


Diabetes affects endothelial cell function and alters fibrin clot formation in a microvascular flow model: A pilot study

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Abstract

Diabetes is a proinflammatory and prothrombotic condition that increases the risk of vascular complications. The aim of this study was to develop a diabetic microvascular flow model that allows to study the complex interactions between endothelial cells, blood cells and plasma proteins and their effects on clot formation. Primary human cardiac microvascular endothelial cells from donors without diabetes or donors with diabetes (type 1 or type 2) were grown in a microfluidic chip, perfused with non-diabetic or diabetic whole blood, and clot formation was assessed by measuring fibrin deposition in real time by confocal microscopy. Clot formation in non-diabetic whole blood was significantly increased in the presence of endothelial cells from donors with type 2 diabetes compared with cells from donors without diabetes. There was no significant difference in clot formation between non-diabetic and diabetic whole blood. We present for the first time a diabetic microvascular flow model as a new tool to study clot formation as a result of the complex interactions between endothelial cells, blood cells and plasma proteins in a diabetes setting. We show that endothelial cells affect clot formation in whole blood, attributing an important role to the endothelium in the development of atherothrombotic complications.

Keywords

Blood coagulation, diabetes mellitus, endothelial cells, microfluidics

Introduction

Diabetes mellitus is a chronic metabolic disease that affected 8.5% of the adult global population in 2014 (422 million). The number of affected individuals has doubled since 1980, and with a predicted number of 522 million people suffering from diabetes by 2030, the disease has developed to a fast-rising global burden that is not restricted to high-income countries anymore.^{1,2} This means that novel preventive, diagnostic and therapeutic approaches are required.

Diabetes is subdivided into type 1 and type 2 diabetes.³ Type 1 diabetes mellitus (T1DM) is an autoimmune disease that causes deficiency of insulin due to the destruction of insulin-producing pancreatic beta cells. In type 2 diabetes mellitus (T2DM), which accounts for the majority of all cases, hyperglycaemia is caused by insulin resistance. Although T1DM and T2DM differ in regard to their causative factors and pathogenesis, both types of diabetes are associated with an elevated risk of macrovascular complications such as cardiovascular disease (CVD).^{4–6} In most

T2DM patients, hyperglycaemia with insulin resistance is accompanied by a cluster of cardiovascular risk factors including obesity, dyslipidaemia and hypertension, summarised as the metabolic syndrome, which is caused by a combination of lifestyle and genetic factors.^{7–10} This cluster of risk factors promotes endothelial dysfunction and procoagulant and proinflammatory changes which contribute to the formation of atherosclerotic plaques and eventually acute thrombotic cardiovascular events.^{11–13}

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Novel approaches to prevent or reduce macrovascular complications of diabetes may target endothelial dysfunction and procoagulant and proinflammatory changes. The complement system, a part of the innate immune system that fights infection but also boosts inflammatory reactions, is emerging as a player in diabetes.^{14,15} The complement system also closely interacts with the coagulation and fibrinolytic system, further promoting clot formation.¹⁶ In diabetes patients, elevated plasma levels of complement proteins and activation products were associated with increased clot formation, denser clot structure and prolonged clot lysis.^{17–19} Similarly, elevated plasma levels of procoagulant factors including fibrinogen and the fibrinolysis inhibitor plasminogen activator inhibitor-1 (PAI-1) were associated with diabetes.^{20,21} Hyperglycaemia has also been shown to alter the function of proteins in plasma (e.g. fibrinogen, plasminogen, complement C3) and on cell surfaces by non-enzymatic glycation, further contributing to the proinflammatory and prothrombotic state.^{12,18,22,23}

As a prerequisite for the exploration of novel preventive and therapeutic targets, diagnostic methods must be in place that can assess whether the desired outcome has been reached. But a complex procoagulant and proinflammatory state in a multifactorial disease such as T2DM may not be reflected by a single biomarker measured in plasma. Novel experimental models that integrate both cellular and humoral components and enable to investigate their interactions and effects on a certain outcome may help to identify novel targets and in the future may also be used as tools to better monitor treatments. A microfluidic model that features microchannels of the dimension of human arterioles coated with a viable monolayer of human endothelial cells was developed to study microvascular occlusion and thrombosis in whole blood in the case of haematological diseases.^{24,25} We have recently shown that activation of the complement system affects blood clot formation in this microvascular whole blood flow model.²⁶

The aim of this study was to further develop this model into a diabetes model. We coated the microchannels with primary human cardiac microvascular endothelial cells from donors without diabetes or donors with T1DM or T2DM. To assess the diabetic phenotype of the endothelial cells, we analysed the expression of markers associated with diabetes by real-time polymerase chain reaction (PCR). The endothelialised microchannels were then perfused at physiological flow and shear rates with freshly drawn whole blood from individuals without diabetes or patients with T1DM or T2DM, and clot formation was observed in real time by confocal microscopy. For the first time, we assessed and compared the effects of endothelial cells and whole blood from T1DM and T2DM patients and donors without diabetes on the extent of clot formation.

Materials and methods

Endothelial cell culture

Primary human cardiac microvascular endothelial cells (HMVECs) from individuals without diabetes and diseased human cardiac microvascular endothelial cells (D-HMVECs) from T1DM and T2DM patients were purchased from Lonza (Basel, Switzerland). Cells were maintained in microvascular endothelial cell growth medium (EGM-2 MV, with SingleQuots™; Lonza) as recommended by the supplier. Cells from T1DM and T2DM donors were cultured with D-glucose (ThermoFisher, Waltham, MA, USA) at a final concentration of 25 mmol/L to sustain the high glucose milieu of a diabetes patient with poor glycaemic control, while cells from donors without diabetes were kept at normal glucose levels (5 mmol/L). All cell lots were negative for mycoplasma as shown by the manufacturer.

