that these animals are more constantly searching for food. Additional Echo-MRI measurements revealed that the main component of weight loss during FMD cycles was loss of lean mass instead of fat mass (figure 4E). The major components of lean mass, total water and muscle mass, were both significantly reduced during the FMD cycle compared to the diabetic mice (figure 4F).

Figure 4. Effects of fasting mimicking diet (FMD) on metabolism and Echo-MRI body composure measurements. (A) Body weight- and **(B)** blood glucose level changes during each FMD cycle of mice in FMD group. **(C)** Respiratory quotient and **(D)** Energy expenditure at day (left) and night (right) during FMD cycles 1 (wk12) and 5 (wk20) in control-, diabetic and FMD mice. **(E)** Average total body mass, fat mass, and lean mass (muscle mass + total water – free water) changes as measured with Echo-MRI in diabetic and FMD groups directly after FMD cycles 1 and 5, respectively. **(F)** Total water measurements and calculated muscle mass (lean mass – (total water- free water)) and % muscle mass (muscle mass/(lean mass +fat mass) x 100) of diabetic and FMD mice. Values are given as mean with standard deviation. Differences between groups were assessed in by ANOVA with Tukey's post-hoc test **(C**,**D),** or by unpaired t-test **(E**,**F)**: $*P < 0.05$, $**P < 0.01$. $***P < 0.001$.


A
Body weight (g)
Days
B
Blood glucose (mmol/L)
Days
FMD cycle 1
FMD cycle 2
FMD cycle 3
FMD cycle 4
FMD cycle 5
C
Respiratory quotient (day)
Respiratory quotient (night)
FMD cycle
Control
Diabetes
FMD
D
Energy expenditure, day (kcal h-1/g lean mass)
Energy expenditure, night (kcal h-1/g lean mass)
FMD cycle
Control
Diabetes
FMD
E
Body weight (g)
Fat mass (g)
Lean mass (g)
FMD cycle
Diabetes
FMD
F
Total water (g)
Muscle mass (g)
% Muscle mass
FMD cycle
Diabetes
FMD

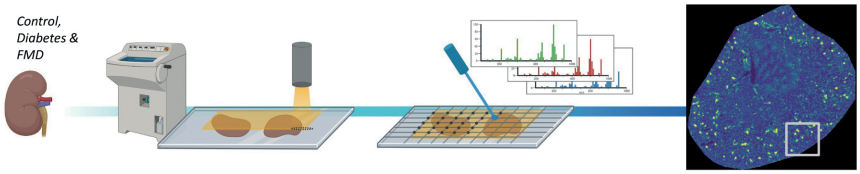
Glomerular metabolic changes upon FMD intervention

Following up on the metabolic changes observed in kidneys between control and diabetic mice [14], we now focused directly on changes in the glomerular areas between control, diabetic and FMD groups (figures 5A,B). Analysis of these three groups at the end of the experiment (week 22), 2 weeks after the final FMD intervention revealed that 31 of the 389 m/z features measured were significantly changed (figure 5C, supplemental table 1 and 2). Heatmap clustering revealed, horizontally, two major clusters; first the control group and a second cluster with both diabetes- and FMD groups. Vertical clusters revealed a large group with decreased metabolites and a small group with increased metabolite expression in the diabetes- and FMD group (figure 5C). Overall, compared to control mice, metabolite levels were all lower in the diabetes- and FMD group. The most pronounced change was observed in protein catabolism breakdown products, which were reduced in both the diabetes- and FMD group (figure 5C, supplemental table 1 and 2). Indications of increased oxidative stress were also observed with increased oxidized sphingolipid (m/z 737.5024; SM(d16:1/20:3(5Z,11Z,14Z)-O(8,9))) and ophthalmic acid, a reduced breakdown product of glutathione turnover. Finally, while the UDP-sugar metabolite UDP-GlcNAc did not change (control, 0.841 ± 0.141; diabetes, 0.819 ± 0.079; FMD, 0.769 ± 0.087), in diabetic mice UDP-GlcUA was significantly reduced (*P = 0.0295*) in diabetes and was not restored after repeated FMD cycles (figure 5C and supplemental table 2).

Figure 5. Glomerular metabolic changes. (A) Experimental workflow for in situ spatial MALDI- mass spectrometry imaging (MALDI-MSI) of control, diabetes, and diabetes mice after 5 cycles of fasting mimicking diet (FMD. **(B)** Experimental workflow for metabolic histologic analysis using fluorescent labeled *Bandeiraea simplicifolia* (BS-I) lectin staining to detect clustered glomerular endothelial cells which coincides with the metabolic m/z value of 566.33, and is used as specific glomerular marker in further analysis. **(C)** Heatmap showing the fractional contribution of significantly changed metabolites between control- (n = 4), diabetic (n = 4) and diabetic apoE-KO mice after fasting mimicking diet (FMD; n = 4), scaled by column. Representative metabolites are given in Annotation column. Differences between groups were assessed by two-way ANOVA with Tukey's post-hoc test. Cartoons were created with BioRender. Heatmap was produced using the R pheatmap package based on the average value of metabolites.

[§]Breakdown product of protein catabolism; [#]Not a natural metabolite, part of exposome; Blue font, metabolites in diabetes and FMD groups are both significantly different from control

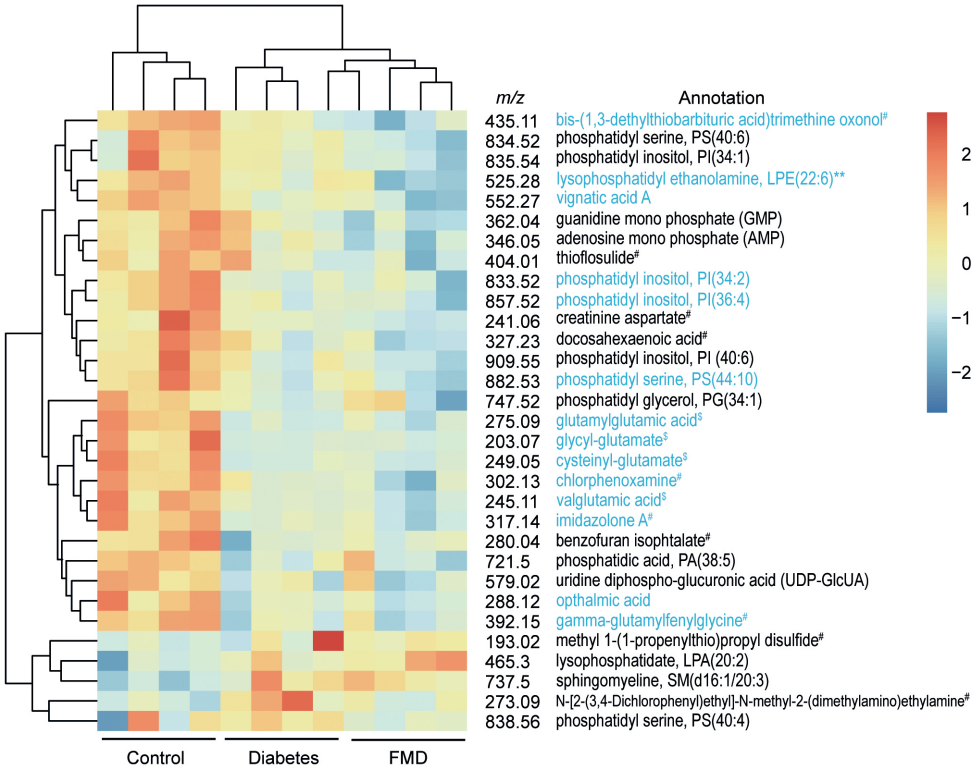
A Spatial MALDI-mass spectrometry imaging



B Immuno fluorescent assisted cluster annotation



C



Discussion

In the present study, we observed that a periodic fasting mimicking diet in a diabetic mouse model reduced overall body weight, partially protected the glomerular endothelial surface glycocalyx layer and prevented glomerular capillary loop destruction. While during the FMD cycles fatty acid as a source of energy completely took over, loss of body mass was observed predominantly through loss of muscle mass instead of fat mass. At renal glomerular level, metabolite presence was not preserved in FMD mice when compared to diabetic and control mice. Here, presence of reduced fatty acids, protein breakdown products and UDP-GlcUA, key glycosaminoglycan substrate, were accompanied by increased oxidative stress.

After inducing diabetes, we observed that periodic FMD was able to protect the glomerular capillaries and its luminal glycocalyx, although with regard to the latter only a partial preservation was found as surface hyaluronan expression was not preserved. Although FMD could have a positive effect in inducing glycocalyx biosynthesis [16], the reduced amounts of one of its substrates, UDP-GlcUA, would prevent optimal hyaluronan synthesis by the main endothelial hyaluronan synthase 2 (HAS2) [21]. Meanwhile, other glycosaminoglycans such as heparan sulfate- and chondroitin sulfate glycosaminoglycans still could benefit, since these molecules are produced intracellularly within the Golgi-system, which exerts specific transporters to keep such substrates in high concentrations [22, 23]. Despite the reduced luminal hyaluronan presence, glomerular capillary rarefaction was prevented, although mice on the periodic FMD intervention in the continuous diabetic environment showed increasing urine production [16, 24].

