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Hypoglycaemia leads to a delayed increase in platelet and coagulation activation markers in people with type 2 diabetes treated with metformin only: Results from a stepwise hypoglycaemic clamp study

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Abstract

Aims: To investigate the effect of hypoglycaemia on platelet and coagulation activation in people with type 2 diabetes.

Materials and methods: This monocentric, open, single-arm, mechanistic trial included 14 people with established type 2 diabetes (four women, 10 men, age 55 ± 7 years, glycated haemoglobin concentration 51 ± 7 mmol/mol) receiving metformin monotherapy. A stepwise hyperinsulinaemic-hypoglycaemic clamp experiment (3.5 and 2.5 mmol/L, for 30 minutes respectively) was performed, aiming to investigate platelet and coagulation activity during predefined plateaus of hypoglycaemia, as well as 1 day and 7 days later.

Results: While platelet activation assessed by light transmittance aggregometry did not significantly increase after the hypoglycaemic clamp procedure, the more sensitive flow cytometry-based measurement of platelet surface activation markers showed hypoglycaemia-induced activation 24 hours (PAC1^{pos}CD62P^{pos}, PAC1^{pos}CD63P^{pos} and PAC1^{pos}CD62P^{pos}CD63^{pos}; $P < .01$) and 7 days after the hypoglycaemic clamp ($P < .001$ for PAC1^{pos}CD63^{pos}; $P < .01$ for PAC1^{pos}CD62P^{pos} and PAC1^{pos}CD62P^{pos}CD63^{pos}) in comparison to baseline. Coagulation markers, such as fibrinogen, D-dimer, plasminogen activator inhibitor-1, von Willebrand factor activity and factor VIII, were also significantly increased, an effect that was most pronounced 24 hours after the hypoglycaemic clamp.

Conclusion: A single event of insulin-induced hypoglycaemia led to an increase in markers of platelet activation and coagulation in people with early stages of type 2 diabetes on metformin therapy. However, the activation occurred with a delay and was evident 24 hours and 7 days after the actual hypoglycaemic episode.

KEYWORDS
clamp, coagulation, hypoglycaemia, hypoglycaemic clamp, metformin, platelet activation, type 2 diabetes

1 | INTRODUCTION

While some studies have demonstrated cardiovascular benefits of intensive glucose control,[1] others were not able to replicate these effects[2-5] or even showed increased mortality rates associated with intensive glucose control.[3] Hypoglycaemic events, which are an integral part of treatment regimens with sulphonylureas or insulin, were suggested to be one potential cause of the increase in mortality seen in the ACCORD trial[3] and several analyses have demonstrated an increase in cardiovascular and overall mortality associated in particular with severe hypoglycaemic events.[6-11]

A couple of pathophysiological theories have been put forward to explain the potential harmful atherothrombotic effects of hypoglycaemic events.[12] One was activation of the sympathetic nervous system, which induces the release of counterregulatory hormones (eg, catecholamines) as a response to the hypoglycaemic state, leading to haemodynamic changes with increased cardiac workload as well as derailments of potassium levels and provoking cardiac arrhythmia, QT interval prolongation and sudden cardiac death.[13-15] The second explanation frequently mentioned is the activation of platelets and the coagulation system by hypoglycaemic episodes, subsequently increasing the risk of atherothrombotic events.[16-19]

Studies investigating the proatherothrombotic effects of hypoglycaemia on platelet activity, however, were mainly performed in healthy individuals or in people with type 1 diabetes, and data on the population with type 2 diabetes remain scarce.[17,20-22] In addition, these studies did not systematically evaluate the hypoglycaemic threshold at which potential platelet and coagulation activation may occur or how sustainable such an activation would be.

In the present study, we aimed to explore platelet activation as well as atherothrombotic and inflammation biomarkers in people at an early stage of type 2 diabetes on metformin monotherapy, without established cardiovascular disease and without platelet aggregation inhibitor therapy during a stepwise hypoglycaemic clamp experiment and a follow-up 7 days after the clamp event.

2 | MATERIALS AND METHODS

2.1 | Participants

The study participants were aged between 18 and 64 years (both inclusive) at screening/enrolment, were diagnosed with type 2 diabetes according to American Diabetes Association criteria,[23] were on either stable monotherapy with metformin for a period of 90 days prior to screening or diet only, had a body mass index (BMI) between 20.0 and 35.0 kg/m^{2} (both inclusive) and a glycated haemoglobin (HbA1c) concentration between 43 and 64 mmol/moL (6.0%-8.0%; both inclusive). The main exclusion criteria were: use of any platelet-inhibiting therapy; treatment with β-blockers or other antiarrhythmic agents; impaired hypoglycaemia awareness; medical history of cardiac arrhythmia or uncontrolled hypertension; previously known established cardiovascular disease and/or past cardiovascular events, or past episodes of congestive heart failure syndrome (New York Heart Association grade II-IV); active smoking; clinically significant abnormal haematology, biochemistry, lipid levels, hormone levels or coagulation values at the screening visit; and use of neuroleptic drugs or intake of illicit substances. If a participant was treated with non-steroidal anti-inflammatory drugs (NSAIDs) or metamizole within 10 days prior to the clamp visits and/or their fasting plasma glucose was higher than 9.0 mmol/L on the day of the clamp investigations, the clamp visit was postponed for 10 to 15 days.

