Effects of acute insulin-induced hypoglycaemia on endothelial microparticles in adults with and without type 2 diabetes

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Aims: To assess whether endothelial microparticles (EMPs), novel surrogate markers of endothelial injury and dysfunction, are differentially produced in response to acute insulin-induced hypoglycaemia in adults with and without type 2 diabetes.

Materials and methods: A prospective, parallel study was conducted in individuals with type 2 diabetes (n = 23) and controls (n = 22). Hypoglycaemia (<2.2 mmol/L: <40 mg/dL) was achieved by intravenous infusion of soluble insulin. Blood samples were collected at baseline and at 0, 30, 60, 120, 240 minutes and 24 hours after hypoglycaemia and analysed for CD31+ (platelet endothelial cell adhesion molecule-1), CD54+ (intercellular adhesion molecule 1), CD62-E+ (E-selectin), CD105+ (endoglin), CD106+ (vascular cell adhesion molecule 1) and CD142+ (tissue factor) EMPs by flow cytometry. The peak elevations (% rise from 0 minutes following hypoglycaemia) seen in CD31+, CD54+, CD62-E+, CD105+ and CD142+ EMPs within 240 minutes were associated with diabetes status after adjustments for all relevant covariates. Individuals with type 2 diabetes showed increased CD31+ EMPs AUC0min–24h (P = 0.014) and CD105+ EMPs AUC0min–24h (P = 0.006) compared with controls, but there were no differences for CD54+ (P = 0.91), CD62-E+ (P = 0.14), CD106+ (P = 0.36) or CD142+ (P = 0.77) EMPs AUC0min–24h.

Conclusions: The associations between peak elevations within 240 minutes after insulin-induced hypoglycaemia for CD31+, CD54+, CD62-E+, CD105+ and CD142+ and diabetes status indicate that the assessment of a panel of EMPs within this timeframe would identify a hypoglycaemic event in this population. The greater overall responses over time (AUCs) for apoptosis-induced CD31+ and CD105+ EMPs suggest that hypoglycaemia exerts greater endothelial stress in type 2 diabetes.

KEYWORDS endothelial dysfunction, endothelial microparticles, hypoglycaemia, insulin, type 2 diabetes mellitus

1 INTRODUCTION

Hypoglycaemia (plasma glucose ≤3.9 mmol/L) has been associated with significant morbidity and mortality.1-3 Evidence from large-scale trials on intensive glycaemic control and complications in type 2 diabetes showed that hypoglycaemia was a severe and common side effect of therapeutic intensification that is associated with increased mortality.4-6 The risk of a severe hypoglycaemic event (requiring assistance for recovery) in insulin-treated type 2 diabetes has been reported to be ~7% within 2 years of insulin therapy initiation and to...
reach up to 31% following ≥10 years of insulin therapy initiation.7–9 The frequency of asymptomatic mild hypoglycaemia is also high; approximately one in two individuals with type 2 diabetes experience at least one event over a 3-day period.10

Hypoglycaemia induces stress responses, which include the sympatho-adrenal activation and the release of glucagon, epinephrine, cortisol and growth hormone.11 Haemodynamic alterations occur to maintain glucose supply to the brain and promote glucose generation from the liver; these alterations include increases in heart rate, systolic blood pressure, myocardial contractility and cardiac output.11 Blood viscosity increases, leading to an elevation in platelet count, aggregation and coagulation.11 At a molecular level, hypoglycaemia causes increased markers of inflammation, leukocytosis, lipid peroxidation, oxidative stress and platelet-monocyte aggregation.12–15 These hypoglycaemia-induced changes may result in the generation of biomarkers that are able to identify a hypoglycaemic event after blood glucose levels have reversed to normal.

Endothelial microparticles (EMPs) are surrogate markers of endothelial injury and dysfunction released by activated or apoptotic endothelial cells.16–17 Microparticles (MPs) are key regulators of cell to cell interactions and, by carrying specific membrane antigens from their source cells, they act as diffusible vectors in the transcellular exchange of biological information.17 EMPs play an important role in maintaining vascular homeostasis, and elevated EMP levels are implicated in the pathogenesis of vascular diseases, cancer, inflammatory, endocrine and metabolic disorders.17–19 Several studies have reported increased EMPs in individuals with diabetes mellitus, as compared to controls without diabetes,20–22 and have explored EMPs as biomarkers of vascular injury, and as potential predictors of cardiovascular outcomes in patients with or without diabetes mellitus.23–26 Given that EMPs are produced at the initial stages of cell injury or as part of membrane remodelling, these markers may be also useful in characterizing endothelial responses to hypoglycaemia; however, their potential as a biomarker in this condition has not been previously investigated in type 2 diabetes.