During the FMD cycles, a metabolic switch to predominant lipid oxidation as energy source was induced, with an extensive reduction in blood glucose levels and body weight during the fasting days. This weight loss was dominantly caused by a reduction in lean mass, which mainly consisted of a significant loss of total water and muscle mass. Negative effects of fasting on muscle mass have been a big concern in fasting methods [25]. Several studies showed that adequate protein intake during weight loss regimes mitigate the loss of lean mass [26, 27]. Moreover, the FMD used in the current study has a very low amino acid/protein content [2, 6], which may enhance the risk of losing muscle mass during the fasting periods [28, 29]. In addition, a great reduction in total water was seen during the FMD cycles. In combination with increased urine production in the FMD mice, it suggest that the diet induces excessive water loss. Interestingly, during the overall 10 week period, the diabetic mice receiving the FMD showed additional weight loss on top of the reduced weight gain observed between diabetes- and control mice. With an overall raise in urine production and reduced muscle mass found, both fluid- and protein homeostasis

affected by the repeated FMD cycles could have exerted a lasting effect beyond the short periods of calorie restriction.

Following the overall total body metabolic assessment, we also used mass spectrometry imaging to specifically identify the glomerular metabolic status [14]. In a subset of control and diabetic kidney samples from our current study, we could determine diabetic related metabolic changes throughout the kidney, especially of small molecule metabolites and glycero(phoso)lipids in downstream nephron segments [14]. Although we observed in the total kidney samples a relative higher acetoacetate expression in both diabetes and FMD-treated mice compared to control (data not shown), focusing on glomeruli we observed in both diabetes- and FMD-treated mice overall lower metabolite levels, reduced protein catabolism breakdown products and increased oxidative stress markers. In particular, ophthalmic acid, a reduced breakdown product of glutathione turnover related to diminished glutathione synthesis, has been found in patients with uncontrolled diabetes [30, 31].

Our study has also several limitations. Despite the earlier positive effects observed in experimental type 1 and type 2 mouse models [5, 6], we did not observe changes in diabetes state nor an overall favourable effect on renal glomerular function. With regard to our STZ induced diabetes model we allocated only stable hyperglycaemic mice after 2 weeks to both the diabetic study arms. In our previous studies in the STZ-induced diabetes model on enriched cholesterol chow, we also did not observe recovery form the diabetes phenotype [12, 13]. Of note, in these previous studies we did observe a lasting increased ACR with increased mesangial surface area expansion and reduced nephrin expression, all for up to week 26. These overall changes coincided with loss of glomerular cationic ferritin and anti-HS antibody staining, intraluminal lectin staining (LEA), and increased glomerular HPSE and CTSL (cathepsin L) presence, all lacking in our current model. We observed that the diabetic model we used previously already displayed increased fatty-acid oxidation in diabetic mouse indiscernible from FMD. By administration of insulin to keep blood glucose levels within a preferred range, i.e. between 15-20mmol/L, we now could observe clear differences in metabolic state between diabetic and FMD mice. Unfortunately, this resulted in a very mild renal phenotype with only changes in glomerular capillary number and in glomerular endothelial glycocalyx coverage. Despite this limitation, our current model provided enough evidence to test for preservation of the glomerular endothelial cells and to observe the limited metabolic changes in the kidney in such a mild diabetic environment.

In conclusion, repeated FMD cycles in a STZ-induced diabetic mouse model, accompanied by periodic metabolic switches to overt lipid oxidation for energy, resulted in overall weight loss and prevented partially glomerular endothelial surface glycocalyx and capillary loss. However, weight loss was mainly found in lean and not fat mass and it did not

prevent glomerular metabolic changes as could be observed in diabetic mice. In diabetic mice after repeated FMD cycles, we still observed increased oxidative stress and reduced UDP-GlcA presence, possibly hampering hyaluronan synthesis.

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Supplemental Table 1. List of significantly different metabolites in mouse kidney samples assigned by the mass measurements using both MALDI-TOF/TOF and MALDI-FTICR MS in negative ion mode.

calibrated m/z from MALDI-TOF/TOF	m/z from MALDI-FTICR	Ion	Annotation*	Theoretical m/z	Formula	ppm
193,0192	NA	[M-H]-	Methyl 1-(1-propenylthio)propyl disulfide	193,0185	C7H14S3	4
203,0676	**NA**	**[M-H]-**	**Glycyl-Glutamate/Alanylaspartic acid/Glutamylglycine/Aspartyl-Alanine/Alanyl-Aspartic acid/gamma-Glutamylglycine/L-beta-aspartyl-L-alanine**	**203,0673**	**C7H12N2O5**	**1**
241,0568	NA	[M-H]-	Creatinine aspartate	241,0578	C8H10N4O5	4
245,1134	**NA**	**[M-H]-**	**Valylglutamic acid/gamma-Glutamylvaline/Glutamylvaline/Leucyl-Aspartate/Isoleucyl-Aspartate/Aspartyl-Leucine/Aspartyl-Isoleucine/ L-beta-aspartyl-L-leucine**	**245,1143**	**C10H18N2O5**	**4**
249,0543	**NA**	**[M-H]-**	**Cysteinyl-Glutamate/Glutamylcysteine/N-gamma-Glutamylcysteine/ gamma-Glutamylcysteine**	**249,0551**	**C8H14N2O5S**	**3**
273,0936	NA	[M-H]-	N-(2-(3,4-Dichlorophenyl)ethyl)-N-methyl-2-(dimethylamino) ethylamine	273,0931	C13H20Cl2N2	2
275,0861	**275,0863**	**[M-H]-**	**Glutamylglutamic acid/D-gamma-Glutamyl-D-glutamic acid/ Thymidine glycol/gamma-Glutamylglutamic acid**	**275,0885**	**C10H16N2O7**	**8**
279,0369	NA	[M-H]-	**Benzofuran isophthalate	279,0299	C16H8O5	25
288,1197	**NA**	**[M-H]-**	**Ophthalmic acid**	**288,1201**	**C11H19N3O6**	**1**
302,1313	**NA**	**[M-H]-**	**Chlorphenoxamine/Retigabine/Phenoxybenzamine**	**302,1317**	**C18H22ClNO**	**1**
317,1441	**NA**	**[M-H]-**	**Imidazolone A**	**317,1467**	**C12H22N4O6**	**8**
327,2301	327,2306	[M-H]-	Docosahexaenoic acid	327,233	C22H32O2	7
346,0498	346,0533	[M-H]-	AMP	346,0558	C10H14N5O7P	17
362,0446	362,0482	[M-H]-	GMP	362,0507	C10H14N5O8P	7
392,1459	**392,1469**	**[M-H]-**	**gamma-Glutamylfelinylglycine**	**392,1497**	**C15H27N3O7S**	**7**
404,0078	404,0094	[M+Cl]-	Thioflosulide	403,9999	C16H13F2NO3S2	24
435,1134	**435,1165**	**[M-H]-**	**Bis-(1,3-diethylthiobarbituric acid)trimethine oxonol**	**435,1166**	**C19H24N4O4S2**	**0**

Supplemental Table 1. List of significantly different metabolites in mouse kidney samples assigned by the mass measurements using both MALDI-TOF/TOF and MALDI-FTICR MS in negative ion mode. *(continued)*

calibrated m/z from MALDI-TOF/TOF	m/z from MALDI-FTICR	Ion	Annotation*	Theoretical m/z	Formula	ppm
465,3012	465,3015	[M-H]-	LPA(20:0)	465,2987	C23H47O7P	5
525,2793	**525,2824**	**[M-H]-**	**LPE(22:6)****	**525,2817**	**C27H44NO7P**	**1**
552,2737	**552,2707**	**[M-H]-**	**Vignatic acid A**	**552,2715**	**C30H39N3O7**	**1**
579,0235	579,0249	[M-H]-	UDP-GlcUA	579,027	C15H22N2O18P2	4
721,5017	721,4737	[M-H]-	PA(38:5)	721,4814	C41H71O8P	11
737,5024	NA	[M-H]-	SM(d16:1/20:3(5Z,11Z,14Z)-O(8,9))/SM(d16:1/20:4(6E,8Z,11Z,14Z)-OH(5S))	737,5239	C41H75N2O7P	12
747,5204	747,5197	[M-H]-	PG(34:1)	747,5182	C40H77O10P	2
833,5226	**833,5206**	**[M-H]-**	**PI(34:2)**	**833,5186**	**C43H79O13P**	**2**
834,5216	834,5285	[M-H]-	PS(40:6)	834,5291	C46H78NO10P	1
835,535	835,5298	[M-H]-	PI(34:1)	835,5342	C43H81O13P	5
838,5605	838,5572	[M-H]-	PS(40:4)	838,5604	C46H82NO10P	4
857,5194	**857,5165**	**[M-H]-**	**PI(36:4)**	**857,5186**	**C45H79O13P**	**2**
909,5529	909,553	[M-H]-	PI(40:6)	909,5499	C49H83O13P	3
882,5253	**882,5216**	**[M-H]-**	**PS(44:10)**	**882,5291**	**C50H78NO10P**	**8**