Expression of diabetic markers in endothelial cells

Total RNA from HMVECs and D-HMVECs (passage 5, approximately 3×10^6 cells per donor) was extracted using an RNAeasy MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's manual. Isolated RNA was transcribed into complementary DNA (cDNA) using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The gene expression of fibronectin (gene name: fibronectin 1, FN1), PAI-1 (gene name: SERPINE1), and advanced glycation end-product-specific receptor (AGE receptor, AGER) was quantified by quantitative PCR (qPCR) performed on a CFX Connect-96 device (Bio-Rad) using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) on customised PrimePCR plates containing lyophilised primer pairs (Bio-Rad Unique Assay ID qHsaCID0012349 for FN1; qHsaCED0043144 for SERPINE1; qHsaCED0037299 for AGER). Results were normalised against *SDHA* expression (succinate dehydrogenase complex, subunit A; Bio-Rad Unique Assay ID qHsaCED0057393). Two technical replicates of each sample were used to determine quantification cycle values (CFX Maestro Version 4.1.2433.1219; Bio-Rad), and the fold-differences between control and diabetic HMVECs were assessed by comparative quantification ($2^{-\Delta\Delta Ct}$).

Patients and blood sampling

A total of 22 T1DM and T2DM patients ($n=11$ each) were recruited during their consultation at the University Clinic for Diabetology, Endocrinology, Nutritional Medicine and Metabolism at the University Hospital of Bern. Inclusion criteria were adult age, either newly diagnosed and non-treated

diabetes or patients with an earlier diagnosis but poor glycaemic control. One T1DM patient and one T2DM patient were excluded from the analysis due to problems with the microvascular flow model and hence implausible clot formation (instead of a smooth curve showing an increasing fluorescence signal over time, the curves were ragged, possibly due to a floating particle such as cell debris). Control subjects without diabetes were recruited by advertisement at the University and University Hospital of Bern. Individuals under anticoagulant and/or antiplatelet treatment, with haemostatic or thrombotic disorders and lactating or pregnant women were excluded. The study was approved by the local ethics committee (reference number 2017-01916), and all patients and control subjects gave informed consent. Demographic and medical data were collected and stored in anonymised form. Whole blood was taken by venipuncture into ethylenediaminetetraacetic acid (EDTA) and trisodium citrate monovettes (1.6 mg EDTA/mL or 0.106 mol/L trisodium citrate; Sarstedt AG, Nümbrecht, Germany). Citrated whole blood (2 mL) was supplemented with corn trypsin inhibitor (CTI), a contact pathway inhibitor (final concentration: 2.86 $\mu\text{mol/L}$; Haematologic Technologies, Essex Junction, VT, USA), and was used for microscopic experiments within 90 min after blood sampling. Platelet-poor plasma was produced from EDTA and citrated whole blood by centrifugation (2900 r/min for 15 min, 4°C) and stored at -80°C for biomarker measurements.

Circulating biomarker measurements

Plasma levels of the complement proteins C3, mannan-binding lectin (MBL) and soluble terminal complement complex (sC5b-9) were measured by commercial ELISA kits (Hycult Biotech, Uden, The Netherlands), according to the manufacturer's protocols. Plasma levels of the coagulation protein fibrinogen and the fibrinolysis inhibitor PAI-1 were also measured by commercial ELISA kits (HYPHEN Biomed, Neuville-sur-Oise, France) following the manufacturer's instructions. Fructosamine levels, a marker of non-enzymatic glycation of plasma proteins, were measured by a commercial colorimetric assay following the manufacturer's protocol (Abcam, Cambridge, UK).

The microvascular flow model

The microvascular flow model was assembled as described earlier.²⁶ Briefly, a silicone photomask with an imprinted microchannel pattern was used as a template to cast identical polydimethylsiloxane (PDMS, Sylgard 184 silicone elastomer kit; Dow Corning, Midland, MI, USA) single-use devices (Figure 1(a)). The microchannels were coated with a 0.005% fibronectin solution (wt/vol., final concentration: 50 $\mu\text{g/mL}$; Sigma Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) (pH 7.4, Life Technologies,

Carlsbad, CA, USA) for 60 min. Passage 5 HMVECs or D-HMVECs grown to full confluency in T25 flasks (4.0×10^4 cells/cm²) were washed and detached with 1 mL of trypsin (0.05% trypsin in Hank's balanced salt solution, 0.53 mmol/L EDTA; Dow Corning), inactivated with 3 mL of EGM-2 MV and centrifuged at 1000g for 10 min. The pellet was resuspended in 100 μL of EGM-2 MV containing dextran (80 mg/mL, $450 \pm 650 \text{ M}_r$; Sigma Aldrich), separated with a cell strainer (35 μm mesh size; Dow Corning) and injected into the microchannels at a concentration of 1×10^7 cells/mL. The device was incubated at 37°C, 5% CO₂ for 75 min. Then, thin tubing (Cole-Parmer, Vernon Hills, IL, USA) was connected to the channels and a constant flow of 2 $\mu\text{L/min}$ of EGM-2 MV was applied for 48 h. After 2 days, the channels were checked by microscopy to determine whether a viable endothelial cell monolayer with 95%–100% confluency had been established (Figure 1(b)).

Microscopic evaluation of clot formation

The HMVEC monolayer was stained with CellMask™ dissolved in EGM-2 MV (final concentration: 1 $\mu\text{L/mL}$, excitation/emission: 554/567 nm, red; Life Technologies) for 5 min. Citrated whole blood was supplemented with pre-stained fibrinogen (final concentration: 50 $\mu\text{g/mL}$, Alexa Fluor 488 conjugate, excitation/emission: 495/519 nm, green; Molecular Probes, Eugene, OR, USA) and recalcified with CaCl₂ (final concentration: 12.5 mmol/L). Subsequently, parallel microchannels were perfused with either diabetes or control blood samples at a flow rate of 2 $\mu\text{L/min}$ (Figure 1(c)). Fibrin deposition was measured for a period of 12.5 min at cycles of 15 s by confocal microscopy at 10 \times magnification (Figure 2) (LSM 710 confocal microscope, Zen software Version 2.1; Carl Zeiss AG, Oberkochen, Baden-Württemberg, Germany) and started 12 min after recalcification of the blood sample. The images were evaluated with ImageJ (version 1.51k; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The extent of clot formation was calculated as mean area under the time curve (AUC) of duplicates measured in parallel channels.