The aim of the present study was to explore the effects of acute insulin-induced hypoglycaemia on EMPs in adults with and without type 2 diabetes.

2 | MATERIALS AND METHODS

A prospective parallel study was performed in the Diabetes Research Centre at Hull Royal Infirmary in adults with type 2 diabetes (n = 25) and controls without diabetes (n = 25). All participants provided their written informed consent before taking part. The trial was approved by the North West - Greater Manchester East Research Ethics Committee (REC number: 16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki.

All participants were white and aged between 40 and 70 years. Participants in the type 2 diabetes group had been diagnosed with type 2 diabetes for <10 years and all were on a stable dose of medication (metformin, statin and/or angiotensin-converting enzyme inhibitor/angiotensin receptor blocker) over the preceding 3 months. Participants in the type 2 diabetes group were excluded if they were on any medications for glycaemic control except metformin, had poor glycaemic control (glycated haemoglobin [HbA1c] levels ≥86 mmol/mol [10%]) or if they had hypoglycaemic unawareness or history of severe hypoglycaemia over the previous 3 months. Participants in the control group were excluded if they had been diagnosed with type 1 or 2 diabetes or if they had HbA1c levels >42 mmol/mol (6%). The following exclusion criteria were applied for both groups: current smokers, body mass index (BMI) <18 or >50 kg/m², excessive alcohol consumption, heart failure, renal or liver disease, history or presence of malignant neoplasms within the last 5 years, diagnosis of psychiatric illness, history of acute or chronic pancreatitis or gastrointestinal tract surgery. Participants on any form of steroids or any medication that can mask hypoglycaemia or cause changes in glucose metabolism in the last 6 months were excluded. Women who were pregnant, breastfeeding or intending to conceive and individuals with contraindications to insulin infusion to achieve hypoglycaemia, including those with ischaemic heart disease, epilepsy or previous history of seizures, drop attacks, history of adrenal insufficiency and treated hypothyroidism, were excluded from participation.

Participants attended three visits. During Visit 1, participants were screened for inclusion and exclusion criteria by medical history, clinical examination, routine blood tests and an ECG. Visit 2 was the main experimental day, followed by Visit 3 the following morning. For Visit 2, participants avoided habitual exercise (defined as brisk walking >20 minutes) for at least 24 hours before their visit and individuals with type 2 diabetes on medication withheld their oral hypoglycemic agents on the morning of the visit. Participants were weighed (Marsden Weighing Machine Group Ltd, Rotherham, UK) and height was taken barefoot using a wall-mounted stadiometer. Blood pressure was measured using a sphygmomanometer (Datascop Duo Masimo Set; Mindray Ltd, Huntingdon, UK) and a blood sample was collected in the fasted state before insulin infusion and used as baseline. Continuous insulin infusion was performed to induce hypoglycaemia. Blood samples were taken at 0, 30, 60, 120 and 240 minutes after hypoglycaemia. After 240 minutes the participants were provided with lunch and were allowed their (morning) diabetes medications. The participants took their evening medication as prescribed that day. For Visit 3 (24 hours from the induction of hypoglycaemia), patients were also allowed to take their medication, once they completed the blood tests in the fasted state, after which breakfast was provided. Prior to discharge, blood glucose was checked using a glucose analyser (HemoCue glucose 201+) to ensure normal levels, together with other vital signs.

2.1 | Insulin infusion

After an overnight fast, bilateral ante-cubital fossa indwelling cannulae were inserted 30 to 60 minutes prior to the commencement of the test (8:30 AM). To induce hypoglycaemia, soluble intravenous insulin (Humulin S; Eli Lilly, Liverpool, UK) was given in a pump starting at a dose of 2.5 mU/kg body weight/min, with an increment of 2.5 mU/kg body weight/min every 15 minutes by a hypoglycaemic clamp,27 until two readings of venous blood glucose measured by a glucose analyser (HemoCue glucose 201+) ≤2.2 mmol/L (<40 mg/dL) or a reading of ≤2.0 mmol/L (36 mg/dL).27 The blood sample schedule was timed subsequently with respect to the time point when hypoglycaemia
occurred. Following the identification of hypoglycaemia, intravenous glucose was given in the form of 150 mL of 10% dextrose and a repeat blood glucose check was performed after 5 minutes if blood glucose was still <4.0 mmol/L. All patients achieved a blood glucose of ≤2.0 mmol/L (36 mg/dL), although the median duration to severe hypoglycaemia was significantly greater in participants with type 2 diabetes compared with controls (54 vs. 30 minutes; Supporting Information Table S1); however, the duration of hypoglycaemia was the same in both groups.