* only one representative lipid name was shown for some m/z features

** lipid isotope

§ Breakdown product of protein catabolism

Not a natural metabolite, part of exposome

Bold; metabolites in diabetes and FMD groups are both significantly different from control

Supplemental Table 2. Measurements of significantly different metabolites in mouse kidney sample

Calibrated m/z from MALDI-TOF/TOF	Peak intensity control (A.U.)						Peak intensity diabetes (A.U.)						Peak intensity FMD (A.U.)					
	1	2	3	4	mean	stdev	1	2	3	4	mean	stdev	1	2	3	4	mean	stdev
193,02	1,679	2,015	2,524	1,809	2,007	0,372	6,114	1,528	3,239	2,018	3,225	2,056	3,235	3,105	2,914	2,592	2,961	0,279
203,07	2,132	3,419	1,895	2,939	2,596	0,708	1,198	1,030	1,091	1,048	1,092	0,076	0,972	1,128	1,179	1,202	1,120	0,104
241,06	4,045	3,296	2,023	2,286	2,912	0,934	1,629	1,944	1,845	1,829	1,812	0,132	1,235	1,322	1,555	1,373	1,371	0,135
245,11	1,090	1,016	0,810	1,202	1,029	0,165	0,639	0,707	0,633	0,670	0,662	0,034	0,462	0,613	0,683	0,552	0,577	0,094
249,05	2,277	3,097	2,399	3,222	2,749	0,480	1,681	1,299	1,360	1,343	1,420	0,175	1,242	1,475	1,580	1,263	1,390	0,165
273,09	0,946	0,841	1,082	0,929	0,949	0,100	1,058	1,232	1,444	1,667	1,350	0,264	0,936	1,152	1,164	0,953	1,051	0,124
275,09	9,283	10,564	9,445	11,023	10,079	0,849	5,692	5,557	6,655	5,537	5,860	0,534	4,806	6,076	7,033	5,583	5,874	0,933
279,04	0,844	0,891	0,761	0,752	0,812	0,067	0,685	0,556	0,676	0,668	0,646	0,061	0,685	0,703	0,717	0,642	0,687	0,033
288,12	0,692	0,745	0,572	0,821	0,708	0,105	0,472	0,398	0,542	0,531	0,486	0,066	0,445	0,480	0,559	0,421	0,476	0,060
302,13	0,929	1,085	0,923	1,096	1,008	0,095	0,726	0,870	0,718	0,736	0,763	0,072	0,508	0,762	0,763	0,617	0,662	0,124
317,14	0,888	0,963	0,805	1,030	0,922	0,097	0,638	0,614	0,626	0,682	0,640	0,030	0,478	0,584	0,697	0,553	0,578	0,091
327,23	0,823	0,741	0,654	0,622	0,710	0,091	0,552	0,702	0,587	0,488	0,582	0,090	0,485	0,488	0,600	0,449	0,505	0,066
346,05	32,576	34,299	24,874	28,267	30,004	4,258	21,570	30,909	20,466	25,398	24,586	4,716	13,517	20,229	15,993	19,903	17,410	3,231
362,04	2,122	2,347	1,849	1,921	2,060	0,224	1,569	2,128	1,830	1,687	1,804	0,241	1,428	1,457	1,364	1,714	1,491	0,154
392,15	0,634	0,641	0,569	0,602	0,611	0,033	0,445	0,414	0,521	0,506	0,472	0,050	0,438	0,478	0,564	0,416	0,474	0,065
404,01	2,344	2,145	1,638	2,093	2,055	0,299	1,299	2,309	1,499	1,602	1,677	0,439	0,857	1,276	1,290	1,568	1,248	0,293
435,11	4,288	4,357	4,086	3,574	4,076	0,354	2,640	3,248	3,380	3,207	3,119	0,327	2,460	2,997	2,517	1,850	2,456	0,471
465,30	1,946	2,057	2,147	1,424	1,894	0,323	2,245	2,242	2,773	2,123	2,346	0,290	2,896	3,025	2,423	2,366	2,677	0,332
525,28	5,464	5,256	5,335	4,847	5,226	0,267	4,844	4,647	4,726	4,260	4,619	0,253	3,923	3,808	4,821	3,671	4,056	0,521
552,27	11,836	11,578	12,412	11,374	11,800	0,450	9,897	10,121	8,729	9,294	9,510	0,627	6,765	7,066	9,486	8,509	7,957	1,272
579,02	0,420	0,446	0,470	0,478	0,453	0,026	0,333	0,345	0,395	0,409	0,371	0,037	0,340	0,380	0,454	0,324	0,375	0,058
721,50	0,814	0,790	0,830	0,814	0,812	0,016	0,753	0,652	0,723	0,679	0,702	0,045	0,699	0,651	0,825	0,687	0,716	0,076
737,50	0,702	0,744	0,748	0,708	0,726	0,024	0,834	0,762	0,891	0,800	0,821	0,055	0,800	0,784	0,857	0,832	0,819	0,033
747,52	4,855	4,402	4,679	5,198	4,783	0,333	3,874	4,209	3,819	3,646	3,887	0,236	3,556	2,928	4,662	4,831	3,994	0,908
833,52	2,075	2,181	1,940	1,787	1,996	0,171	1,650	1,613	1,559	1,478	1,575	0,074	1,537	1,239	1,511	1,700	1,497	0,191
834,52	5,732	5,874	6,343	4,578	5,632	0,749	5,199	5,209	5,183	4,825	5,104	0,187	4,288	3,914	4,606	4,472	4,320	0,301
835,54	3,669	3,726	4,253	2,946	3,649	0,537	3,286	3,344	3,315	3,017	3,240	0,151	2,792	2,535	2,937	2,952	2,804	0,193
838,56	5,012	6,482	7,568	3,916	5,744	1,607	6,564	6,103	6,872	6,131	6,417	0,369	5,691	5,602	5,491	5,165	5,487	0,230
857,52	5,157	5,341	4,819	4,395	4,928	0,416	4,073	4,181	4,342	3,671	4,067	0,286	3,689	3,225	4,006	4,034	3,738	0,376
909,55	2,819	2,170	1,886	1,871	2,186	0,443	1,840	1,869	1,341	1,198	1,562	0,343	1,217	1,031	1,521	1,242	1,253	0,202
882,53	0,944	0,836	0,799	0,764	0,836	0,078	0,713	0,720	0,665	0,618	0,679	0,048	0,606	0,574	0,755	0,642	0,644	0,079

Bold metabolites in diabetes and FMD groups are both significantly different from control

CHAPTER 7

Summary and General Discussion

Summary

The endothelial glycocalyx covers the endothelium throughout the whole vasculature. After years of research, the important functional role of the glycocalyx in vascular homeostasis and several pathophysiological processes is widely accepted. In diabetes, perturbation of the glycocalyx is related to the pathogenic diabetic milieu with elevated glucose, increased lipids and circulating inflammatory stimulants. Sustained exposure to this diabetic milieu facilitates activation of endothelial cells, with influx of macrophages in response to MCP-1 and heparan sulphate breakdown by HPSE-1, inducing alterations in the endothelial glycocalyx [1, 2]. Consequently, alterations within the endothelial glycocalyx is proposed as an early hallmark for vascular dysfunction. Detecting and monitoring these changes can be used to identify early risk for the development of vascular complications or monitor the effects of therapeutic interventions. As some ethnic groups are more prone to develop vascular complications [3, 4], unravelling the pathogenesis of these cardiovascular health disparities and investigating interventions aimed at reducing cardiovascular risk could lead to improved cardiovascular risk management.

This thesis studied several aims: (1) can detection of endothelial glycocalyx changes be used as a diagnostic marker, (2) explore the relation between the two biomarkers, HPSE-1 and MCP-1, and microvascular glycocalyx changes in type 2 diabetes mellitus (T2DM) and (3) whether dietary interventions result into measurable microvascular restoration in diabetes.

Study findings

One of the first signs of cardiovascular disease are changes in the microcirculation. In **chapter 2** we showed that obese individuals at high risk for developing cardiovascular disease, defined by the Framingham risk score, were characterized by changes in the microvasculature. A new software system allowed the measurements of capillary red blood cell velocity and analyzed the capillaries according to diameters class (each 1 µm). These measurements were incorporated into new microvascular parameters. We explored if correcting the PBR for red blood cell velocity minimized the variability in the estimated PBR. However, we still found high variability and overlap in the PBR between the Framingham risk groups. It would be expected that the PBR was increased in individuals in the high-risk group, reflecting degradation of the endothelial glycocalyx. The high variability within- and between the risk groups may be to individual variability in the PBR or compensatory increased glycocalyx synthesis. Although the risk groups had overlap in microvascular parameters, the highest risk group was characterized by loss of the smallest capillaries. This was accompanied by an increase in red blood cell velocity indicating

impaired capillary recruitment in the high risk group. Within this study, we show that detecting early microvascular changes using SDF-imaging, can be a useful tool to monitor individuals before the development of cardiovascular disease.