Statistical analysis

Statistical analysis was performed with IBM® SPSS® Statistics, version 26.0; IBM Corporation, Armonk, NY, USA). Numeric data are shown as mean (\pm SD) and were tested for normal distribution with the Shapiro–Wilk test. Normally distributed variables [body mass index (BMI), fructosamine, fibrinogen, PAI-1, C3] were compared between groups with the *t*-test, and data that were not normally distributed (age, HbA1c, diabetes duration, MBL, sC5b-9, AUC of clot formation) were compared with the Mann–Whitney *U*-test. Clot formation data from the

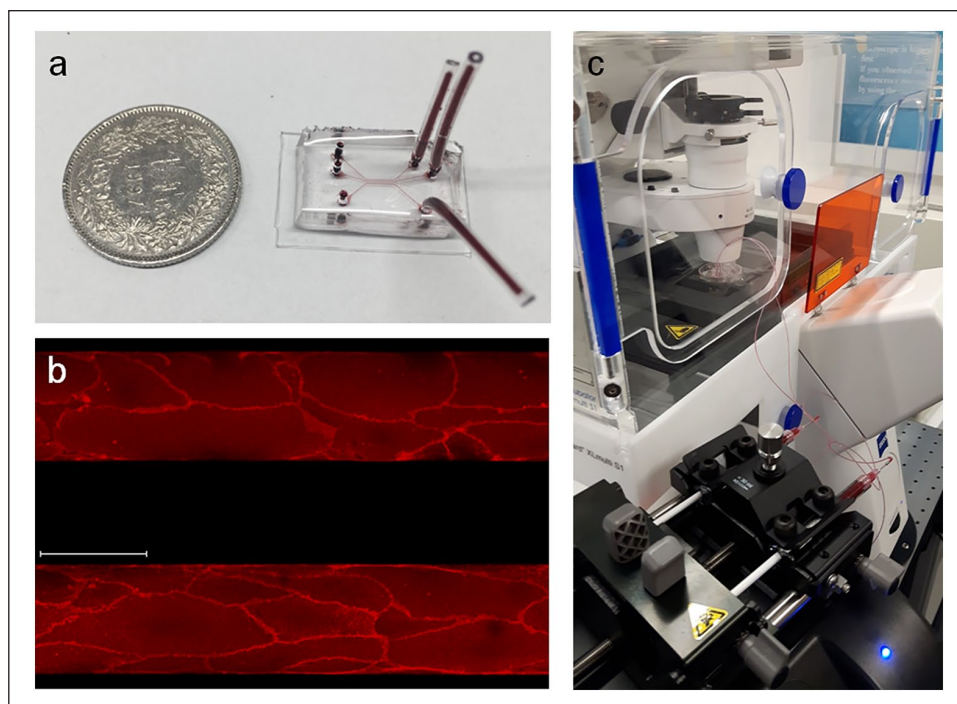


Figure 1. The microvascular flow model. (a) Exemplary picture of a PDMS straight channel device with three microchannels filled with red dye to visualise the channel size. For size comparison, the device is shown next to a Swiss 1 Franc coin with a diameter of 2.32 cm. [Figure 1(a), from Jenny et al.²⁶ is subject to the creative commons licence (www.creativecommons.org) and has not been changed.] (b) Two microchannels coated with a human microvascular endothelial cell monolayer, 48h after the channels were seeded. Scale bar = 100 μ m. (c) Experimental setup of a straight channel device being perfused with diabetic whole blood while observing the blood flow by laser scanning confocal microscopy.

microvascular flow model (AUC) were not always normally distributed and are therefore shown as median and interquartile range (IQR). Paired variables were tested with the paired *t*-test or with the Wilcoxon signed-rank test. Bivariate correlations were assessed with the Spearman correlation coefficient. The distributions of gender and number of smokers between groups were assessed with the chi-square test.

Results

In this study, we developed a diabetic microvascular flow model to investigate the effects of diabetic endothelial cells and diabetic whole blood on clot formation. We determined typical cellular and circulating biomarkers associated with diabetes to characterise the primary endothelial cells and blood samples we used in the microvascular flow model.

Expression of diabetes markers in primary HMVECs from donors with and without diabetes

The primary HMVECs were from seven deceased donors, among them were two individuals without diabetes (controls), three T1DM patients and two T2DM patients. The

donor information supplied by the company is shown in Table 1. We quantified the gene expression of three cell markers associated with diabetes and diabetes-associated cardiovascular disease: fibronectin, PAI-1 and AGE receptor. Fibronectin was reported to be highly upregulated in endothelial cells from diabetes patients, and cellular fibronectin released into plasma was associated with prothrombotic clot properties.^{27–29} Similarly, expression of PAI-1 in endothelial cells was increased several fold in diabetes, in addition to the well-established elevation of PAI-1 plasma levels thought to be a link to the increased thrombotic risk in diabetes.^{28,30} Hyperglycaemia causes the formation of AGEs which act on several cell types, including endothelial cells, through the AGE receptor (AGER or RAGE). AGEs and AGER have been linked to endothelial dysfunction and vascular complications of diabetes.^{31–33} As shown in Figure 3, we observed a high interindividual variation of the expression in diabetic cells relative to the non-diabetic control cells. Among the two T2DM donors, all three diabetes markers were increased in the cells from one donor compared with the cells from the donors without diabetes, while the cells of the second T2DM cell donor showed a lower expression of all three marker genes. The gene expression in the endothelial cells from the three T1DM donors compared with the non-diabetic cells was also very heterogeneous and showed no consistent pattern.