The Ethics Committee of the Medical University of Graz, Austria, approved the study (Ethics Committee number: EK30-012ex17/1) and all participants gave written consent prior to any study-related procedures. The study was conducted in accordance with the Declaration of Helsinki as well as the guidelines laid down by the International Conference on Harmonization for Good Clinical Practice (ICH GCP E6 guidelines). Participants were recruited using a local recruitment registry (Graz Diabetes Registry for Biomarker Research, GIRO). The study was registered at clinical trials.gov (https://clinicaltrials.gov/ct2/show/NCT03460899 | NCT03460899).

2.2 | Study design

All participants underwent a hyperinsulinaemic-euglycaemic and subsequently a hyperinsulinaemic-hypoglycaemic clamp experiment, separated by a washout phase of 10 to 24 days in order to avoid overlapping effects of outcome parameters. Both clamp investigations were performed at the Clinical Trials Unit in Graz, Austria, after a 10-hour overnight fasting period. The morning metformin dose, if applicable, was not administered on the days the clamps were performed. During both clamp experiments, two intravenous cannulas were inserted in antecubital veins of both forearms for both serial sampling of arterialized plasma glucose, platelet, inflammation and hormone levels and, on the opposite side, for insulin (40 IU Actrapid,
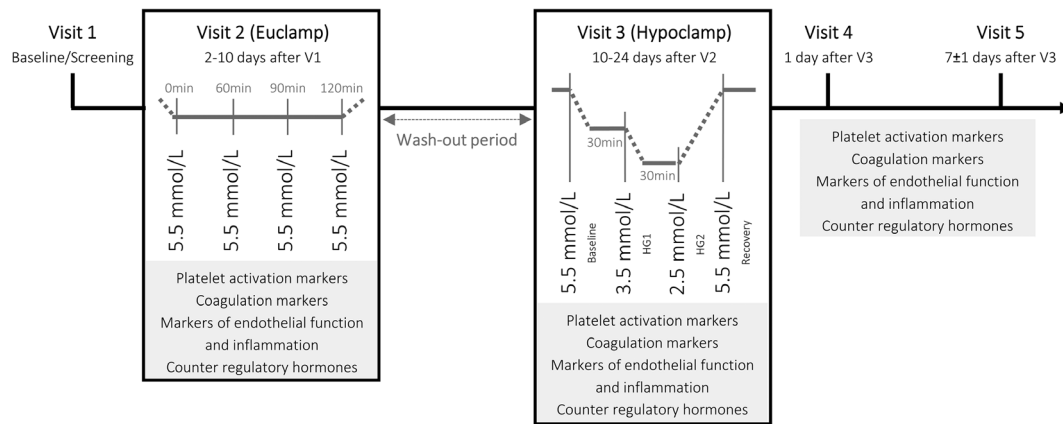
FIGURE 1 Study design. HG 1, hypoglycaemic plateau 1 (3.5 mmol/L); HG 2, hypoglycaemic plateau 2 (2.5 mmol/L); V, visit

100 IU/mL in 99.6 mL saline) or glucose infusion. For safety reasons, serum potassium was measured before and after the clamps. The study design is shown in Figure 1.

2.3 | Hyperinsulinaemic-euglycaemic clamp experiment

To rule out activation of platelets and coagulation by hyperinsulinaemia rather than insulin-induced hypoglycaemia, we perfomed a hyperinsulinaemic-euglycaemic clamp experiment. A variable intravenous infusion of glucose 20% or human rapid-acting insulin was initiated in order to obtain a plasma glucose target of 5.5 mmol/L ± 10%. After achieving this glucose target, a continuous insulin infusion (2.5 mU/kg/min) was applied for 120 minutes, together with a variable infusion of glucose 20% to maintain serum glucose concentrations at the prespecified level. Blood samples for measurement of plasma glucose concentrations were obtained at 5-minute intervals. For measurements of platelet and coagulation activation, blood samples were collected at timepoints 0, 60, 90 and 120 minutes.

Having analysed the results we amended the protocol and performed another euglycaemic clamp experiment in people willing to participate again, and measured platelet and coagulation activation also 1 day and 7 days after the clamp experiment.

2.4 | Hyperinsulinaemic-hypoglycaemic clamp experiment

After initial stabilization of plasma glucose at 5.5 mmol/L using a variable insulin or glucose 20% infusion, a constant insulin infusion of 2.5 mU/kg/min was initiated. Variable glucose 20% infusion was applied to maintain the target plasma glucose level of 5.5 mmol/L for 30 minutes (baseline plateau). Subsequently, by discontinuation of glucose infusion, plasma glucose was allowed to further decline to 3.5 mmol/L, a level that was again kept for 30 minutes. Thereafter, the procedure was repeated to reach and maintain a second hypoglycaemic plateau at 2.5 mmol/L for 30 minutes. After 15 minutes of the second hypoglycaemic plateau, insulin infusion was terminated

and glucose infusion was tapered off to allow a spontaneous recovery from hypoglycaemia, aiming to achieve plasma glucose of 5.5 mmol/L.

Samples for platelet activation and coagulation markers as well as values for markers of endothelial function, inflammation and counterregulatory hormones were obtained 30 minutes after reaching the designated plateau as well as after recovery from hypoglycaemia at a plasma glucose level of 5.5 mmol/L.

To ensure safety, participants were monitored with an ECG throughout both clamp experiments.