2.2 | Blood sample preparation and biochemical analyses

Venous blood samples collected during the screening visit were analysed for serum insulin, total cholesterol, triglycerides, HDL cholesterol, high-sensitivity C-reactive protein (hsCRP) and glycated haemoglobin (HbA1c). Samples were placed in sodium citrate anticoagulant at a concentration of 0.109 M (3.2%) (BD UK Ltd, Plymouth, UK) for EMP analysis.

Serum blood samples were centrifuged at 3500 g for 15 minutes at 5°C. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer’s DPC Immulite 2000 analyser (Euro/DPC, Llanberis, UK), with a coefficient of variation of 6%, and no stated cross-reactivity with proinsulin. Total cholesterol, triglycerides, HDL cholesterol, and hsCRP levels were measured enzymatically on a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, UK) with coefficients of variation of <4.9%, 0.9%, 1.6% and 8.4%, respectively. LDL cholesterol was calculated using the Friedewald equation. Plasma blood samples were analysed under the same conditions and for HbA1c on a Menarini Diagnostics HB9210 premier (A.Menarini Diagnostics Ltd, Winnersh-Wokingham, UK).

2.3 | EMP assessment and characterization

Platelet-free plasma was prepared within 2 hours of blood sample collection using an initial centrifugation at 1000 g for 10 minutes, followed by a second centrifugation of the supernatant at 12 000 g for 10 minutes. All assays were performed on a BD Accuri C6 Plus flow cytometer (BD Biosciences, Detroit, Michigan). The EMP gates were established using a blend of beads of four diameters (0.16, 0.2, 0.24 and 0.5 μm; Megamix-Plus SSC; BioCytex, Marseilles, France) and set between 0.3 and 0.8 μm. The platelet-free plasma samples (25 μL) were directly incubated for 30 minutes in the dark with 5 μL of fluorescent isothiocyanate-conjugated monoclonal antibodies against cell-type specific antigens. EMPs were identified using CD31 (platelet endothelial cell adhesion molecule-1; BD Biosciences), CD54 (intercellular adhesion molecule 1; Bio-Rad, Watford, UK), CD62-E (E-selectin; Bio-Rad), CD105 (endoglin; BD Biosciences), CD106 (vascular cell adhesion molecule 1; BD Biosciences) and CD142 (tissue factor; Bio-Rad). After incubation, the samples were diluted in 300 μL of phosphate-buffered saline that had been filtered through a sterile 0.1-μm syringe filter (Minisart, Scientific Laboratory Supplies, Nottingham, UK). A total of 25 μL of counting beads with an established concentration (AccuCheck Counting Beads; Life Technologies Corp., Paisley, UK) were added to each sample to calculate EMPs as absolute numbers per microlitre.