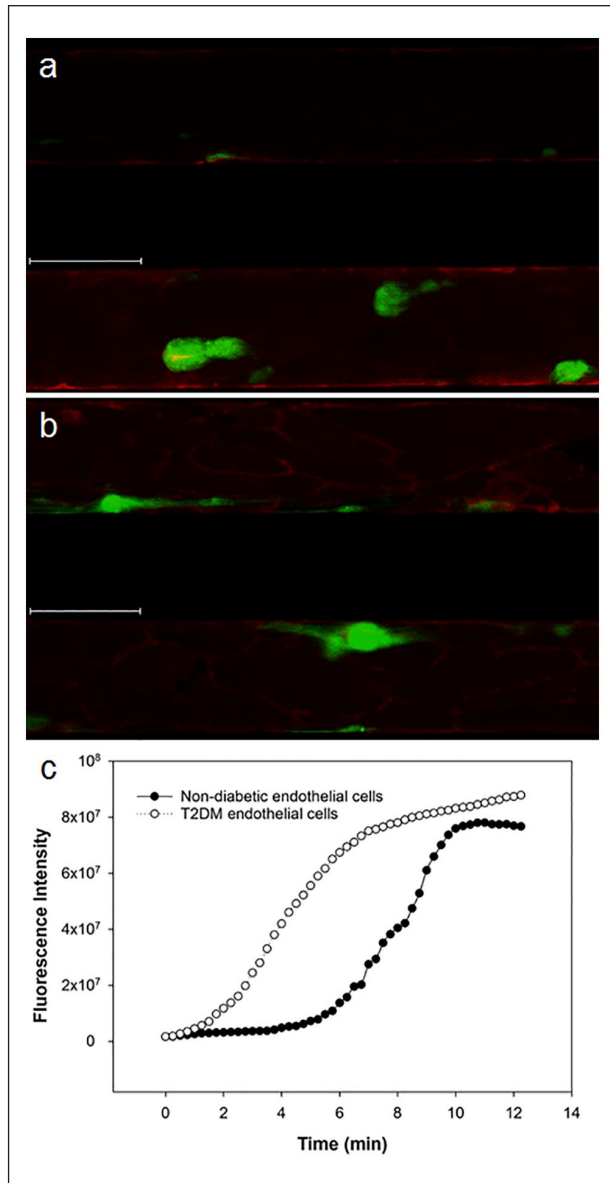


Figure 2. Exemplary images from one clot formation experiment in the microvascular flow model. The images illustrate how clot formation was detected in the microvascular flow model and how the quantitative evaluation was performed. Microchannels were coated in duplicates with (a) non-diabetic or (b) type 2 diabetic human microvascular endothelial cells. Microscopic images were taken at 10 \times magnification, 25.5 min after the blood was recalcified. Red stain: endothelial cells; green stain: deposited fibrin. Scale bar = 100 μ m. (c) Corresponding fluorescence intensity curve over time, with each point being the mean of duplicates.

Demographic and medical data of diabetes patients and control subjects

We included 10 patients with T1DM, 10 patients with T2DM and 20 control subjects without diabetes into the analysis. Characteristics of the diabetes patients and control

subjects are shown in Table 2. All three groups were predominantly male and contained a high number of smokers. As expected, T2DM patients were significantly older than T1DM patients and controls. T2DM patients also had the highest BMI and HbA1c levels.

Circulating biomarker analysis in non-diabetic and diabetic plasma samples

We measured several proteins of the coagulation/fibrinolysis (fibrinogen, PAI-1)^{20,21} and complement system (MBL, C3, sC5b-9)^{14,15} that are associated with diabetes and reflect the procoagulant and proinflammatory state. Fructosamine is a marker for non-enzymatic protein glycation. As shown in Table 2, fructosamine levels were significantly higher in diabetes patients than in controls. Fibrinogen, PAI-1 and sC5b-9 levels were highest in patients with T2DM. On the contrary, fibrinogen, PAI-1, MBL and C3 levels were lowest in patients with T1DM.

Clot formation in the diabetic microvascular flow model

As illustrated schematically in Figure 4, clot formation experiments with the microvascular flow model were always performed with one freshly drawn blood sample (from a control individual without diabetes, a T1DM patient, or a T2DM patient) that was used to perfuse parallel microchannels coated with non-diabetic endothelial cells and diabetic (type 1 or type 2) endothelial cells in duplicates.

In the T1DM model (Table 3), when non-diabetic blood was used, perfusion of microchannels coated with endothelial cells from T1DM donors showed a significantly lower clot formation compared to microchannels coated with non-diabetic cells. However, when whole blood from T1DM patients was used, there was no difference in clot formation compared with non-diabetic blood, neither in microchannels coated with non-diabetic endothelial cells nor in microchannels coated with T1DM endothelial cells.

In the T2DM model (Table 4), clot formation in non-diabetic whole blood was significantly increased in the presence of T2DM endothelial cells compared with non-diabetic endothelial cells. Again, there was no difference in clot formation between non-diabetic blood and blood from T2DM patients, neither in microchannels coated with non-diabetic endothelial cells nor in microchannels coated with T2DM endothelial cells.

When we compared the three ‘pure’ combinations non-diabetic (i.e. non-diabetic cells and non-diabetic blood), T1DM (i.e. T1DM cells and T1DM blood), and T2DM (i.e. T2DM cells and T2DM blood), the T2DM setting showed a tendency towards increased clot formation compared

Table 1. Cell donor information obtained from Lonza.

Donor	Cell lot	Type	Age (years)	Gender	Ethnicity	Smoking	BMI	Medication	Duration of diabetes (years)	Cause of death
1	0000473674	No diabetes	35	M	Hispanic	Y	–	–	–	Stroke
2	0000550176	No diabetes	55	M	Black	N	–	–	–	Accident
3	0000239347	T1DM	63	F	Caucasian	N	30	Insulin	50	Stroke
4	0000239350	T1DM	29	F	Caucasian	Y	30	Insulin	15	Stroke
5	0000287834	T1DM	28	M	Black	Y	44	Insulin	13	Stroke
6	0000233247	T2DM	60	M	Caucasian	N	27	Insulin, sitagliptin, metformin	3	Asthma attack
7	0000239346	T2DM	63	F	Black	N	45	Metformin	9	Stroke

T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus.