MCP-1 and HPSE-1 are upregulated in patients with diabetic nephropathy [5-7]. Through increasing influx of monocytes to areas of inflammation and modifying the HS glycosaminoglycans, these factors can perturb the glomerular filtration barrier, inducing urinary protein leakage. Besides possible increased plasma levels, urinary levels of MCP-1 and HPSE-1 activity could reflect the degree of inflammation and glomerular damage in the diabetic kidney. As there are striking ethnic differences in the development and progression of diabetic nephropathy, we studied such ethnic differences in a multi-ethnic cohort of individuals with T2DM measuring urinary MCP-1 and HPSE-1 activity levels in **chapter 3.** Of the 6 ethnicities (Dutch, South Asian- and African Surinamese, Ghanaian, Moroccan and Turkish), Moroccan and South-Asian Surinamese participants had higher urinary MCP-1 levels compared to the Dutch, independently of confounding factors. In addition, urinary MCP-1 levels were associated with albumin-creatinine levels in all ethnic minorities, confirming the link between renal inflammation and albuminuria in diabetic nephropathy. These associations were the strongest in diabetic individuals from South-Asian and African Surinamese descent. In contrast, low urinary HPSE-1 activity levels were found in all participants. Compared to Dutch, African Surinamese and Ghanaian had lower urinary HPSE-1 activity levels. Only in Dutch participants, HPSE-1 activity levels correlated with the degree of albuminuria.

In **chapter 4,** we investigated two dietary interventions, a supplement to strengthen the endothelial glycocalyx or a fasting mimicking diet (FMD) to strengthen cellular response, in South-Asian Surinamese patients with T2DM. At baseline, we observed that South-Asian Surinamese T2DM patients have a reduced functional capillary network compared to the pre-clinical cohort in chapter 2, reflecting poor microvascular health in South Asian individuals. After 3 months of supplementation with glycocalyx mimetics, the RBC-velocity independent $PBR_{dynamic}$ and overall microvascular health score improved in the South Asian Surinamese T2DM patients. No changes were seen in clinical markers such as blood pressure and albuminuria, or in glycocalyx degrading markers such as MCP-1 and HPSE-1 activity. This showed that supplementation with glycocalyx mimetics is able to improve the endothelial glycocalyx beyond the HPSE-1 inhibiting effects that has been shown *in vitro*.

In contrast, after 3 months of a monthly 5-day FMD regime, BMI and Hba1c levels were significantly lower compared to baseline. However, endothelial markers worsened after the diet intervention, revealed by an increase in $PBR_{dynamic}$ and a decrease in capillary blood volume and microvascular health score. No differences were seen in albumin-creatinine

ratio, MCP-1 or HPSE-1 levels after the fasting mimicking diet. As one patient experienced an temporary decline of the kidney function after the FMD cycle, the FMD should be used with caution in patients with decreased kidney function.

Parallel to this clinical intervention study we also tested both dietary interventions in a diabetic mouse model, with specific emphasis on the possible effects on the diabetic kidney. In **chapter 5 and 6,** we investigated the effect of the supplement and fasting mimicking diet on the glomerular endothelial glycocalyx in a streptozotocin-induced diabetic nephropathy mouse model in ApoE-KO mice on a standard diet enriched with cholesterol. After 10 weeks of supplementation with glycocalyx mimetics, the glomerular endothelial surface coverage with heparan sulfate and hyaluronan was preserved and capillary loop rarefaction was prevented compared to control diabetic mice (**chapter 5**). These effects were accompanied with a reduction of CD11b positive renal tissue macrophages and dendritic cells, while the major myeloid cell population macrophage-like dendritic cells were not affected. During this 10-week period in both diabetic control mice and diabetic mice receiving the glycocalyx mimetics, increased levels of albuminuria and HPSE-1 activity returned to normal levels, without significance between the two groups. In **chapter 6** we showed that the diabetic ApoE-KO mice switched to lipid metabolism during the fasting mimicking cycles, which was accompanied with reduced glucose levels and loss of lean mass. After 5 FMD cycles, glomerular endothelial glycocalyx breakdown and the loss of capillaries was partially prevented (not for hyaluronan presence). However, metabolite analysis revealed increased oxidative stress and reduced presences of hyaluronan precursors, suggesting negative effects on kidney metabolism after the FMD cycles. In contrast to our previous studies with this mouse model, we monitored and maintained blood glucose levels to preferred set glucose levels (15-20 mmol/L) throughout the study in chapter 5 and 6. These set glucose levels were used since pilot experiments revealed that STZ-induced diabetes in the apoE-KO mice was accompanied by blood glucose levels above the detection limit and a total body metabolic switch already to predominant lipid oxidation. While our strategy prevented the preliminary shift to lipid usage for energy metabolism, it resulted in a much milder nephropathic disease phenotype. Along with reduced morphologic changes, maintaining set glucose levels also could have played a role in reducing proteinuria during the experimental period.

Endothelial glycocalyx as a diagnostic marker for microvascular health

In this thesis, we show that changes in the sublingual endothelial glycocalyx can be detected before the onset of clinical signs of cardiovascular disease. In recent years, changes in the sublingual microcirculation has been studied in specific patient groups and correlated with disease severity. In sepsis patients, microvascular changes are more prominent and sublingual microvascular changes have a high correlation with disease severity [8]. In patients with diabetes, microvascular changes were associated with an increased coronary

artery calcium score [9] and with the degree of albuminuria [10]. We revealed that several microvascular changes, such as loss of the smallest sublingual capillaries, can be detected in a pre-clinical cohort with individuals at risk for developing cardiovascular disease. This loss of capillaries is a phenomenon that is called capillary rarefaction, i.e. a decrease in functional capillary density. Functional rarefaction is a reversible state with arteriolar vasoconstriction leading to less perfusion of capillaries whereas structural rarefaction is the irreversible anatomic loss of capillaries. It is considered to be a sign of endothelial dysfunction and has been linked to cardiovascular disease [11]. A lower functional and total perfused capillary density in several microvasculature beds has been found in patients with hypertension, chronic heart failure, diabetes and chronic kidney disease [12-14]. It would be interesting to investigate whether the microvascular parameters that we found can be used for risk stratification for developing cardiovascular disease in the future. One study with a 6 year follow up in a cohort without established cardiovascular disease was the first study that showed that the PBR_{static} was associated with the risk of future cardiovascular events even after adjustment for conventional atherosclerotic risk factors [15]. However, more studies need to be conducted in the future to corroborate these findings.

In the current study, the new software included measurements of the longitudinal RBC velocity (V_{RBC}). This resulted in several new parameters such as: $PBR_{dynamic}$ (PBR corrected for variation in local V_{RBC}), V_{RBC} in capillaries and in feed vessels (> 10µm), perfused capillary density, capillary blood volume. While current calculations are still dependent on per-group slope calculations between V_{RBC} and PBR_{static} to get $PBR_{dynamic}$, new recording and analysis strategies are under investigation to allow these parameters calculated from intra-person measurements. This will result in a more person specific estimate of endothelial glycocalyx health.

In addition, for use in clinical practice, specific cut off points for interpretation of the microvascular parameters have yet to be determined. This will make it more accessible to use the technique in daily practice.

Concluding, there is a role for the endothelial glycocalyx to be used as a diagnostic marker for microvascular health. Ultimately, it may even have a role in the cardiovascular risk management (CVRM) in current practice, identifying and monitoring individuals at risk for developing cardiovascular disease.

MCP-1 and HPSE-1 in type 2 diabetes mellitus

Monocyte chemoattractant protein 1 (MCP-1) and heparanase-1 (HPSE-1) are upregulated in diabetes and result in endothelial activation and glycocalyx perturbation. Several studies found that MCP-1 and HPSE-1 are released in renal tissue and urine of patients with T2DM, correlating with the degree of albuminuria and kidney function [5-7, 16-19].

The ROADMAP study even showed that serum and urinary MCP-1 levels were a strong independent predictor for the development of albuminuria over time, suggesting that elevated MCP-1 levels may be an early indicator of diabetic nephropathy [20]. In the HELIUS study in chapter 3, we corroborated this association with urinary MCP-1 levels and degree of albuminuria in type 2 diabetic individuals from different ethnic origins. These findings reveal that urinary MCP-1 activity can also be used as a biomarker for diabetic nephropathy in these ethnic groups and indicate that these patients may also benefit from therapeutic interventions aimed at inhibiting MCP-1 activity, such as Emapticap [21, 22].

