# Early Microvascular Changes with Loss of the Glycocalyx in Children with Type 1 Diabetes

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**Objective** To evaluate the microcirculation of children with type 1 diabetes mellitus who demonstrate no clinical signs of diabetic microangiopathy for the presence of microvascular alterations and glycocalyx perturbation.

**Study design** Images of sublingual vessels were obtained in 14 children with diabetes (ages 12.8 ± 2.8 years, diabetes duration 6.7 ± 4.3 years) and 14 control patients (ages 11.8 ± 2.8 years) by the use of sidestream dark field imaging and analyzed for total vessel density, vessel surface coverage, vessel diameter distribution, mean flow index, and glycocalyx thickness. Wilcoxon rank sum test and Pearson correlation were used for statistical analysis.

**Results** We observed profound microcirculatory changes in children with diabetes compared with control patients, with a significant reduction of glycocalyx thickness (0.38 μm vs 0.60 μm; $P = .013$), which was inversely correlated with blood glucose levels ($r = -0.55$; $P = .003$). Furthermore, the percentage of large vessels (>20 μm diameter) was significantly increased (11% vs 6%; $P = .023$) at the expense of capillaries (<10 μm diameter) with consequent increase in total vessel surface coverage (30% vs 26.0%; $P = .041$). No differences were seen in total vessel density and mean flow index.

**Conclusions** Microvascular alterations, including changes in microvessel distribution and loss of the glycocalyx, can be detected in children with type 1 diabetes mellitus before clinically apparent vascular complications. Our results disclose the glycocalyx as a possible monitoring measurement for earlier detection of diabetic microangiopathy and may provide a basis for new therapeutic strategies aiming at protection or restoration of the glycocalyx. *(J Pediatr 2014;164:584-9)*.

The long-term outcome of patients with type 1 diabetes mellitus (T1DM) is governed by the occurrence of vascular sequelae such as nephropathy, retinopathy, and cardiovascular disease, which are leading causes of morbidity and premature mortality.[1] The prevention and early identification of vascular complications are a central issue in the care of patients with diabetes.

The pathogenesis of vascular complications still remains incompletely understood; however, endothelial dysfunction (ED) is thought to play a central role in the development of diabetic microangiopathy and macroangiopathy.[2] There is some evidence that endothelial-dependent responses of the microvasculature and macrovasculature are impaired in children with T1DM when vascular complications are still subclinical.[3,4] These studies are supported by reports of increased markers of endothelial activation and perturbation such as intercellular adhesion molecule 1, E-selectin, and von Willebrand factor,[5] along with carotid intima-media thickening indicating beginning arteriosclerosis.[6,7]

The mechanisms leading to ED in diabetes mellitus are multifactorial.[8] Large clinical trials have underscored the causative role of hyperglycemia,[9,10] which contributes to ED by increasing production of reactive oxygen species and oxidative stress, activation of protein kinase C, and generation of vasoactive and proinflammatory substances. In recent years, the endothelial glycocalyx has emerged as an important target of hyperglycemia-induced vascular damage.[11] This complex layer of proteoglycans, glycoproteins, glycosaminoglycans, and attached plasma proteins covers the endoluminal vessel surface and has been recognized as a critical regulator of vascular integrity and endothelial function.[12] Acute and chronic elevation of blood glucose levels cause perturbation of the glycocalyx, leading to ED and increased microvascular permeability in vitro and in vivo.[13,14] Greater levels of hyaluronan, a major constituent of the endothelial glycocalyx, were found in the circulation of adults with diabetes, where they correlated with increased intima media thickness.[15] These data suggest that loss of the endothelial glycocalyx or alterations in the composition of the glycocalyx may present a common mechanism underlying the development

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| BP | Blood pressure |
| ED | Endothelial dysfunction |
| HbA1c | Glycosylated hemoglobin A1c |
| MFI | Mean flow index |
| SDF | Sidestream dark field |
| T1DM | Type 1 diabetes mellitus |
| TVD | Total vessel density |

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of both microvascular and macrovacscular complications in diabetes. To date, it is not known whether such alterations of the glycocalyx occur in children with diabetes. Therefore, we assessed the microcirculation and the microvascular endothelial glycocalyx in children with T1DM and control patients.