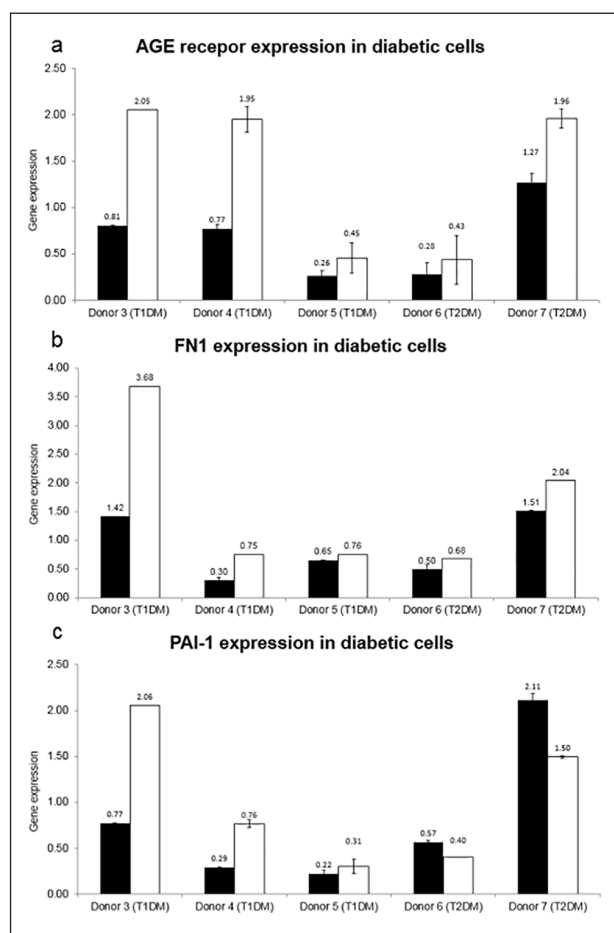


Figure 3. Expression of diabetic marker genes in diabetic and non-diabetic human microvascular endothelial cells. Individual gene expression in the cells from the donors with diabetes is shown relative to the expression in control cells from donor 1 without diabetes (depicted as black bars) and relative to donor 2 without diabetes (white bars). Error bars represent the standard deviation from two technical replicates. AGE receptor: advanced glycation end-product-specific receptor; FN1: fibronectin; PAI-1: plasminogen activator inhibitor-1; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus.

with the non-diabetic and T1DM setting (Figure 5), but interindividual variability was high and the differences did not reach statistical significance.

Taken together, our results show for the first time that diabetic endothelial cells have a significant effect on clot formation when evaluated together with non-diabetic whole blood. This effect was, however, overridden when non-diabetic blood was replaced by diabetic blood, which may be attributed to the large interindividual variation observed in particular in diabetes patients or to certain characteristics in the blood that have a stronger effect on clot formation than the effects of diabetic endothelial cells. We, therefore, also investigated possible correlations between the analysed circulating biomarkers and clot formation. Yet none of the analysed biomarkers showed a significant correlation with the extent of clot formation (data not shown).

Discussion

Diabetes is a major cause of cardiovascular disease. In particular in T2DM, metabolic risk factors cluster and lead to a proinflammatory and prothrombotic state. But also T1DM is associated with an increased cardiovascular risk, and changes in plasma and cell surface proteins due to non-enzymatic glycation also occur as a response to hyperglycaemia in both diabetes types, as shown in our study by the elevated levels of fructosamine in both T1DM and T2DM patients versus individuals without diabetes. Taken together, diabetes is associated with elevated plasma concentrations of proinflammatory proteins (including complement components and activation products) and procoagulant/antifibrinolytic proteins, functional changes of plasma proteins, increased expression of certain cell surface proteins (e.g. adhesion proteins), hyperreactive thrombocytes, and endothelial dysfunction and endothelial damage. How all these changes interact and eventually affect blood clot formation in diabetes has to our knowledge never been studied in a model that integrates endothelial cells, blood cells and plasma in a close-to-physiological system with a physiological geometry and blood flow.

Table 2. Demographic data, diabetes indices, coagulation and complement markers.

	Controls without diabetes (n=20)	T1DM patients (n=10)	T2DM patients (n=10)	p-value
Gender (male:female)	12:8	10:0	7:3	n.s.
Age (years)	33.9 ± 13.6	34.3 ± 8.2	52.6 ± 11.9	Co vs T2DM: 0.002 T1DM vs T2DM: 0.003
Number of smokers	8 (40%)	4 (36%)	7 (64%)	n.s.
BMI (kg/m ²)	n.d.	23.5 ± 3.6	35.3 ± 7.6	T1DM vs T2DM: 0.001
Duration of diabetes (years)	n.a.	17 ± 10	9 ± 9	n.s.
HbA _{1c} (%)	n.d.	7.3 ± 1.1	8.6 ± 1.4	T1DM vs T2DM: 0.015
Fructosamine (μM)	10.3 ± 1.1	15.2 ± 1.9	13.6 ± 3.6	Co vs T1DM: >0.001 Co vs T2DM: 0.017
Fibrinogen (mg/mL)	4.79 ± 0.88	3.89 ± 0.97	5.56 ± 1.15	Co vs T1DM: 0.017 Co vs T2DM: 0.053 T1DM vs T2DM: 0.003
PAI-I (ng/mL)	22.8 ± 14.3	12.7 ± 6.4	36.8 ± 16.6	Co vs T1DM: 0.012 Co vs T2DM: 0.023 T1DM vs T2DM: >0.001
MBL (μg/mL)	0.94 ± 0.73	0.40 ± 0.43	0.59 ± 0.44	Co vs T1DM: 0.005
C3 (mg/mL)	1.06 ± 0.25	0.75 ± 0.12	1.07 ± 0.25	Co vs T1DM: >0.001 T1DM vs T2DM: 0.002
sC5b9 (U/mL)	1.33 ± 0.30	1.73 ± 0.42	2.32 ± 0.83	Co vs T1DM: 0.006 Co vs T2DM: 0.002

Numeric data are shown as mean ± SD. Co: controls; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; n.d.: not determined; n.a.: not applicable; n.s.: not significant; BMI: body mass index; MBL: mannan-binding lectin; PAI-I: plasminogen activator inhibitor-1.