2.5 | Follow-up visits

Participants were instructed to return to the study centre 1 and 7 days after the hypoglycaemic clamp experiment in order to have blood drawn to assess platelet activity, coagulation, endothelial function and inflammatory markers as well as counterregulatory hormones to evaluate delayed or sustainable effects of the hypoglycaemic event.

2.6 | Analytical methods

2.6.1 | Platelet activity measures

Platelet activity was determined in the course of blood sampling at visits 2, 3, 4 and 5. In both clamp experiments, platelet activity markers were measured at four time points. The blood samples were collected in sodium citrate tubes, kept at ambient temperature, and transferred immediately to the Clinical Institute for Medical and Chemical Laboratory Diagnostics at the University Hospital of Graz and the Centre for Biomarker Research in Medicine.

The number of platelets was determined on the Sysmex XE-2100TM Automated Hematology System (Sysmex Austria GmbH, Vienna, Austria). Fibrinogen (Multifibren U reagent; Siemens Healthcare Diagnostics GmbH, Vienna, Austria) was measured on a Behring Coagulation System BCS XP Analyser (Siemens Healthcare Diagnostics GmbH). D-dimer (Innovance D-Dimer; Siemens Austria GmbH, Vienna, Austria), von Willebrand factor activity (vWF; Innovance vWF activity assay) and factor VIII (Coagulation Factor VIII

Deficient Plasma; Siemens Austria GmbH) were measured on the Coagulation Analyser BCS XP.

Light transmission aggregometry (LTA) was performed on a Chronolog 700 Lumi-Aggregometer (Chronolog Corp, Havertown, Pennsylvania) using standard agonists of platelet aggregation (Collagen, ADP, arachidonic acid, TRAP) in order to stimulate platelet aggregation in platelet-rich plasma.[24,25]

PFA-200 was measured on a Platelet Function Analyser-200 (Siemens Healthineers, Marburg, Germany) using collagen/epinephrine cartridges to monitor closure time in vitro.

2.6.2 | Fluorescence-activated cell sorting: Quantification of platelet function and activation by flow cytometry

Blood samples were processed for multiparameter flow cytometric (fluorescence-activated cell sorting [FACS]) analysis within 10 minutes after collection. Aliquots of whole blood were treated with 200 μM ADP sodium salt (Sigma-Aldrich, Steinheim, Germany) for 2 minutes at room temperature to achieve platelet stimulation. In both stimulated and unstimulated blood samples, surface antigens were stained with the following fluorochrome-conjugated monoclonal antibodies: anti-CD41 PE (Thermo Fisher Scientific, Waltham, Massachusetts), anti-CD42b PE-CF594 (BD Biosciences, Franklin Lakes, New Jersey), anti-CD62P APC (Thermo Fisher Scientific), anti-CD63 PE-Cyanine 7 (Thermo Fisher Scientific) and anti-PAC-1 FITC (BD Biosciences). Single platelets were identified according to the size and granularity parameters and the expression of CD41 and CD42b. We analysed only free platelets and excluded CD45^{pos}CD42b^{pos} platelet-leukocyte aggregates from the analyses. The percentage of postitive cells (positive for the single or combined activation markers PAC1, CD62P or CD63) always represents the percentage within the population of CD41CD42b positive platelets.

We used previously described surface platelet activation markers; CD62P (P-selectin) is stored within the platelet and released upon activation of the platelets, as is CD63, which represents a lysosomal protein. PAC-1 antibodies bind to activated GP IIb/IIIa complex on the platelet surface. Platelet activation markers were quantified using a BD LSRFortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

The concentration of soluble semaphorin 4D (Sema4D) in freshly thawed EDTA plasma aliquots was quantified using a commercially available kit ELISA (Biomedica, Vienna, Austria) as recommended by the manufacturer. The intra- and interassay coefficients of variation were given as ≤8% and ≤ 11%, respectively.

Further analytical methods are described in the Supporting Information.

2.7 | Statistical analyses

The statistical analyses were performed with SAS software version 9.4 (SAS Institute, Cary, North Carolina). Demographic and baseline characteristics were summarized and tabulated as means ± SD or median and minimum and maximum for quantitative variables and frequencies and percentages for categorical variables.

The primary endpoint, changes in platelet activation measured by light transmittance aggregometry based on ADP activation from baseline to the end of the hypoglycaemia phase (ie, 2.5 mmol/L for 30 minutes) at visit 3, was analysed using a paired t test. In addition, for the primary and secondary endpoints, linear models for repeated measurements were used to explore changes over time during euglycaemia (time points: baseline euclamp vs 60, 90 and 120 minutes) and during hypoclamp (visit 3) up to 7 days after visit 3 (time points: baseline hypoclamp vs glucose plateau at 3.5 mmol/L [HG1] and glucose plateau at 2.5 mmol/L [HG2], recovery, day 1 and day 7). Descriptive statistics (mean ± SE for each variable over time) are presented graphically. A two-sided P value of <.05 was considered to indicate statistical significance. To correct for multiple testing, for the secondary endpoints only P values <.01 were considered significant.

2.8 | Sample size calculation

We have shown previously that LTA based on ADP activation in a population with type 2 diabetes is 70 ± 12%.[26] For a clinically relevant difference in LTA from baseline to hypoglycaemia of 10% and assuming a standard deviation of the differences of 12%, 14 participants were needed to achieve a power of 80% using a paired t test with a .05 two-sided significance level. One participant who dropped out was replaced in order to retain adequate power.