## Methods

Children with T1DM were recruited during their routine outpatient visits at the Dr. von Hauner Children's Hospital, University of Munich Medical Center. All patients had been diagnosed with T1DM for at least 1 year, and the diagnosis was made according to the current criteria of the American Diabetes Association: (1) symptoms of diabetes; (2) random plasma glucose or 2-hour plasma glucose during oral glucose tolerance testing of 200 mg/dL (11.1 mmol/L) or greater or fasting plasma glucose of 126 mg/dL (7 mmol/L) or greater; (3) detection of diabetes-specific autoantibodies; and (4) increased glycosylated hemoglobin A1c (HbA1c) levels. All patients were kept on daily subcutaneous insulin regimens. Two children were treated with L-thyroxin for autoimmune thyroiditis. One patient was suffering from paroxysmal seizures of unknown origin, and one patient had arterial prehypertension (blood pressure [BP] values ranging from the 90th to the 94th size-adjusted percentile). None of the children suffered from celiac disease.

Patients were screened routinely for the presence of microalbuminuria by measurement of the urinary albumin/creatinine ratio in first urine of the morning and for retinopathy by an annual mydriatic ophthalmoscopy examination performed from 11 years of age with ≥5 years of diabetes' duration, with none of the children demonstrating pathologic results. Pubertal status in patients with diabetes was documented twice a year, and there was no delay in pubertal development.

Age- and sex-matched control patients were recruited from the surgical outpatient clinic at the Dr. von Hauner Children's Hospital, University of Munich Medical Center where they presented for minor elective surgical procedures, including dermatologic, urologic, and orthopedic interventions. The presence of comorbidities likely to affect the (micro-) circulation in controls, such as hypertension, diabetes mellitus, acute infections, lead to the control patient's exclusion from the study. For all patients with TIDM and control patients, fasting plasma glucose levels, leukocyte count, and hematocrit were determined by the use of standard laboratory tests. Furthermore, the current HbA1c value of patients was measured, and the average HbA1c level during the preceding 2 years was retrieved from the patients' records. Level of HbA1c was measured by DCA 2000 Control (Bayer AG, Leverkusen, Germany), based on specific inhibition of latex immunoagglutination. Normal values of HbA1c as established in our laboratory range from 4.0% to 6.0%.

Blood was collected through peripheral venous puncture via the use of routine blood-collection tubes (S-Monovetten; Sarstedt, Nuembrecht, Germany), and samples were immediately transferred to the clinical laboratory for further processing. Written informed consent was obtained from at least one parent or legal guardian before the patient's inclusion in the study. The study was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the local ethics committee.

The sublingual microvasculature was visualized by use of the sidestream dark field (SDF) imaging technique as previously described.[16] Images (phase alternating line format, 1.35 $\mu$m/pixel) were recorded at a frame rate of 25 frames/s with a handheld video microscope (MicroScan; Microvision Medical, Amsterdam, The Netherlands) with a 5-fold objective connected to a computer and monitor via an analog-to-digital converter. The recordings were performed at the central area under the patient's tongue with patient lying on their back and took approximately 5-10 minutes. Care was taken to avoid movement artifacts and pressure on the tissue, as seen by increased background illumination attributable to greater reflection of light. To minimize measurement bias attributable to variability of the microvasculature within the sublingual area, at least 5 video sequences of 10 seconds' duration for each participant were obtained and stored digitally for later offline analysis. All recordings were performed by the same investigator to preclude interobserver variability.

Video sequences of the sublingual microcirculation underwent blinded offline analysis with the Automated Vascular Analysis software (AVA; Version 3.0, University of Amsterdam, Amsterdam, The Netherlands). Vessels were classified according to their diameter into small (<10 $\mu$m), medium (10-20 $\mu$m), and large (>20 $\mu$m). The following measures were calculated automatically: (1) total vessel density (TVD), defined as the total vessel length (mm) per image area ($mm^2$); (2) vessel diameter distribution, describing small, medium, and large vessels as percentage of total vessel length; and (3) vessel surface coverage, defined as the percentage of the total image area that is covered by vessel surface. Because the latter integrates both TVD and vessel diameter distribution, it is a sensitive measure of early, more subtle microcirculatory changes. Furthermore, blood flow velocity was assessed semiquantitatively according to the classification of Boerma. For this purpose, recordings of the sublingual microvasculature were divided into 4 quadrants, and a Boerma score was assigned to each quadrant depending on its predominant flow characteristics: no flow = 0, intermittent flow = 1, sluggish flow = 2, and continuous flow = 3. The mean flow index (MFI) was then calculated as average of the 4 quadrants.

Estimates of the endothelial glycocalyx dimension were obtained with a method that is based on the linear theory model as published by Nieuwdorp et al.[17] This method takes into account that flowing red blood cells are unable to compress or enter the intact glycocalyx and leukocytes traveling through a capillary are more rigid and cause compression of the glycocalyx. This allows the following red blood cells to get closer to the vessel wall, leading to a transient widening of the red blood cell column. By measuring the diameter of the red blood cell column before and after passage of the leukocytes ($\Delta$ vessel diameter), one can derive