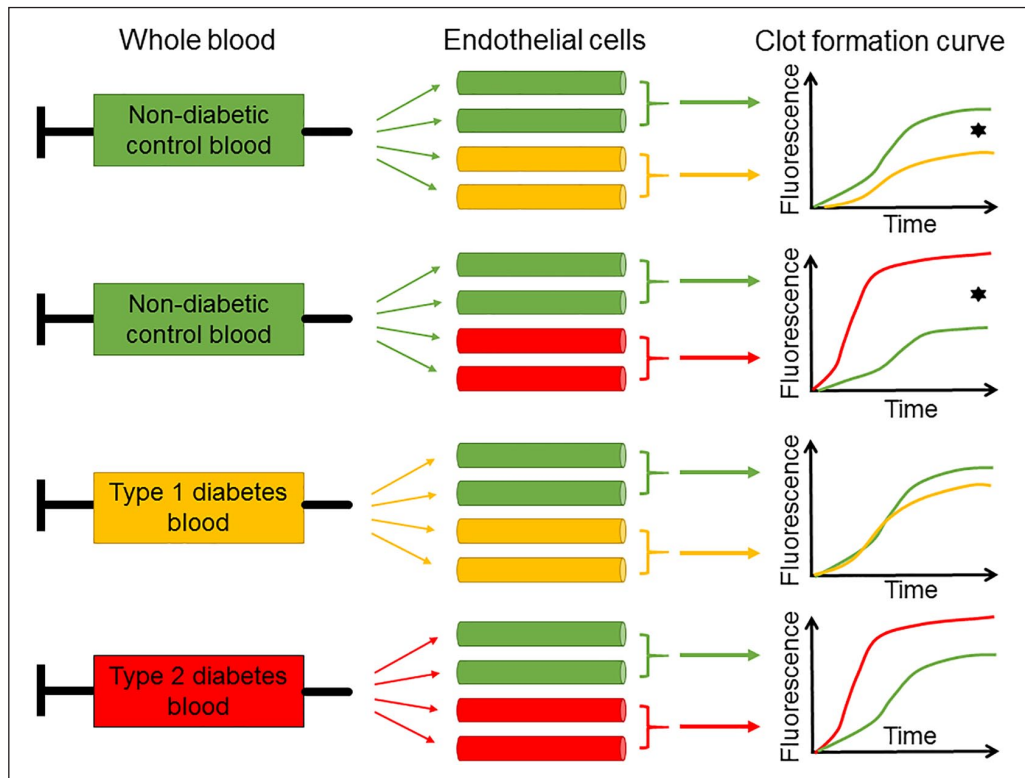


Figure 4. Schematic illustration of clot formation experiments in the diabetic microvascular flow model. This figure explains the complex study design but does not accurately reflect the results. Non-diabetic (green), type 1 diabetic (yellow) or type 2 diabetic (red) whole blood was used to perfuse parallel microchannels coated with non-diabetic (green), type 1 diabetic (yellow) or type 2 diabetic (red) primary human cardiac microvascular endothelial cells. Clot formation was recorded over time. The black star symbolises statistical significance.

Table 3. Clot formation in type 1 diabetes.

Microchannel coating	Non-diabetic blood (n = 10)	T1DM blood (n = 10)	p-value of T1DM vs non-diabetic blood
Non-diabetic cells	8.48×10^8 (9.78×10^8)	9.46×10^8 (1.43×10^9)	n.s. ^a
T1DM cells	5.43×10^8 (4.76×10^8)	5.36×10^8 (8.87×10^8)	n.s. ^a
p-value of T1DM vs non-diabetic cells	0.028 ^b	n.s. ^b	

Microchannels coated with non-diabetic or T1DM endothelial cells were perfused with non-diabetic or T1DM blood, and the total clot formation over time (calculated as AUC) is shown as median (IQR). n.s. = not significant; T1DM: type 1 diabetes mellitus; AUC: area under the time curve; IQR: interquartile range.

^aMann–Whitney *U*-test.

^bWilcoxon signed-rank test.

Table 4. Clot formation in type 2 diabetes.

Microchannel coating	Non-diabetic blood (n = 10)	T2DM blood (n = 10)	p-value of T2DM vs non-diabetic blood
Non-diabetic cells	3.50×10^8 (1.04×10^9)	6.00×10^8 (1.24×10^9)	n.s. ^a
T2DM cells	1.56×10^9 (1.39×10^9)	9.31×10^8 (1.99×10^9)	n.s. ^a
p-value of T2DM vs non-diabetic cells	0.005 ^b	n.s. ^b	

Microchannels coated with non-diabetic or T2DM endothelial cells were perfused with non-diabetic or T2DM blood and the total clot formation over time (calculated as AUC) is shown as median (IQR). n.s. = not significant; T2DM: type 2 diabetes mellitus; AUC: area under the time curve; IQR: interquartile range.

^aMann–Whitney *U*-test.

^bWilcoxon signed-rank test.

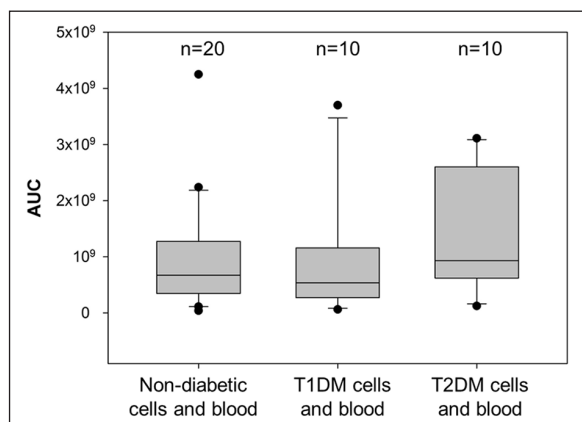


Figure 5. Comparison of clot formation between the pure non-diabetic, type 1 and type 2 diabetic models. Boxplots (representing median and interquartile range, with error bars showing the 10th and 90th percentile and black dots representing outliers) show the clot formation observed in non-diabetic cells perfused with non-diabetic blood, T1DM cells perfused with T1DM blood and T2DM cells perfused with T2DM blood.

Our main novel finding was the significant effects of diabetic HMVECs on clot formation. It was striking that T2DM HMVECs led to a stronger clot formation, while the presence of T1DM cells reduced clot formation when perfused with non-diabetic whole blood. The procoagulant effect

observed with T2DM HMVECs can be explained by endothelial dysfunction persisting even in cell culture due to the metabolic memory of endothelial cells caused by epigenetic modifications.^{11,34} But why did the T1DM HMVECs in our study show an opposite effect? Considering that T1DM is mostly diagnosed and controlled in early years – supported also by the fact that our T1DM blood donors had lower HbA1c, coagulation and inflammatory biomarkers than the T2DM donors – the epigenetic changes in T1DM HMVECs might be less pronounced than in T2DM HMVECs and therefore did not exhibit a procoagulant phenotype.

In order to characterise the primary HMVECs used in this study, we measured the expression of three markers, AGE receptor, fibronectin and PAI-1, which are differently expressed in and associated with diabetes.^{27–33} However, the high interindividual variation and small number made it difficult to draw a final conclusion.