TABLE 1 Baseline characteristics (n = 14)

Characteristic	
Age, y	55 ± 7
Men, n (%)	10 (71.4)
BMI, kg/m^2	28.9 ± 3.3
Weight, kg	86.4 ± 15.1
SBP, mmHg	133 ± 13
DBP, mmHg	83 ± 8
Fasting plasma glucose, mmol/L	7.2 ± 0.9
Triglycerides, mmol/L	2.02 ± 1.33
Cholesterol, mmol/L	5.22 ± 1.19
HDL cholesterol, mmol/L	1.19 ± 0.36
LDL cholesterol, mmol/L	3.13 ± 1.01
Diabetes duration, years	5 ± 4
Daily metformin dose, mg	1336 ± 599
HbA1c, mmol/mol (%)	51 ± 7 (6.8 ± 2.8)
ACE inhibitors, n (%)	4 (28.6)
Angiotensin-II receptor antagonists, n (%)	5 (35.7)
Calcium antagonists, n (%)	3 (21.4)
Diuretics, n (%)	1 (7.1)
Statins, n (%)	4 (28.6)

Note: Data are presented as mean ± SD unless otherwise indicated. Abbreviations: ACE, angiotensin-converting-enzyme; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; SBP, systolic blood pressure.

3 | RESULTS

In total, 19 volunteers were screened for the study, of whom four did not qualify for enrolment (one with steroid replacement therapy, three with HbA1c values outside the inclusion range). One participant was excluded from the trial after the second clamp procedure when we became aware that he was not fulfilling all inclusion criteria (participant was using insulin intermittently). This participant was also excluded from the final analysis. Finally, 14 participants (10 men and four women, age 55 ± 7 years, BMI 28.9 ± 3.3 kg/m^2) were included in the statistical analysis. Detailed baseline characteristics and demographic data are summarized in Table 1. All clamp experiments were conducted without any related adverse events.

3.1 | Blood glucose and counterregulatory hormones

The baseline glucose values prior to the initiation of the clamp experiments were 7.2 ± 1.2 mmol/L (hypoglycaemic clamp) and 7.1 ± 1.0 mmol/L (euglycaemic clamp). Detailed glucose levels achieved during the hyperinsulinaemic-hypoglycaemic clamp experiments are shown in Table S1.
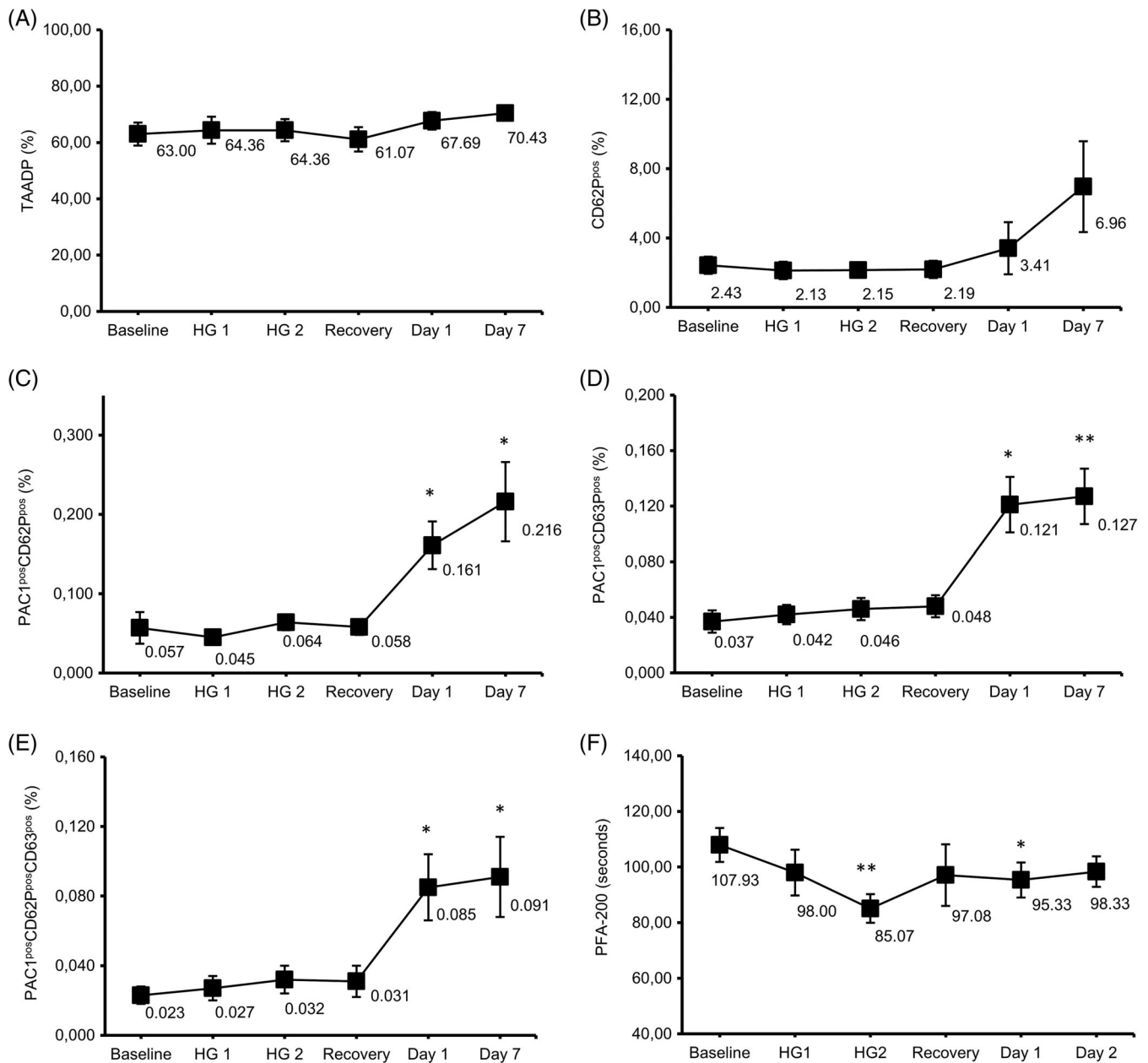