2.4 | Statistical analysis

All variables were checked for extreme outliers (>3 times interquartile range above the third quartile or <3 times interquartile range below the first quartile) graphically. Participants who were indicated as extreme outliers for >3 EMPs (out of six EMPs studied) at least at one time point for each EMP were excluded from analysis (type 2 diabetes, n = 2; control group, n = 3). Total analysis was performed using the data from individuals with type 2 diabetes (n = 23) and controls (n = 22). All data were checked for normality according to the Shapiro–Wilk test. A two-way analysis of variance with repeated measures was used to determine main and interaction effects for EMPs’ responses to hypoglycaemia. Non-normally distributed data were log-transformed prior to this analysis. Significant main or interaction effects were followed by Bonferroni’s post hoc analysis. By using the percentage data from 0 minutes following hypoglycaemia for each time point, total and partial areas under the curve (AUC0-min, AUC0–240min) were calculated. An independent t test or the Mann–Whitney test were used to detect differences in baseline characteristics and AUCs between groups. A step-wise multiple regression analysis was performed to explore whether significant overall responses (AUC) were predicted by age, sex, weight, height, duration of diabetes, BMI, systolic blood pressure, diastolic blood pressure, HbA1c, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, insulin levels and hsCRP. Statistical significance was set at P ≤ 0.05. We performed additional statistical analyses to examine the clinical utility of EMPs in predicting hypoglycaemia. Our data showed that, after hypoglycaemia, the levels of EMPs increased in both patients with diabetes and controls (Figure 1). We hypothesized that this increase reflects an endothelial injury and that those with diabetes are likely to have greater elevations after acute hypoglycaemia, hence, this should be useful in detecting hypoglycaemic episodes among these patients. We used the highest elevation in each EMP within 240 minutes after insulin-induced hypoglycaemia, and calculated the percentage rise from 0 minutes after hypoglycaemia in both cases and controls. This percentage change for each EMP was then modelled using a regression model with the following independent variables: diabetes status, age, sex, BMI, baseline HbA1c, insulin and total cholesterol levels. All statistical analyses were performed using IBM-SPSS version 24.0 (Chicago, Illinois) and R version 3.4.1.

3 | RESULTS

3.1 | Demographic and clinical characteristics

The main demographic and clinical characteristics of the individuals with and without type 2 diabetes are presented in Table 1.

3.2 | EMP responses to insulin-induced hypoglycaemia

There were no significant differences in the baseline concentrations of any EMPs between individuals with type 2 diabetes and controls (all P values from 0.11 to 0.93; Figure 1).
CD31+ EMPs increased between 120 minutes and baseline (P = 0.008), 0 minutes (P = 0.006), 30 minutes (P = 0.005) and 240 minutes (P = 0.001) after hypoglycaemia. CD31+ EMPs at 240 minutes were increased compared with all other time points (all P values <0.001). No differences were shown between groups at any time point (time × group interaction effect; P = 0.081 [Figure 1]). CD31+ EMPs AUC0–240min (P = 0.029) and AUC0min–24h (P = 0.014) were higher in the type 2 diabetes group compared with the control group (Figure 2). Stepwise regression analysis showed that only diabetes status and HbA1c significantly predicted CD31+ EMPs AUC0–240min (R² = 0.186, P = 0.013) and AUC0min–24h (R² = 0.295, P = 0.001). The percent rise in CD31+ EMPs (P = 0.03) was significantly higher in participants with diabetes compared with controls (Table 2).

There was an increase in CD54+ EMPs at 120 minutes compared with baseline (P = 0.009), 0 minutes (P = 0.002) and 240 minutes (P = 0.001) following hypoglycaemia. A higher number of CD54+ EMPs was seen at 240 minutes after hypoglycaemia compared with all other time points (all P values <0.0001). CD54+ EMP responses after hypoglycaemia did not differ between groups for any time point (time × group interaction effect; P = 0.75). CD54+ EMPs AUC0–240min (P = 0.62) or AUC0min–24h (P = 0.91) were not different between groups (Figure 2). The percent rise in CD54+ EMPs (P = 0.04) was significantly higher in those with diabetes compared with controls (Table 2).

Elevations were seen for CD62-E+ EMPs between 120 minutes and baseline (P = 0.005), 0 minutes (P < 0.001), 240 minutes (P = 0.019) and 24 hours (P < 0.001) after hypoglycaemia. CD62-E+ EMPs at 240 minutes were greater compared with all other time points (BASE, P < 0.0001; 0 minutes, P < 0.0001; 30 minutes, P < 0.0001; 60 minutes, P < 0.0001; 120 minutes, P = 0.002 and 24 hours, P < 0.0001). There were no differences between groups at any time point (time × group interaction effect P = 0.083; Figure 1). Overall responses in CD62-E+ EMPs did not differ between adults with and without type 2 diabetes (CD62-E+ EMPs AUC0–240min
P = 0.28 or AUC0min–24h P = 0.14; Figure 2). The percent rise in CD62+ EMPs (P = 0.03) was significantly higher in those with diabetes compared with controls (Table 2).