We also observed a high interindividual variation in the circulating biomarkers. Our results are consistent with reports of elevated plasma levels of fibrinogen, PAI-1³⁰ and the complement activation marker sC5b-9 in diabetes.^{35,36} In contrast to published reports, including own results from an earlier study, that had shown elevated levels of C3^{17,18} and MBL^{37,38} in diabetes, we observed lower MBL and C3 levels in plasma samples from T1DM patients and similar levels in T2DM samples compared to the control group.

This unexpected finding might be explained by strong complement activation and consequently partial depletion of complement proteins, a hypothesis supported by high levels of sC5b-9 that showed an inverse correlation with MBL levels in our T1DM samples. Complement activation in diabetes can occur as a consequence of constant systemic inflammation in diabetes^{39,40} and also on glycated cell surface proteins which have been shown to activate the complement lectin pathway.⁴¹ In addition, MBL plasma levels depend to a large extent on genetic polymorphisms, and MBL deficiency has been reported in up to 10%–20% of the Caucasian population.^{42,43} Whether the similarity and lack of differences in MBL and C3 levels between control individuals without diabetes and T2DM patients were also connected to lack of difference in clot formation between non-diabetic and T2DM blood seems unlikely but cannot be excluded. We also observed unexpectedly low levels of fibrinogen and PAI-1 in the T1DM group and relatively high levels in the control group. There may be several possible explanations, as these three proteins are all influenced by multiple environmental, acquired and genetic factors. Fibrinogen and also C3, of course, are acute phase proteins and undergo acute changes upon exposure to pathogens. PAI-1 levels are affected by the common 4G/5G promoter polymorphism. Our T1DM patients may also have a less pronounced cardiovascular risk profile due to a deliberately healthier lifestyle and good medical care. A major factor, however, may be the small sample size which is of course more sensitive to extreme values than a larger patient population. We hypothesise that the latter, a high interindividual variation combined with the small sample size, may be the main reason for the unusual biomarker results.

Our study has of course several limitations. Ideally we would have tested endothelial cells and blood from the same patient, but it is not possible to obtain cardiac microvascular endothelial cells from living patients. We therefore used cardiac microvascular endothelial cells from deceased donors who had no diabetes or diagnosed T1DM or T2DM. These primary endothelial cells proved perfectly viable in cell culture and grew to confluent monolayers in our microchannels. However, only two donors had died of non-vascular causes (accident and asthma attack), and the other donors including one control individual without diabetes had succumbed to stroke. Unfortunately we have no information on the type of stroke; however, an underlying arterial disease is very likely. We can also not exclude the possibility that death has affected the endothelial cells and may explain the inconsistent expression patterns of the cell markers we studied. Another limitation is the small number of different cell and blood donors, and with a high interindividual variation this makes it more difficult to reach consistent and significant results. On the other hand, our study setting may be closer to ‘real-life’ where the average patient does not exist.

Diabetes is a complex multifactorial disease that is difficult to express in only a few biomarkers. We believe that a comprehensive diagnosis, which also takes the proinflammatory and procoagulant state into account for an estimation of the imminent or future risk of cardiovascular complications, will become more important in the management of diabetes in the future. Medicine is moving towards personalised or precision medicine, and this is also under discussion for the management of diabetes.^{44,45} However, personalised medicine for a complex multifactorial disease such as T2DM is a great challenge.⁴⁵ Novel diagnostic systems and models similar to the one we have described here may help to assess an individual patient’s status and risk of complications and monitor multitargeted therapies.

Conclusion

We present for the first time a diabetic microvascular flow model as a new tool to study the complex interactions between endothelial cells, blood cells and plasma proteins and their effects on clot formation under physiological flow conditions. We have shown that endothelial cells affect clot formation in whole blood, with cells from T2DM donors promoting a stronger clot formation compared with cells from T1DM donors and donors without diabetes. In the future, models similar to ours may contribute to a personalised management of diabetes and identify patients at risk of thrombotic events, but more research is clearly needed to validate such models.

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Author contributions

L.J. designed and performed the experiments, analysed the data and wrote the manuscript. A.M. recruited the diabetes patients and reviewed the manuscript. M.L. reviewed the manuscript. E.T.H. produced the wafers used for the device and reviewed the manuscript. W.L. developed the original microvascular flow model and reviewed the manuscript. V.S. designed the study, analysed the data and wrote the manuscript.