FIGURE 2 Effects of hypoglycaemia on platelet function. A, Platelet activation adenosine diphosphate (TAADP). B, CD62P^{pos}. C, PAC1^{pos}CD62P^{pos}. D, PAC1^{pos}CD63^{pos}. E, PAC1^{pos}CD62P^{pos}CD63^{pos}. F, PFA-200. *P < .01, **P < .001. Data are displayed as mean ± SE. HG 1, hypoglycaemic plateau 1 (3.5 mmol/L); HG 2, hypoglycaemic plateau 2 (2.5 mmol/L)
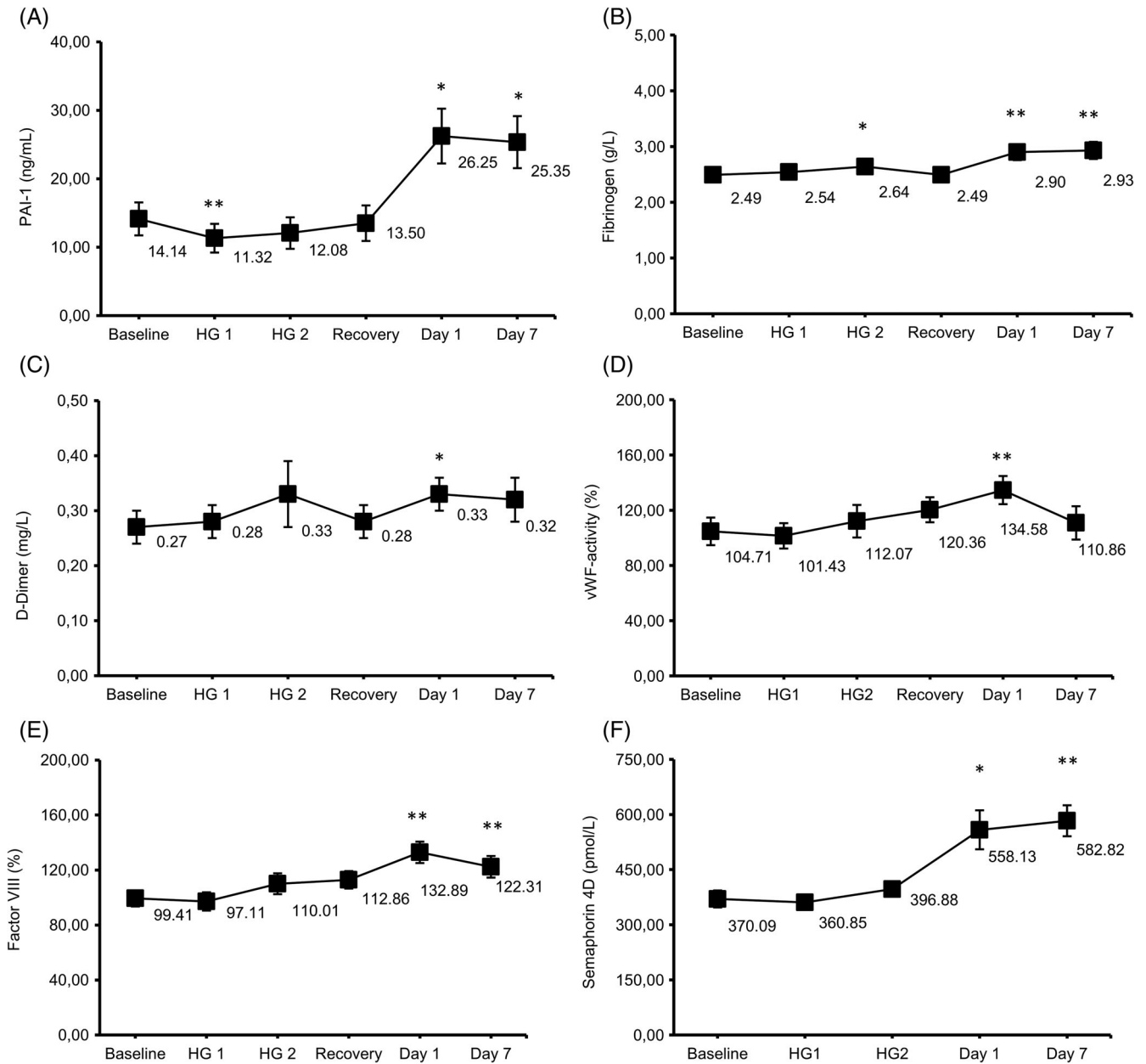
FIGURE 3 Effects of hypoglycaemia on coagulation markers. A, Plasminogen activator inhibitor-1 (PAI-1). B, Fibrinogen. C, D-Dimer. D, Von Willebrand factor (vWF) activity. E, Factor VIII. F, Semaphorin 4D. $*P < .01$, $**P < .001$. Data are displayed as mean ± SE. HG1, hypoglycaemic plateau 1 (3.5 mmol/L); HG2, hypoglycaemic plateau 2 (2.5 mmol/L)

As expected, adrenaline, cortisol and glucagon significantly increased during the stepwise hypoglycaemic clamp procedure (Figure S1).