CD105+ EMPs at 240 minutes were higher compared with those at baseline, 0, 30, 60 minutes and 24 hours after hypoglycaemia (all P values <0.0001). There was a significant time × group interaction (P = 0.023), but post hoc analysis did not reveal any significant differences between groups at any time point (P values from 0.077 to 0.60;
Figure 1). CD105+ EMPs AUC0–240min (P = 0.051) and AUC0–24h (P = 0.006) were higher in the type 2 diabetes group compared with controls (Figure 2). Stepwise regression analyses did not reveal any variable that can significantly predict CD105+ EMPs AUC0–240min and AUC0min–24h. The percent rise in CD105+ EMPs (P = 0.006) was significantly higher in those with diabetes compared with controls (Table 2).

CD106+ EMPs increased at 120 minutes from 0 minutes (P = 0.011) after hypoglycaemia. CD106+ EMPs were also higher at 240 minutes compared with all time points (baseline, P < 0.0001; 0 minutes, P < 0.0001; 30 minutes, P < 0.0001; 60 minutes, P = 0.002; 120 minutes, P = 1.0 and 24 hours, P = 0.001). No significant differences were shown between groups at any time point for CD106 EMPs (time × group interaction effect, P = 0.32; Figure 1). CD106+ EMPs AUC0–240min (P = 0.79) or AUC0min–24h (P = 0.36) were not different between groups (Figure 2).

CD142+ EMPs were higher at 120 minutes compared with baseline (P = 0.004), 0 minutes (P = 0.006) and 240 minutes (P = 0.035) following hypoglycaemia. CD142+ EMPs at 240 minutes appeared to be increased compared to all other time points (baseline, P < 0.0001; 0 minutes, P < 0.0001; 30 minutes, P < 0.0001; 60 minutes, P < 0.0001; 120 minutes, P = 0.035 and 24 hours, P < 0.0001). No differences were shown between groups at any time point (time × group interaction effect, P = 0.40; Figure 1). CD142+ EMPs AUC0–240min (P = 0.77) or AUC0min–24h (P = 0.77) did not differ between individuals with type 2 diabetes and controls (Figure 2). The percent rise in CD142+ EMPs (P = 0.001) was significantly higher in those with diabetes compared with controls (Table 2).

### TABLE 1  Demographic and clinical characteristics of the study participants

| Baseline                  | Type 2 diabetes (n = 23) | Controls (n = 22) | P     |
|---------------------------|--------------------------|-------------------|-------|
| Age (y)                   | 62 ± 7                   | 55 ± 10           | <0.0001|
| Men/Women                 | 12/11                    | 10/12             | 0.77  |
| Weight (kg)               | 90.9 ± 11.4              | 79.0 ± 8.5        | <0.0001|
| Height (cm)               | 167 ± 14                 | 169 ± 5           | 0.64  |
| BMI (kg/m²)               | 32 ± 4                   | 28 ± 3            | <0.0001|
| Systolic blood pressure (mm Hg) | 131 ± 8               | 122 ± 8           | 0.001 |
| Diastolic blood pressure (mm Hg) | 81 ± 7              | 75 ± 6            | 0.003 |
| Duration of diabetes (y) | 4.5 ± 2.9                | N/A               |       |
| Insulin (μU/mL)           | 47.5 ± 86                | 9.8 ± 8.1         | 0.001 |
| HbA1c (mmol/mol)          | 52.6 ± 10.9              | 37.4 ± 2.2        | <0.0001|
| HbA1c (%)                 | 6.8 ± 1.0                | 5.6 ± 0.2         | <0.0001|
| Total cholesterol (mmol/L)| 4.2 ± 1.0                | 4.8 ± 0.7         | 0.014 |
| Triglycerides (mmol/L)    | 1.7 ± 0.7                | 1.3 ± 0.6         | 0.055 |
| HDL cholesterol (mmol/L)  | 1.1 ± 0.3                | 1.5 ± 0.4         | 0.001 |
| LDL cholesterol (mmol/L)  | 2.2 ± 0.8                | 2.7 ± 0.8         | 0.051 |
| CRP (mg/L)                | 3.1 ± 2.8                | 5.3 ± 11.0        | 0.66  |

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HbA1c, glycated haemoglobin.

Data are presented as mean ± 1 SD.

### 4 | DISCUSSION

In the present study we characterized and compared the effects of acute insulin-induced hypoglycaemia on EMPs in individuals with and without type 2 diabetes. A similar pattern of changes was reported in both groups; EMP levels were increased at 240 minutes following hypoglycaemia and returned to their baseline values within 24 hours. The elevations (% rise from 0 minutes hypoglycaemia) seen in CD31+.