In our studies, low urinary HPSE-1 activity levels were found in diabetic patients from different ethnic origins (chapter 3) and our cohort of South Asian Surinamese patients (chapter 4). Increased glomerular and tubular HPSE-1 expression has been found in T2DM patients, but the histological findings have not been correlated with the urinary HPSE-1 activity levels [5, 23]. It therefore needs to be established whether urinary HPSE-1 activity levels actually reflect the local HPSE-1 expression in kidney and how much of this activity is still detectable in urine. Given previous results in literature, we expected higher HPSE-1 urinary and plasma levels in patients with T2DM than we observed in the clinical studies in chapter 3 and 4. We used a TAKARA HPSE ELSA kit, a sandwich ELSA that measures HPSE activity by detecting cleaved HS fragments in supernatant. A probable disadvantage of this method is that HPSE-1 could already be bound to shedded HS, from other downstream renal tubular or bladder cells, as every cell in the urinary tract expresses heparan sulphates. In the presence of high HS levels in urine samples the current ELSA possibly will give an underestimation of the amount of active HPSE-1. Therefore, a standard test to detect active HPSE-1 and inactive pro-HPSE-1 directly in urine is needed in future research [1, 24]. One such approach could be the use of activity-based probes (ABPs). Activity-based probes are designed to covalently bind to the active cleavage site of the target molecule, allowing detection, visualization and even inactivation of the enzymatic activity. A β-glucuronidase specific ABP has been developed for rapid and quantitative visualization of HPSE-1 in biological samples [25]. This ABP has the ability to label active HPSE-1 but also the inactive pro-HPSE-1, even in the presence of shed HS. Labelling and quantifying both active and non-active HPSE-1 is of importance as it gives a better understanding on real HPSE-1 release in urine or tissue. The current method to detect and analyse urinary HPSE-1 may not be sufficient and is not yet suitable as a biomarker in type 2 diabetes.

Dietary interventions to restore the endothelial glycocalyx

In the introduction, we elucidated on several therapeutic interventions aimed at restoring the endothelial glycocalyx, by inhibiting heparanase activity or supplementing glycocalyx substitutes. Several of those interventions, mainly heparanase inhibitors, are not yet approved for clinical trials. Only sulodexide, a heparan sulphate mimetic, has been studied in clinical trials [26, 27].

We investigated if two dietary interventions were able to restore the endothelial glycocalyx or reduce glycocalyx breakdown in a clinical trial with South-Asian Surinamese with type 2 diabetes and in a mouse model of diabetes. The first intervention was a food supplement, consisting of glycocalyx components such as hyaluronic acid and fucoidan, a heparan sulphate mimetic. In the experimental study, we showed that supplementation with glycocalyx mimetics preserved the glomerular endothelial surface coverage of heparan sulfate and hyaluronan and capillary loop rarefaction was prevented. In the clinical study, the PBR, an indirect marker of the endothelial glycocalyx, improved after supplementation with glycocalyx mimetics. These result suggest that the supplement is able to partially restore the endothelial glycocalyx, or prevent further damage to the glycocalyx induced by the diabetic environment. We previously showed that fucoidan was able to restore the endothelial glycocalyx and permeability barrier in human pulmonary microvascular endothelial cells *in vitro* [28]. The *in vitro* study in chapter 4 showed that fucoidan was able to dose dependently inhibit HPSE-1 activity in glomerular endothelial cells. Despite this, in both the experimental and clinical study, no significant improvement in HPSE-1 or albumin-creatinine levels were detected. Interestingly, in 2 patients with macro-albuminuria at baseline, albuminuria levels dropped to normo-albuminuria after 3 months of daily supplementation with glycocalyx mimetics. Larger studies with more patients are needed to corroborate these findings, investigating if fucoidan supplementation can restore the glomerular barrier function and reduce albuminuria in individuals with diabetes. Long-term follow-up studies are needed to investigate if the supplement is able to slow down the progression of cardiovascular disease. An advantage of supplementation with glycocalyx mimetics is that the supplement consists of natural ingredient as opposed to other therapeutic interventions, with no major side effects in the clinical setting. If proven effective in larger trials, it might be used as an add-on therapeutic intervention in the battle against development of cardiovascular disease in patients with diabetes.

Fasting interventions have become increasingly popular over the years for their potential to improve longevity and cardiometabolic risk factors [29-31]. We investigated a periodic 5 day fasting regime that previously revealed to improve cardiovascular and diabetes risk factors in a healthy population [32]. Besides this, it is believed that cellular pathways that regulate autophagy and reduce inflammation are upregulated after periods of fasting [31].

In the clinical study, 3 FMD cycles showed beneficial effects on BMI and Hba1c levels. In contrary to the earlier published clinical study with the FMD in healthy persons, systolic blood pressure, inflammatory markers such as hs-CRP or HPSE-1 and albuminuria were not affected. In one patient in our study, kidney function deteriorated on day 5 of one FMD cycle. Kidney function recovered after intravenous fluid resuscitation, pointing at dehydration as the probable cause of this decline in kidney function. Interestingly, in the mouse model, water and muscle loss was the main source of weight loss during the FMD

cycles. The loss of water during fasting may be due to glycogen depletion in the liver and muscles, which is mainly bound to water. More than half of the weight loss that can occur after fasting periods are a result of the loss of this excess water, which has been found by several studies [33, 34]. In addition, no positive effects were seen on the microvasculature, as the PBR worsened after the FMD cycles. In the mouse model, in dept metabolite analysis revealed negative effects on kidney metabolism with increased oxidative stress and reduced UDP-GlcA levels, a precursor of hyaluronan. Reduced levels of UDP-GlcA could prevent optimal hyaluronan synthesis, impairing endothelial glycocalyx integrity.

An advantage of this FMD is that it is easier to adhere compared to continues diet regimes, while still having positive effects on weight loss and glucose levels. However, we observed that these effects disappeared when the diet regime was discontinued. This is a common phenomenon for diet intervention studies when individuals are not monitored as intensively as in a clinical trial, which underlies challenges for the use of lifestyle interventions in daily practice [35, 36]. Overall, FMD is not suitable as an intervention to restore the endothelial glycocalyx and may even have adverse effects on the microvasculature and glycocalyx integrity. Furthermore, we advise that the FMD should not be used or used with caution in patients with CKD due to the possibility of dehydration.

Other interventions aimed at reducing HPSE-1 activity may be more promising to target the endothelial glycocalyx. A recent study showed that the use of HPSE-2 protein and peptides prevented streptozotocin-induced kidney injury, showing possible HPSE-1 inhibiting effects in this diabetic nephropathy mouse model [37]. The above mentioned activity-based probes also could be a possible therapeutic intervention, as the probes can covalently and irreversible bind to the active HPSE-1 enzyme and inhibit its activity. Recently, the first promising proof of principle experimental study with a selective covalently binding HPSE-1 inhibitor has been published in the field of cancer research [38]. It remains to be seen if these therapeutic interventions emerge as a possible therapy in type 2 diabetes.

Challenges for South-Asian Surinamese individuals

In the current thesis, we investigated individuals with type 2 diabetes from South-Asian Surinamese descent. As mentioned in the introduction, South-Asians are more prone to develop diabetes and cardiovascular complications, despite standard therapeutic management. The onset and progression of cardiovascular complications remains higher compared to individuals from European descent. Type 2 diabetes affects South-Asians at an earlier age and at a lower BMI, they have a higher rate of myocardial infarction, a higher prevalence of proteinuria and a faster decline in eGFR compared to individuals of European descent [39, 40]. The cardiovascular health burden of South-Asians has been

well described over the years, however, representation in clinical trials, adequate risk calculators and therapeutic strategies are lacking.

South-Asians are a difficult group to recruit for randomized controlled trials, especially for lifestyle intervention studies. Conducting lifestyle intervention studies in the South-Asian population has been proven to be extremely difficult due to low response rates, high drop-out rates and conflicting effects. An intensive 1 and 2 year targeted lifestyle intervention in general practice revealed no significant weight loss or improvement in metabolic profiles in South-Asian Surinamese participants in The Hague [41, 42]. In our study, we also experienced a low response rate but did achieve significant improvement of BMI and Hba1c after 3 months. A meta-analysis with randomized controlled trials also showed that lifestyle interventions can be successful to reduce the risk of diabetes in South-Asian individuals [43]. A large part of the current studies conducted with South-Asians are directed towards preventive lifestyle interventions [44, 45]. Other studies are directed towards pathophysiological explanations for the disparities in cardiovascular or diabetes risk. Altered high-density lipoprotein composition [46] or different inflammatory pathways [47] are examples of recent discovered differences between South-Asian and European individuals. Therefore, unravelling the differences in pathophysiology of cardiovascular complication development may lead to more tailored and successful therapeutic interventions for South-Asians in the future.