3.2 | Platelet activation markers

Platelet activation markers remained unchanged during the euglycaemic clamp experiment (Figure S2).

Changes in platelet activation, measured by LTA based on ADP activation, remained unchanged during hypoglycaemia, recovery and on follow-up at days 1 and 7 as well as during the euglycaemic clamp compared to baseline levels. However, platelet activation measured

by flow cytometric methods demonstrated a significant rise in PAC1posCD62Ppos, PAC1posCD63pos, PAC1posCD62PposCD63pos positive cells the day after the hypoglycaemic clamp, with a sustained increase also seen 7 days after (Figure 2C-E). Closure time in the PFA-200 assay significantly decreased at the glucose level of 2.5 mmol/L and remained reduced the day after the clamp experiment (Figure 2F).

3.3 | Markers of coagulation

During the euglycaemic clamp coagulation markers remained stable (D-dimer, von Willebrand factor activity, factor VIII) or numerically
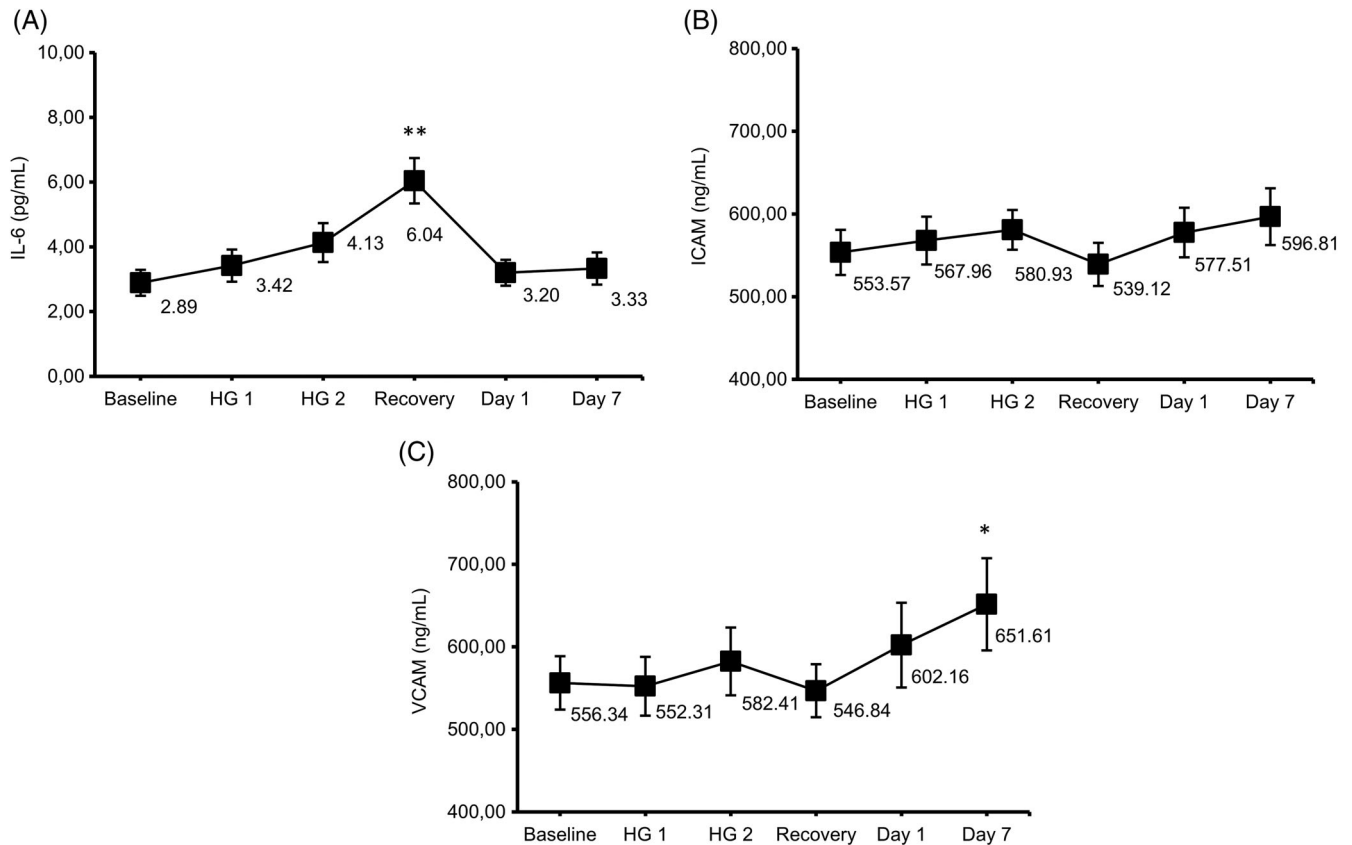
FIGURE 4 Effects of hypoglycaemia on inflammatory markers. A, Interleukin-6 (IL-6). B, Intracellular adhesion molecule (ICAM). C, Vascular cell adhesion molecule (VCAM). *$P < .01$, **$P < .001$. Data are displayed as mean ± SE. HG 1, hypoglycaemic plateau 1 (3.5 mmol/L); HG 2, hypoglycaemic plateau 2 (2.5 mmol/L)

decreased (fibrinogen, plasminogen activator inhibitor-1 [PAI-1]) over time (Figure S3).