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References

- WHO. Global report on diabetes: WHO Library Cataloguing-in-Publication Data Global report on diabetes, 2016, https://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=C7C2E7032D2371D285BA2E852174D4C3?sequence=1
- Whiting DR, Guariguata L, Weil C, et al. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res Clin Pract* 2011; 94: 311–321.
- American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33(Suppl. 1): S62–S69.
- de Ferranti SD, de Boer IH, Fonseca V, et al. Type 1 diabetes mellitus and cardiovascular disease: a scientific statement from the American Heart Association and American Diabetes Association. *Diabetes Care* 2014; 37: 2843–2863.
- Low Wang CC, Hess CN, Hiatt WR, et al. Clinical update: cardiovascular disease in diabetes mellitus. *Circulation* 2016; 133: 2459–2502.
- Fan W. Epidemiology in diabetes mellitus and cardiovascular disease. *Cardiovasc Endocrinol* 2017; 6: 8–16.
- Martin-Timon I, Sevillano-Collantes C, Segura-Galindo A, et al. Type 2 diabetes and cardiovascular disease: have all risk factors the same strength. *World J Diabetes* 2014; 5: 444–470.
- Shin JA, Lee JH, Lim SY, et al. Metabolic syndrome as a predictor of type 2 diabetes, and its clinical interpretations and usefulness. *J Diabetes Investig* 2013; 4: 334–343.
- Cornier MA, Dabelea D, Hernandez TL, et al. The metabolic syndrome. *Endocr Rev* 2008; 29: 777–822.
- Katakami N, Kaneto H, Funahashi T, et al. Type 2 diabetes and atherosclerosis: focusing on metabolic syndrome. *Diabetol Int* 2013; 4: 143–148.
- Roberts AC and Porter KE. Cellular and molecular mechanisms of endothelial dysfunction in diabetes. *Diab Vasc Dis Res* 2013; 10: 472–482.
- Schneider DJ. Factors contributing to increased platelet reactivity in people with diabetes. *Diabetes Care* 2009; 32: 525–527.
- Dunn EJ, Ariens RA and Grant PJ. The influence of type 2 diabetes on fibrin structure and function. *Diabetologia* 2005; 48: 1198–1206.
- Hertle E, Stehouwer CDA and van Greevenbroek MM. The complement system in human cardiometabolic disease. *Mol Immunol* 2014; 61: 135–148.
- Ajjan RA and Schroeder V. Role of complement in diabetes. *Mol Immunol* 2019; 114: 270–277.
- Conway EM. Reincarnation of ancient links between coagulation and complement. *J Thromb Haemost* 2015; 13: S121–132.
- Schroeder V, Carter AM, Dunne J, et al. Proinflammatory and hypofibrinolytic phenotype in healthy first-degree relatives of patients with Type 2 diabetes. *J Thromb Haemost* 2010; 8: 2080–2082.
- Hess K, Alzahrani SH, Mathai M, et al. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. *Diabetologia* 2012; 55: 1103–1113.
- Jenny L, Ajjan R, King R, et al. Plasma levels of MASP-1 and MASP-2 are elevated in type 1 diabetes and correlate with glycaemic control. *Clin Exp Immunol* 2015; 180: 227–232.
- Grant PJ. Diabetes mellitus as a prothrombotic condition. *J Intern Med* 2007; 262: 157–172.
- Alzahrani SH and Ajjan RA. Coagulation and fibrinolysis in diabetes. *Diab Vasc Dis Res* 2010; 7: 260–273.
- Austin GE, Mullins RH and Morin LG. Non-enzymic glycation of individual plasma proteins in normoglycemic and hyperglycemic patients. *Clin Chem* 1987; 33: 2220–2224.
- Ajjan RA, Gamlen T, Standeven KF, et al. Diabetes is associated with posttranslational modifications in plasminogen resulting in reduced plasmin generation and enzyme-specific activity. *Blood* 2013; 122: 134–142.
- Tsai M, Kita A, Leach J, et al. In vitro modeling of the microvascular occlusion and thrombosis that occur in hematologic diseases using microfluidic technology. *J Clin Invest* 2012; 122: 408–418.
- Mannino RG, Myers DR, Ahn B, et al. Do-it-yourself in vitro vasculature that recapitulates in vivo geometries for investigating endothelial-blood cell interactions. *Sci Rep* 2015; 5: 12401.
- Jenny L, Dobo J, Gal P, et al. MASP-1 of the complement system enhances clot formation in a microvascular whole blood flow model. *Plos One* 2018; 13: e0191292.
- Kanters SD, Banga JD, Algra A, et al. Plasma levels of cellular fibronectin in diabetes. *Diabetes Care* 2001; 24: 323–327.
- Moradipoor S, Ismail P, Etemad A, et al. Expression profiling of genes related to endothelial cells biology in patients with type 2 diabetes and patients with prediabetes. *Biomed Res Int* 2016; 2016: 1845638.
- Konieczynska M, Bryk AH, Malinowski KP, et al. Interplay between elevated cellular fibronectin and plasma fibrin clot properties in type 2 diabetes. *Thromb Haemost* 2017; 117: 1671–1678.
- Yarmolinsky J, Bordin Barbieri N, Weinmann T, et al. Plasminogen activator inhibitor-1 and type 2 diabetes: a systematic review and meta-analysis of observational studies. *Sci Rep* 2016; 6: 17714.
- Rhee SY and Kim YS. The role of advanced glycation end products in diabetic vascular complications. *Diabetes Metab J* 2018; 42: 188–195.
- Gao X, Zhang H, Schmidt AM, et al. AGE/RAGE produces endothelial dysfunction in coronary arterioles in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol* 2008; 295: H491–148.
- Leung SS, Forbes JM and Borg DJ. Receptor for advanced glycation end products (RAGE) in type 1 diabetes pathogenesis. *Curr Diab Rep* 2016; 16: 100.

34. Reddy MA and Natarajan R. Epigenetic mechanisms in diabetic vascular complications. *Cardiovasc Res* 2011; 90: 421–429.
35. Mellbin LG, Bjerre M, Thiel S, et al. Complement activation and prognosis in patients with type 2 diabetes and myocardial infarction: a report from the DIGAMI 2 trial. *Diabetes Care* 2012; 35: 911–917.
36. Saini PK, Saluja M, Meena SR, et al. Study of plasma fibrinogen level in type 2 diabetes mellitus and its association with microalbuminuria and glycemic control. *Curr Med Res Pract* 2016; 6: 113–116.
37. Hansen TK, Thiel S, Knudsen ST, et al. Elevated levels of mannan-binding lectin in patients with type 1 diabetes. *J Clin Endocrinol Metab* 2003; 88: 4857–4861.
38. Mellbin LG, Hamsten A, Malmberg K, et al. Mannose-binding lectin genotype and phenotype in patients with type 2 diabetes and myocardial infarction: a report from the DIGAMI 2 trial. *Diabetes Care* 2010; 33: 2451–2456.
39. Mangge H, Schauenstein K, Stroedter L, et al. Low grade inflammation in juvenile obesity and type 1 diabetes associated with early signs of atherosclerosis. *Exp Clin Endocrinol Diabetes* 2004; 112: 378–382.
40. Pitsavos C, Tampourlou M, Panagiotakos DB, et al. Association between low-grade systemic inflammation and type 2 diabetes mellitus among men and women from the ATTICA study. *Rev Diabet Stud* 2007; 4: 98–104.
41. Fortpied J, Vertommen D and Van Schaftingen E. Binding of mannose-binding lectin to fructosamines: a potential link between hyperglycaemia and complement activation in diabetes. *Diabetes Metab Res Rev* 2010; 26: 254–260.
42. Goicoechea de Jorge E, Lopez Lera A, Bayarri-Olmos R, et al. Common and rare genetic variants of complement components in human disease. *Mol Immunol* 2018; 102: 42–57.
43. Keizer MP, Wouters D, Schlapbach LJ, et al. Restoration of MBL-deficiency: redefining the safety, efficacy and viability of MBL-substitution therapy. *Mol Immunol* 2014; 61: 174–184.
44. Marshall SM. Precision diabetes: a realistic outlook on a promising approach. *Diabetologia* 2017; 60: 766–768.
45. Gloyn AL and Drucker DJ. Precision medicine in the management of type 2 diabetes. *Lancet Diabetes Endocrinol* 2018; 6: 891–900.