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CHAPTER 8

Nederlandse samenvatting
List of publications
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Dankwoord

Nederlandse samenvatting

Patiënten met diabetes hebben een verhoogde kans op het ontwikkelen van complicaties in de bloedvaten. Dit kan zowel in de grote- (macrovasculaire) als in de kleine (microvasculaire) bloedvaten zijn. De haarvaten, de kleinste bloedvaten van het lichaam, zijn onderdeel van het microvasculaire stelsel, waar de uitwisseling van voedingstoffen en zuurstof met het omliggende weefsel plaatsvindt. Aan de binnenkant van alle bloedvaten liggen de endotheelcellen, een cellaag die wordt bedekt door een belangrijke suikerlaag, de glycocalyx, welke onder andere bescherming biedt tussen het stromende bloed en het bloedvat. Door jaren van onderzoek is de belangrijke rol van de endotheliale glycocalyx in de gezondheid van de bloedvaten duidelijk geworden. Bij patiënten met diabetes wordt de microcirculatie beschadigd door een verstoord intern milieu als gevolg van hoge bloed glucosewaarden, circulerende inflammatoire cytokinen en oxidatieve stress. Aanhoudende blootstelling aan dit diabetische milieu activeert de endotheelcellen, waarbij er een verhoogde interactie is met circulerende witte bloedcellen en bloedplaatjes. Onder invloed van monocyt chemoatractant-1 (MCP-1) worden monocyten aangetrokken en door heparanase-1 (HPSE-1) wordt heparaan sulfaat in de glycocalyx afgebroken, wat leidt tot structurele veranderingen in de endotheliale glycocalyx. Deze veranderingen in de endotheliale glycocalyx worden gezien als de eerste kenmerken van microvasculaire dysfunctie. Het detecteren van deze vroege veranderingen in de microcirculatie kan worden gebruikt om het risico op het ontwikkelen van cardiovasculaire complicaties in te schatten, of om het effect van therapeutische interventies te monitoren. Veranderingen in de microcirculatie en endotheliale glycocalyx kunnen worden gedetecteerd met een camera, die door middel van de “sidestream darkfield imaging” (SDF-beeldvorming) techniek rode bloedcellen (RBCs) kan visualiseren. Deze camera kan de sublinguale microcirculatie, een bloedvat netwerk onder de tong, in beeld brengen waarbij er wordt gekeken naar het aantal vaten waardoor RBCs bewegen. Door een nieuwe software kan de snelheid van RBCs bepaald worden, terwijl berekeningen van de zijwaartse beweging van deze RBCs een indicatie geeft van de endotheliale glycocalyx gezondheid, uitgedrukt in “perfused boundary region” (PBR), een omgekeerde maat voor glycocalyx dikte en toegankelijkheid. Een hoge PBR reflecteert hierbij een ongezonde glycocalyx en daarmee mogelijke functionele veranderingen van het bloedvat.

Mensen van Surinaams-Hindoestaanse afkomst hebben meer kans op het ontwikkelen van diabetes mellitus type 2. We zien dat diabetes patiënten van Surinaams-Hindoestaanse afkomst erg vatbaar zijn voor het ontwikkelen van vasculaire complicaties. Het is belangrijk om de oorzaak van deze verhoogde vatbaarheid te achterhalen, zodat er interventies ontwikkelt kunnen worden die de snelle progressie van vasculair complicaties in deze groep tegen kunnen gaan.

Dit proefschrift onderzoekt een aantal verschillende aspecten: (1) kan de detectie van glycocalyx veranderingen worden gebruikt als een diagnostische marker, (2) wat is de rol van HPSE-1 en MCP-1 als biomarker (indicator) in patiënten met type 2 diabetes mellitus (T2DM) en (3) kunnen dieetinterventies leiden tot een meetbaar herstel van de glycocalyx bij patiënten met T2DM.

In **hoofdstuk 2** onderzoeken we of individuen met een verhoogd risico op het ontwikkelen van cardiovasculaire ziekte, gedefinieerd door de Framingham risico score, gekenmerkt worden door veranderingen in de sublinguale microcirculatie. In de nieuwe software is het nu mogelijk om de snelheid van RBCs in de microcirculatie te meten en deze bloedvaatjes te analyseren op basis van diameterklasse (elke 1 µm, van 4- tot 25 µm). Met deze metingen is het mogelijk om de PBR voor de RBC snelheid te corrigeren, wat de variabiliteit in de geschatte PBR minimaliseert. Echter, we vonden nog steeds grote variabiliteit en overlap tussen de Framingham risico groepen. Hoewel de hoge variabiliteit binnen en tussen de risicogroepen te wijten kan zijn aan individuele variabiliteit in de PBR, of aan een compensatoire verhoogde synthese van de glycocalyx, zien we nu wel duidelijk dat ten opzichte van de laagste risico groep, de kleinste doorbloede haarvaten verloren gaan bij individuen met een hoog risico op het ontwikkelen van cardiovasculaire complicaties. Dit ging gepaard met een toename van de RBC snelheid, wat wijst op een verminderde rekrutering van haarvaten, en mogelijk verlies van de haarvaten in de individuen in de hoog risico groep. Met dit onderzoek tonen we aan dat het detecteren van vroege microvasculaire veranderingen met behulp van SDF-beeldvorming een nuttig hulpmiddel kan zijn om individuen te monitoren voor het ontwikkelen van cardiovasculaire complicaties.

Patiënten met diabetische nefropathie hebben een verhoogde activiteit van MCP-1 en HPSE-1 in plasma en in de urine. MCP-1 zorgt voor een instroom van monocyten in de nierfilters (glomeruli), waardoor een ontstekingsreactie optreedt. HPSE-1 breekt de heparaan sulfaten in de glycocalyx binnen deze glomeruli af, waardoor de glomerulaire filtratie barrière wordt verstoord, wat kan leiden tot eiwit lekkage in de urine. We onderzochten of de MCP-1 en HPSE-1 gehaltes in de urine de mate van ontsteking en schade in de glomeruli bij patiënten met diabetische nefropathie kan voorspellen. Omdat er opvallende etnische verschillen zijn in de ontwikkeling en progressie van diabetische nefropathie, hebben we in **hoofdstuk 3** de urine gehaltes van MCP-1 en HPSE-1 gemeten in een cohort van de HELIUS studie met individuen van verschillende etnische achtergronden met T2DM. Van de 6 verschillende etnische groepen (Nederlands, Surinaams Hindoestaans, Surinaams Afrikaans, Ghanees, Marokkaans en Turks) hadden Marokkaanse en Surinaams Hindoestaanse individuen een hogere MCP-1 activiteit in de urine in vergelijking met de Nederlanders, onafhankelijk van confounding factoren (variabelen die zowel de afhankelijke variabele als de onafhankelijke variabele beïnvloeden). Bovendien was de MCP-1 activiteit geassocieerd met de gevonden albumine-creatinine levels (maat voor eiwitlekkage in de

urine) in alle etnische groepen behalve de Nederlanders, wat de link tussen inflammatie en het ontstaan van eiwitlekkage in de diabetische nier bevestigd. Deze associatie was het sterkst in individuen van Surinaams Hindoestaanse- en Afrikaanse afkomst. Daarentegen werd in alle deelnemers lage HPSE-1 activiteit in de urine gevonden. In vergelijking met Nederlanders hadden Afrikaanse Surinamers en Ghanezen een beduidend lagere HPSE-1 activiteit in de urine. Alleen bij de Nederlanders correleerde HPSE-1 activiteit met de mate van eiwitlekkage.

In **hoofdstuk 4** onderzochten we of twee dieet interventies de endotheliale glycocalyx konden versterken of herstellen bij patiënten van Surinaams-Hindoestaanse afkomst met T2DM. De eerste interventie was een supplement met componenten van de glycocalyx (glycocalyx mimetica), met onder andere een zeewier extract (fucoidan) met een vergelijkbare structuur als heparaan sulfaat, en hyaluronzuur. De tweede interventie was een dieet van 5 dagen waarbij het effect van periodiek vasten wordt nagebootst (fasting mimicking diet; FMD), om de cellulaire weerbaarheid te verhogen. Aan het begin van de studie zagen we dat de Surinaams-Hindoestaanse patiënten minder functionele capillairen hadden in vergelijking met het cohort van hoofdstuk 2, wat wijst op een slechte microvasculaire gezondheid van de Surinaams-Hindoestaanse patiënten. Na 3 maanden dagelijks gebruik van het supplement verbeterde de snelheid onafhankelijke PBR (dynamische PBR) en de microvasculaire gezondheidsscore. Er werden geen veranderingen waargenomen in klinische parameters zoals bloeddruk, albumine-creatinine ratio, of MCP-1 en HPSE-1 activiteit in plasma en urine.

Na 3 maanden een maandelijks dieet van 5 dagen te volgen, verbeterde de body mass index (BMI) en Hba1c waarde significant in vergelijking met het begin van de studie. Echter, de microvasculaire parameters verslechterde na deze dieet interventie, waarbij een toename van de dynamische PBR en een afname van de microvasculaire gezondheidsscore werd gezien. We zagen geen verschil in bloedruk, MCP-1 of HPSE-1 activiteit na het dieet. In één patiënt werd een tijdelijke achteruitgang van de nierfunctie waargenomen op dag 5 van het dieet, dit herstelde na intraveneuze vocht toediening.

Parallel aan deze klinische interventie studies hebben we beide dieet interventies ook onderzocht in een experimenteel muis model, met speciale nadruk op mogelijke effecten in de diabetische nier, in **hoofdstuk 5 en 6**. Na 10 weken van glycocalyx mimetica suppletie aan het voer (**hoofdstuk 5**) werd de bedekking van heparaan sulfaat en hyaluronzuur in het glomerulaire endotheel oppervlak behouden en werd het verlies van de haarvaten voorkomen in vergelijking met controle muizen met diabetes. Deze effecten gingen gepaard met een vermindering van CD11b-positieve macrofagen en dendritische cellen. Zowel in controle muizen met diabetes als in de muizen met diabetes die het supplement

kregen, verminderde de albumine-creatinine ratio's en HPSE-1 activiteit na 10 weken, zonder verschil tussen de twee groepen.