### TABLE 2  Associations of diabetes status with peak elevations (% rise from 0 min following hypoglycaemia) in endothelial microparticles

| Peak elevations within 240 min (% rise from 0 min after hypoglycaemia) | β    | SE   | P    |
|-----------------------------------------------------------------------|------|------|------|
| CD31+ EMPs                                                             | −0.101 | 0.046 | 0.033 |
| CD54+ EMPs                                                             | −0.084 | 0.040 | 0.042 |
| CD62+ EMPs                                                             | −0.099 | 0.046 | 0.038 |
| CD105+ EMPs                                                             | −0.141 | 0.049 | 0.007 |
| CD106+ EMPs                                                             | −0.017 | 0.046 | 0.72  |
| CD142+ EMPs                                                             | −0.133 | 0.0373| 0.001 |

Abbreviations: BMI, body mass index; EMP, endothelial microparticle; HbA1c, glycated haemoglobin.

Regression models accounted for age, diabetes status, sex, BMI, baseline HbA1c, insulin and total cholesterol levels as covariates.
and CD105+ EMPs to hypoglycaemia were more marked in participants with type 2 diabetes compared with controls. Taken together, our findings indicate that hypoglycaemia exerts endothelial stress in individuals with and without diabetes, but this stress may be more pronounced in type 2 diabetes.

Significant increases in EMPs did not occur until 120 minutes after the hypoglycaemic event. Given that the process of EMPs shedding is active in nature, this time delay in the release of EMPs is unsurprising and consistent with previous research exploring conditions that impose physiological stress on the endothelial cells (i.e., hypoglycaemia, hyperglycaemia or hypoxia). The greatest elevations in all EMPs occurred at 240 minutes after hypoglycaemia. As a result of the blood sampling schedule of the present study, we were unable to provide further insight into the time course of these changes, which should be the focus of future studies. Nevertheless, the increased levels of all EMPs determined indicate activation and apoptosis of endothelial cells. Indeed, apoptosis-induced EMPs are likely to express CD31 and CD105, whilst activation-induced EMPs appear to be positive for CD54, CD62-E and CD106. Data from 24 hours after insulin-induced hypoglycaemia indicated a reduction of EMPs to baseline values, suggesting the recovery of the endothelium.

Individuals with type 2 diabetes and controls both reached a peak of endothelial stress (240 minutes) and subsequent recovery within a similar timeframe (24 hours). With the goal of assessing the clinical usefulness of EMPs and their potential for detecting hypoglycaemic episodes among these patients, we expressed the peak elevation for each EMP within 240 minutes as percentage rises from 0 minutes after hypoglycaemia and modelled this using a regression model with a number of covariates, namely, diabetes status, age, sex, BMI, baseline HbA1c, insulin and total cholesterol levels. We showed that the peak percentage rises from 0 minutes after hypoglycaemia for CD31, CD54, CD62, CD105 and CD142 EMPs were associated with diabetes status after adjustments for these covariates. These results have important clinical implications and suggest that EMPs have the potential to be used as diagnostic biomarkers in clinical practice in the future. This is important given that, for many patients, the most feared complication of intensified diabetes therapy and the main barrier to achieving optimal glycaemic control to prevent complications is the increased risk of hypoglycaemia. As such, the identification and standardization of novel, minimally invasive biomarkers with the ability to determine whether hypoglycaemia has occurred several hours after the event has taken place could help in confirming the clinical suspicion of healthcare staff and allow more objective optimization of glycaemic control such as in patients with impaired hypoglycaemic awareness.

When data were expressed as AUCs, overall responses for CD31 and CD105 EMPs to hypoglycaemia were more marked in participants with type 2 diabetes compared with healthy controls, perhaps a sign of increased apoptosis of endothelial cells and atherosclerosis in this group. These results suggest that the endothelium in type 2 diabetes may be more susceptible to injury and dysfunction, and it is speculated that increased EMPs may provide a mechanistic link between hypoglycaemia and increased risk of vascular complications. Indeed, both CD31 and CD105 have been suggested to play a role in atherogenesis; CD105 expression has been demonstrated in atherosclerotic vessels predominantly in endothelial cells in both preclinical and clinical studies and CD31 EMPs have been demonstrated to contribute to atherosclerotic lesion formation in regions of disturbed blood flow.