Fibrinogen, PAI-1, D-dimer, von Willebrand factor activity and factor VIII were significantly increased 1 day after the hypoglycaemic clamp. All but D-dimer and von Willebrand factor activity remained significantly elevated 7 days after the hypoglycaemic clamp (Figure 3A-E).

3.4 | Markers of endothelial function and inflammation

During the hyperinsulinaemic-euglycaemic clamp, interleukin-6 (IL-6) remained unchanged, while vascular cell adhesion molecule (VCAM) and intercellular cell adhesion molecule (ICAM) gradually declined over time (ICAM was significantly lower at the end of the clamp experiment; $P < .001$ [Figure S4A-C]).

Levels of VCAM and ICAM did not change during the hypoglycaemic clamp experiment, but increased thereafter, reaching a peak at day 7, the increase for VCAM reaching statistical significance ($P < .01$). IL-6 acutely and significantly increased during the hypoglycaemic clamp and reached a peak at the end of the clamp ($P < .001$ compared to baseline). This increase was not sustained and no increases were observed 24 hours or 7 days after hypoglycaemia (Figure 4A-C).

Figure S2 shows markers for endothelial function and inflammation during the euglycaemic clamp.

Sema4D remained unchanged during acute hypoglycaemia; however, it significantly increased during the follow-up 1 day later and was even more pronounced 7 days after the antecedent hypoglycaemic event (Figure 3F).

4 | DISCUSSION

This experimental study investigated acute and sustainable effects of a single stepwise hypoglycaemic episode on platelet activation and coagulation as well as on endothelial function and inflammation markers in human volunteers with type 2 diabetes on metformin monotherapy.

Light transmission aggregometry is accepted as a valuable test for platelet function and it was chosen as the primary endpoint on which to base sample size calculation for the present study because previous baseline data in a similar diabetes cohort were available. However, other methods, such as those using flow cytometry to measure platelet activation by antibody binding to specific surface proteins are more sensitive to detect a moderate change in the activation pattern. CD62P represents P-selectin, a cell adhesion molecule that is released to the surface upon activation, as is CD63, which is stored lysosomally in non-activated platelets. PAC-1 antibodies bind to the activated GP

IIb/IIIa complex on the platelet surface, while they do not if the complex is quiescent.[27] In the present study we were able to show activation of platelets expressed by those activation markers, or a combination of them. Platelet function, assessed by flow cytometry or PFA-200 measurement, demonstrated activation in particular 1 day after the hypoglycaemic clamp, which was sustained over 1 week.

Our data support and extend the study findings by Chow et al[28] demonstrating increased platelet reactivity after hypoglycaemia. However, the present study used different measurements to assess platelet reactivity. While Chow et al performed two consecutive hypoglycaemic clamps with a 2.5-mmol/L glucose target (60 minutes in the morning and 60 minutes in the afternoon) in each participant, we used a stepwise hypoglycaemic clamp with 30 minutes of 3.5 mmol/L and 30 minutes of 2.5 mmol/L glucose plateaus to investigate whether a moderate hypoglycaemic plateau already leads to platelet or coagulation activation. Interestingly, in the preset study, the increase in platelet reactivity was mainly observed the day after the hypoglycaemic clamp and was sustained over the next 6 days. While Chow et al[28] included people who were on various glucose-lowering agents including insulin, with two of them also taking aspirin, our study participants had a short duration of diabetes and were all on metformin only and none was taking any antiplatelet agents.

Kahal et al[29] investigated the effects of a single hypoglycaemic (2.8 mmol/L) episode on platelet reactivity in 10 people with type 2 diabetes. They demonstrated reduced platelet sensitivity to prostacyclin 24 hours after hypoglycaemia. That study supports our finding of platelet activation 24 hours after the hypoglycaemic stimulus.

In contrast to platelet activation, the markers of the coagulation system showed a consistent activation response to hypoglycaemia. It is striking that this activation was not observed during the hypoglycaemic event itself, but consistently 1 day after the clamp procedure. This activation seems to be sustained for most of the variables assessed for 1 week after the hypoglycaemic episode.

vWF activity is a surrogate marker for endothelial function inducing platelet-monocyte aggregation, and during the present study we noticed a significant increase in this marker following hypoglycaemia. These results are in line with data shown previously which demonstrated similar vWF properties in people with type 1 diabetes but not in healthy individuals.[16]

Zakai et al[30] recently suggested elevated factor VIII levels to be predictive of coronary heart disease and stroke, and this risk was more pronounced in patients with known diabetes. In the present experiment, factor VIII was significantly increased after hypoglycaemia and remained elevated for 1 week.