In **hoofdstuk 6** tonen we aan dat de muizen overgingen op vetverbranding tijdens het FMD, wat gepaard ging met lagere glucosewaardes en verlies van voornamelijk spiermassa en water, maar niet het vetgehalte. Na 5 dieet-cycli werd de afbraak van de glomerulaire glycocalyx en het verlies van haarvaten gedeeltelijk voorkomen. Echter, metabole analyse onthulde verhoogde oxidatieve stress en verminderde aanwezigheid van hyaluronzuur precursors, wat wijst op negatieve metabole effecten na het dieet.

In tegenstelling tot eerdere studies met dit muismodel, hebben we in deze studie de glucose waardes op een bepaald niveau gehouden (15-20 mmol/L) gedurende de studie om te voorkomen dat de muizen zelf al over gingen op vetverbranding. Dit resulteerde in een veel mildere diabetische nefropathie model met weinig schade in de glomeruli. Ook kan het houden van de glucosewaardes op een bepaald niveau hebben geleid tot een spontane verminderding van de eiwitlekkage, door verminderde glucosetoxiciteit in de nier.

De endotheliale glycocalyx als diagnostische marker voor microvasculaire gezondheid

In dit proefschrift tonen we aan dat veranderingen in de sublinguale endotheliale glycocalyx kan worden gedetecteerd vóór het optreden van klinische tekenen van hart- en vaatziekten. In de afgelopen jaren zijn veranderingen in de sublinguale microcirculatie bestudeerd bij specifieke patiëntengroepen en gecorreleerd aan ernst van de ziekte. Bij patiënten met sepsis zijn microvasculaire veranderingen snel zichtbaar en hebben een sterke correlatie met de ernst van de sepsis. Bij patiënten met diabetes worden microvasculaire veranderingen geassocieerd met een verhoogde calciumafzetting in de kransslagaders en de mate van eiwit verlies in de urine. Wij hebben aangetoond dat verschillende microvasculaire veranderingen, zoals het verlies van de kleinste sublinguale haarvaten, kunnen worden gedetecteerd in een preklinisch cohort van individuen met een verhoogd risico op het ontwikkelen van hart- en vaatziekten. Een lagere dichtheid van de capillairen kan komen door structureel anatomisch verlies van de capillairen of door vasoconstrictie, wat leidt tot verminderde perfusie van de capillairen. Een lagere dichtheid van de capillairen wordt gezien als een teken van microvasculaire dysfunctie en is gerelateerd aan het ontwikkelen van hart- en vaatziekten. Het is interessant om te onderzoeken of de microvasculaire veranderingen die wij hebben gevonden gebruikt kunnen worden bij de risico-inschatting voor het ontwikkelen van hart- en vaatziekten. Eén studie met een follow-up van 6 jaar in een cohort zonder vastgestelde hart- en vaatziekten was de eerste studie die aantoonde dat de PBR geassocieerd was met het risico op toekomstige cardiovasculaire gebeurtenissen, zelfs na correctie voor atherosclerotische risicofactoren. In de toekomst zijn meer studies nodig om deze bevindingen te bevestigen.

In de onze studie is een verbeterde software gebruikt die ook de longitudinale RBC snelheid (VRBC) kan meten. Dit resulteerde in nieuwe microvasculaire parameters zoals de dynamische PBR, VRBC in haarvaten en in aanvoerende vaten (> 10µm), perfusiedichtheid- en bloedvolume van haarvaten. De huidige berekening van de dynamische PBR maakt gebruik van de helling tussen VRBC en de statische PBR gebaseerd op de waardes van de groep. Een toekomstige software aanpassing is momenteel in ontwikkeling die de dynamische PBR per individu kan berekenen. Dit zal resulteren in een individuele schatting van de microvasculaire gezondheid.

Voor het gebruik van de microvasculaire parameters in de klinische praktijk is het belangrijk dat er specifieke afkappunten worden vastgesteld om de interpretatie van de parameters toegankelijker te maken.

Concluderend is er een rol weggelegd voor de endotheliale glycocalyx als diagnostische marker voor de microvasculaire gezondheid. Mogelijk kan het uiteindelijk een rol krijgen in de cardiovasculaire risicobeheersing (CVRM) in de huidige praktijk, waarbij individuen met een risico op het ontwikkelen van hart- en vaatziekten kunnen worden geïdentificeerd en gemonitord door middel van een SDF-meting.

MCP-1 and HPSE-1 in diabetes mellitus type 2

Verschillende studies hebben aangetoond dat MCP-1 en HPSE-1 activiteit kan worden aangetoond in nierweefsel en urine van patiënten met T2DM, waarbij de activiteit correleert met de mate van eiwit verlies in de urine en de nierfunctie. De ROADMAP-studie toonde zelfs aan dat serum- en urine MCP-1 gehaltes sterke onafhankelijke voorspellers waren voor de ontwikkeling van eiwitlekkage, wat suggereert dat aanwezigheid van MCP-1 een vroege indicator kan zijn van diabetische nefropathie. In de HELIUS-studie in hoofdstuk 3 bevestigden we deze associatie met MCP-1 in de urine en de mate van eiwitlekkage bij individuen met T2DM van verschillende etnische afkomst. Deze bevindingen tonen aan dat MCP-1 activiteit ook kan worden gebruikt als een marker voor diabetische nefropathie in deze etnische groepen en suggereert dat deze patiënten baat kunnen hebben bij therapeutische interventies gericht op het remmen van MCP-1 productie, zoals Emapticap, een MCP-1 remmer.

In al onze klinische studies werd een lage HPSE-1 activiteit gevonden, dit is in tegenstelling tot eerdere studies gepubliceerd met T2DM patiënten waar hogere plasma en urine waardes werden gevonden. Verhoogde glomerulaire en tubulaire HPSE-1 expressie werden gevonden bij T2DM patiënten, maar deze histologische bevindingen waren niet gecorreleerd met de HPSE-1 activiteit in de urine. Hoeveel van de HPSE-1 activiteit in de urine de lokale HPSE-1 expressie in de nier weerspiegeld, moet nog worden vastgesteld. In onze studies gebruiken wij de TAKARA HPSE-1 kit, een indirecte ELISA die activiteit meet door

de hoeveelheid geknipte heparaan sulfaat fragmenten te detecteren. Een mogelijk nadeel van deze methode is dat HPSE-1 al gebonden zou kunnen zijn aan heparaan sulfaten aanwezig in de urine en afkomstig van de tubulaire cellen, gezien elke cel in het urinestelsel heparaan sulfaat tot expressie brengt. In aanwezigheid van hoge heparaan sulfaat concentraties in urinemonsters kan de huidige test een onderschatting geven van de hoeveelheid actief HPSE-1. In toekomstig onderzoek zal het nodig zijn om een test te ontwikkelen die onderscheid maakt tussen actief HPSE-1 en het inactieve pro-HPSE-1 zodat een betere indicatie verkregen kan worden van het totaal aan HPSE-1 dat vrijkomt. Een mogelijkheid om dit te doen is door het gebruik van activiteitsgebonden probes (ABP's).

ABP's kunnen binden aan de actieve bindingsplaats van een molecuul, waardoor detectie, visualisatie en zelfs inactivering van het molecuul mogelijk is. Er is een β-glucuronidase-specifieke ABP ontwikkeld die het meten van HPSE-1 in biologische monsters mogelijk maakt. Deze ABP heeft het vermogen om actieve HPSE-1 maar ook het inactieve pro-HPSE-1 te labelen. Het labelen en meten van zowel actief als niet-actief HPSE-1 is belangrijk omdat het een beter inzicht geeft in de daadwerkelijke HPSE-1 activiteit in urine of weefsel. Gezien de huidige methode die wordt gebruikt om HPSE-1 te detecteren niet adequaat is, is HPSE-1 activiteit nog niet geschikt als biomarker.

Dieet interventies om de endotheliale glycocalyx te herstellen

De afgelopen jaren zijn er verschillende therapeutische interventies ontwikkeld gericht op het herstellen van de glycocalyx. De meeste interventies zijn gericht op het remmen van HPSE-1 activiteit, maar zijn nog niet goedgekeurd voor klinische trials. Alleen sulodexide, een heparaan sulfaat mimeticum, is bestudeerd in klinisch onderzoek maar faalde om eiwitverlies in de urine te verminderen in T2DM patiënten.