PAI-1, a platelet-derived protein, represents an important key component of the haemostatic system, inhibiting the cleavage of plasminogen to plasmin and hence inhibiting fibrinolysis. In the present study, PAI-1 concentrations were significantly increased 24 hours after hypoglycaemia and remained elevated over the following 6 days, suggesting reduced fibrinolytic activity after a prior hypoglycaemic episode. Data from the Framingham Heart Study identified increased PAI-1 as a strong predictive marker for cardiovascular events even after adjusting for established risk factors.[31] Our finding again

corresponds with the results reported by Chow et al[28] showing delayed clot lysis after hypoglycaemia. Interestingly, we observed a drop in PAI-1 in all clamps shortly after the initiation of the insulin infusion, a finding that was reported previously, suggesting a direct insulin mediated PAI-1-lowering effect.[21,28]

Soluble Sema4D, also known as Cluster of Differentiation 100 (CD100), represents an integral membrane surface protein expressed mainly by T cells where it supports B cell development[32]; its main function comprises the support of intracellular signal transduction and axon guidance in the central nervous system.[33] However, Sema4D has also been shown to be expressed by platelets and, at this stage, it might be involved in repairing of vascular injury by inducing angiogenic responses from endothelial cells and interfering with monocyte migration.[34] Sema4D was identified to act as a potential biomarker for acute heart failure,[35] and a loss of expression was described to reduce platelet hyperactivity and consequently might prevent the development of atherosclerosis and cardiovascular disease.[36] However, Sema4D levels during hypoglycaemia in people with type 2 diabetes have not yet been investigated. The present study provides the first evidence that an acute hypoglycaemic event leads to an increase in Sema4D levels, an effect which was most pronounced 7 days after hypoglycaemia. The role of Sema4D in atherothrombosis in people with diabetes mellitus will need to be further explored.

The present study has some limitations, including the fact that 14 people with diabetes could be considered a small patient group; however, this study, together with other small mechanistic studies performed in patients with type 2 diabetes,[28,29] provides evidence of platelet and coagulation activation after a single hypoglycaemic event. Given the unpleasant side effects involved in participating in a hypoglycaemic clamp procedure, sample sizes need to be kept at a minimum for this type of mechanistic study. Furthermore, we used light aggregometry as the primary outcome measure, based on which we powered the analysis. However, we believe that flow cytometry-based platelet activation assays are more sensitive to moderate changes observed during hypoglycaemia.

In addition, given the delayed activation of platelet and coagulation markers in the present study, we still cannot determine at which glycaemic threshold this activation occurs. To build on the knowledge obtained from the present study and the recent publication by Chow et al,[28] further studies are needed with separate hypoglycaemic clamps using different hypoglycaemic targets to identify the threshold at which platelet and coagulation activation occurs.

Given that the activation in platelet and coagulation markers was delayed, we were not able to rule out activation occurring after the euglycaemic clamp as a result of the insulin infusion as well. Hence, after the data were available, we invited the participants again for another euglycaemic clamp, with subsequent follow-up 1 day and 1 week thereafter (Figure S5). Six participants were willing to participate. While we did not observe a statistically significant platelet or coagulation activation 1 or 7 days after the euglycaemic clamp procedure, a numerical increase in some of the markers from the end of the clamp to day 1 was evident (Figures S6 and S7). In contrast to the

hypoglycaemic clamp where platelet activation was sustained or even further increased over the following 6 days, all platelet activation markers returned even numerically to baseline levels 1 week after the euglycaemic clamp. Hence, given the sample size of six people in the additional euglycaemic clamp experiment we can not entirely rule out an insulin infusion-induced short-term activation of platelets 1 day after the clamp, as has been shown previously in other settings.[37] However, clearly the activation observed after the hypoglcaemia is more pronounced and sustainable for >1 week after the hypoglycaemic event. Based on the present study we cannot fully explain the mechanisms by which the platelet and coagulation activation after hypoglcaemia occurs, but considering the lifespan of platelets being ~7 to 10 days, the observed sustained activation even 1 week after hypoglcaemia seems to be triggered by a subsequent mechanism following the blood glucose drop (eg, stress hormone release), which lasts longer than the low glucose level itself.

The main strength of the present study lies in its design, which included the stepwise hypoglycaemic clamp, and its homogenous patient group who were all on metformin only, with no participant taking antiplatelet drugs or other agents known to affect platelet activity at any time during the trial.

This study provides evidence for the activation of platelet reactivity and coagulation after hypoglycaemia. The observed effect seems to be sustained for 1 week after the event. Our data support the hypothesis that hypoglycaemic events could be one reason for the detrimental effects observed with intensive glucose-lowering using drugs with high hypoglycaemic potency such as in the ACCORD trial.[7,8] Future trials investigating intensive glucose-lowering using novel drugs with no or very low risk of hypoglycaemia are needed to clarify the open question of the best glycaemic treatment target in people with type 2 diabetes.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

H.S. has received speaker's honoraria and is on the advisory board for Amgen, Astra Zeneca, Böhringer Ingelheim, Eli Lilly, MSD, NovoNordisk and Sanofi-aventis. No other potential conflicts of interest relevant to this article are reported.

AUTHOR CONTRIBUTIONS

H.S. and F.A. designed the study protocol. H.K. recruited the participants. P.N.P., N.J.T., A.M.O., B.P., S.K. and M.B. made substantial contributions to acquisition of data. H.S., F.A. and P.N.P. wrote the clinical protocol and obtained authorization from the Ethics Committee and wrote the manuscript. T.S., H. Scharnagl, B.P., S.K. and F.P. performed coagulation, inflammation and platelet function testing. C.S. and E.N. performed the patient visits. R.R., F. Aziz and A.O. were responsible for statistical analyses. H.S. was the principal investigator and the guarantor of the trial. All authors read and approved the final manuscript. H.S. and F.A. are responsible for the content of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.