We hebben onderzocht of dieetinterventies in staat waren de endotheliale glycocalyx te herstellen of de afbraak van de glycocalyx te verminderen in een klinische studie met Surinaams-Hindoestaanse T2DM patiënten en in een experimenteel diabetes muismodel. Het voedingssupplement bevat glycocalyx componenten zoals hyaluronzuur en fucoidan uit zeewier, wat een heparaan sulfaat mimeticum is. In de muizen studie hebben we aangetoond dat het supplement de afbraak van de glomerulaire hyaluronzuur en heparaan sulfaat in de glycocalyx tegen hield en dat de capillaire dichtheid werd behouden. In de klinische studie verbeterde de PBR, een indirecte marker van de endotheliale glycocalyx, na dagelijks gebruik van het supplement. Deze resultaten suggereren dat het supplement in staat is om de endotheliale glycocalyx gedeeltelijk te herstellen of verdere schade aan de glycocalyx die wordt veroorzaakt door de diabetische omgeving te voorkomen. Eerder hebben we laten zien dat fucoidan *in vitro* de endotheliale glycocalyx en de permeabiliteitsbarrière kon herstellen in pulmonale microvasculaire endotheelcellen. De *in vitro* studie in **hoofdstuk 4** toonde aan dat fucoidan HPSE-1 activiteit in glomerulaire

endotheelcellen dosisafhankelijk kon remmen. Ondanks deze bevindingen werden in zowel de muizenstudie als de klinische studie geen significante verbeteringen in HPSE-1 of albumine-creatinine waardes gevonden.

Bij twee patiënten in de klinische studie daalde de albumine-creatinine ratio van macro-albuminurie bij het begin van de studie naar normo-albuminurie na 3 maanden dagelijkse inname van het supplement. Grotere studies met meer patiënten zijn nodig om deze bevindingen te bevestigen en om te onderzoeken of fucoidan de glomerulaire barrièrefunctie kan herstellen en eiwitlekkage kan verminderen bij mensen met diabetes. Ook zijn er langetermijnstudies nodig om te onderzoeken of het supplement in staat is de progressie van hart- en vaatziekten te vertragen door het beschermen van de endotheliale glycocalyx. Een voordeel van het supplement is dat het bestaat uit natuurlijke ingrediënten en geen ernstige bijwerkingen heeft in de klinische setting, in tegenstelling tot andere medicamenteuze interventies.

Dieetinterventies zijn de afgelopen jaren steeds populairder geworden vanwege positieve effecten op het verouderingsproces en cardiometabole risicofactoren. In dit proefschrift deden we onderzoek naar een dieet van 5 dagen, ook wel periodiek vasten genoemd. Dit dieet liet in een gezonde populatie positieve effecten zien op cardiovasculaire risico factoren. Naast deze effecten wordt gedacht dat periodiek vasten ook positieve effecten heeft op de cellulaire afweer, waarbij ontstekingsreacties worden geremd en beschadigde delen in de cel worden opgeruimd. In de klinische studie toonden we aan dat 3 cycli van het dieet gunstige effecten had op BMI en Hba1c waardes. In tegenstelling tot de eerder gepubliceerde studie in gezonde personen, zagen wij echter geen effect op systolische bloeddruk en inflammatie markers zoals hs-CRP, MCP-1 en HPSE-1 activiteit. In één patiënt verslechterde de nierfunctie op dag 5 van het dieet, dit werd per toeval gemeten bij een poliklinische controle van de patiënt. De nierfunctie herstelde na intraveneuze vochttoediening, wat wijst op uitdroging als de oorzaak voor de achteruitgang van de nierfunctie. In onze muizenstudie zagen we ook dat het gewichtsverlies tijdens het dieet voornamelijk bestond uit het verlies van water en spieren. Het verlies van water tijdens vasten is een bekend effect als gevolg van glycogeen omzetting in de lever en spieren, wat voornamelijk gebonden is aan water. Meer dan de helft van het gewichtsverlies dat kan optreden na periodes van vasten is het gevolg van het verlies van dit overtollige water wat vrijkomt bij de afbraak van glycogeen. Ook zagen we geen positieve effecten op de microcirculatie, aangezien de PBR en microvasculaire gezondheidsscore verslechterde na 3 cycli van het dieet. In het muismodel waren er aanwijzingen bij metabole analyse dat er een verhoogde oxidatieve stress was in de nier. Ook werden er minder hoge concentraties van UDP-GlcA gevonden, een substraat van hyaluronzuur. Verminderde concentraties van UDP-GlcA kunnen er voor zorgen dat er geen optimale synthese is van hyaluronzuur, wat de opbouw en integriteit van de endotheliale glycocalyx kan verstoren.

Een voordeel van dit dieet is dat het makkelijker is vol te houden in vergelijking tot continue diëten, terwijl er toch positieve effecten worden gezien op glucose levels en gewichtsverlies. We hebben echter waargenomen dat deze effecten verdwenen wanneer het dieetregime werd stopgezet. Dit is een veelvoorkomend fenomeen bij dieet interventie studies waarbij personen intensief gemonitord worden tijdens de studie, wat uitdagingen met zich meebrengt voor het gebruik van levensstijlinterventies in de dagelijkse praktijk.

Concluderend is het dieet niet geschikt als interventie om de endotheliale glycocalyx te herstellen en kan het zelfs nadelige effecten hebben op de microcirculatie en samenstelling van de glycocalyx. Ook adviseren we dat het dieet met voorzichtigheid moet worden gebruikt in patiënten met chronische nierschade vanwege de mogelijke kans op uitdroging tijdens het dieet.

Uitdagingen voor personen van Surinaams-Hindoestaanse afkomst

In het huidige proefschrift hebben we individuen met type 2 diabetes van Surinaams Hindoestaanse afkomst bestudeerd. Van oorsprong komt deze bevolkingsgroep uit Zuid-Azië, met name uit Noord-Oost India. Individuen van Surinaams-Hindoestaanse afkomst hebben een hogere kans op het ontwikkelen van diabetes en cardiovasculaire complicaties. Ondanks standaard medicamenteuze behandelingen blijft de prevalentie en de progressie van cardiovasculaire complicaties hoger in vergelijking met individuen van Europese afkomst. Individuen van Zuid-Aziatische afkomst krijgen diabetes op een jongere leeftijd en bij een lagere BMI, hebben een verhoogd risico op het krijgen van een hartinfarct, een hogere prevalentie van eiwitlekkage en een snellere afname van de nierfunctie. De hoge cardiovasculaire gezondheidslast van personen van Zuid-Aziatische afkomst, zoals de Hindoestanen, is de afgelopen jaren duidelijk onderzocht en beschreven, maar de vertegenwoordiging van deze etnische groep in klinische studies, adequate risico calculators en therapeutische strategieën ontbreken.

Patiënten van Zuid-Aziatische afkomst zijn moeilijk om te werven voor gerandomiseerde onderzoeken, met name voor leefstijl interventiestudies. Het uitvoeren van leefstijl studies in de Zuid-Aziatische bevolking blijkt een uitdaging wegens weinig reacties op uitnodigingen, hoge uitvalspercentages en tegenstrijdige uitkomsten uit de onderzoeken. Een intensieve 1- en 2-jarige leefstijl interventiestudie in huisartsenpraktijken leidde niet tot significante gewichtsverlies of verbetering van metabole parameters bij deelnemers van Surinaams-Hindoestaanse afkomst in Den Haag. In onze studie hadden we ook te maken met weinig reacties op de uitnodigingen, maar we hebben wel significante verbeteringen laten zien na 3 maanden. Een meta-analyse van gerandomiseerde onderzoeken toonde ook aan dat leefstijl interventies ook in individuen van Zuid-Aziatische afkomst succesvol kunnen zijn in het verlagen van het risico op het krijgen van diabetes. Een groot deel van de studies die nu worden uitgevoerd is gericht op preventieve leefstijl interventies.

Andere studies richten zich op het vinden van pathofysiologische verklaringen voor de verschillen in het cardiovasculaire risico. Een andere samenstelling van high-density lipoproteïnen of verschillen in genetische inflammatoire systemen zijn voorbeelden van recent ontdekte verschillen tussen Zuid-Aziatische en Europese individuen. Het ontrafelen van pathologische verklaringen voor het ontwikkelen van cardiovasculaire complicaties, kan in de toekomst leiden tot meer op maat gemaakte interventies voor personen van Zuid-Aziatische afkomst.

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Curriculum Vitae

Anouk Ilse Maria van der Velden was born on the 13th of October 1991 in Egmond aan den Hoef. She completed her pre-universitiy education at the Petrius Canisius College in Alkmaar in 2010. Her classes focused on Natural Sciences and Economy during high school. In the same year she started with her medicine study at the Leiden University Medical Center (LUMC). Her master research project was at the department of Gerontology and Geriatrics at the LUMC under supervision of Prof. dr. S.P. Mooijaart. Besides her study, Anouk was actively engaged within the student association L.M.D Forestus and worked as a research assistant in several projects.

In 2017, she finished her medical master's degree at the University of Leiden. After her graduation she started her PhD project, of which the results are described in this thesis, at the department of Nephrology under supervision of prof. dr. A.J. Rabelink.

After her PhD project, Anouk started working in the Spaarne Gasthuis as a medical doctor in the department of internal medicine. In 2021, after travelling for 4 months in South-America, she worked as a medical doctor in the Alrijne hospital and got accepted into the internal medicine program. She began her residency program to become an internist at the Haaglanden Medical Center (HMC+) and Leiden University Medical Center in 2023.

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