Few experimental studies have explored the effects of acute hypoglycaemia on MPs expressed by endothelial or other cells (i.e., platelets, mononuclear cells) in humans. In individuals with and without type 1 diabetes, Joy et al demonstrated an increase in VCAM (CD106), ICAM (CD54), E-selectin (CD62-E), P-selectin (CD62-P) and vascular endothelial growth factor, in response to hypoglycaemia relative to euglycaemia. In another study by Wright et al, hypoglycaemia induced an increase in CD40 expression on mononuclear cells and plasma concentration of CD40L and P-selectin (CD62-P), with a trend towards an increase in von Willebrand factor concentrations. In these studies, glucose clamps were used to equate glucose at hypoglycaemic levels of 2.5 mmol/L for 60 minutes and at 2.9 mmol/L for 120 minutes, respectively. Notably, greater increases in proinflammatory factors were reported by Joy et al compared with those by Wright et al, confirming that the duration of hypoglycaemia is an important characteristic of a hypoglycaemic stimulus. The effects of hypoglycaemia in the present study were even more pronounced; this may be explained by the way hypoglycaemia was achieved (insulin infusion), which caused a rapid decrease in blood glucose, which, as evident in previous research, results in a rapid release of catecholamines and initiation of inflammation.

Although the mechanisms that underlie the rise in EMPs in response to hypoglycaemia remain unclear, these may involve the release of pro-inflammatory factors, oxidative stress and shear stress. Indeed, insulin and counter-regulatory hormones trigger increases in pro-inflammatory mediators including tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8, and plasminogen activator inhibitor type 1 (PAL-1), which have been shown to provoke the release of MPs in vitro. Further actions of these hormones involve enhanced lipolysis and elevated levels of triglycerides and non-esterified fatty acids, which may also explain a rise in EMP release. Other mechanisms which have been implicated in the regulation of EMPs include the activated sympathetic nervous system, which through haemodynamic alterations exerts shear stress on blood vessels, disruptions in the redox balance of cells, and oxidative stress.

Upon their expression on endothelial cells, MPs have direct effects on intracellular signalling to trigger cellular responses. For instance, CD31 is expressed by endothelial cells, but also platelets and leukocytes, and plays important roles in angiogenesis, platelet function, thrombosis, mechanosensation of shear stress and leukocyte migration. CD54, an adhesion molecule, enables leukocytes rolling within vasculature and leukocyte-endothelial cells interactions for the regulation of vascular permeability. CD62-E originates exclusively by endothelial cells and allows the binding of neutrophils, monocytes, and T-cell subpopulations at sites of inflammation. CD105 regulates...
TGF-β signalling in endothelial cells and is involved in haematopoiesis, angiogenesis and nitric oxide-dependent vasodilatation. It has a key role in cellular transmigration, this notion supported by studies showing that CD105 also regulates the expression of extracellular matrix molecules such as fibronectin, collagen, PAI-1 and lumican. CD106 is a major regulator of leukocyte transmigration and a modulator of endothelial signalling through NADPH oxidase-generated reactive oxygen species. Finally, CD142, expressed by endothelial cells and leukocytes, initiates the extrinsic pathway of blood coagulation, and increased CD142 levels have been associated with thrombotic events. Taken together, the EMPs that were elevated in response to hypoglycaemia in the present study play a critical role in vascular inflammation and affect the coagulation pathway. Available literature suggests the roles of EMPs are more complex than initially thought and it remains uncertain whether these EMP-mediated alterations aim to maintain vascular homeostasis in response to stimuli such as hypoglycaemia or if they contribute to endothelial dysfunction and the development of both macro- and microvascular complications in individuals with diabetes.

In conclusion, acute hypoglycaemia increased EMP levels, indicating the induction of endothelial stress, and their appearance was maximal at 240 minutes, suggesting that these EMPs, alone or in combination, may have utility as biomarkers for post hypoglycaemia, especially in patients with impaired hypoglycaemic awareness. The greater overall responses of CD31+ and CD105+ EMPs (AUCs) to hypoglycaemia in adults with type 2 diabetes suggest that the endothelium in diabetes may be sensitive to hypoglycaemia-induced injury and dysfunction and could provide a mechanistic link between hypoglycaemia and increased risk of vascular complications; however, clarity is needed on the mechanisms mediating EMP expression and the associated EMP effects related to hypoglycaemia duration and severity.

ACKNOWLEDGMENT
We thank all study participants for their commitment to this study.

CONFLICT OF INTEREST
None declared.

Author contributions
A.A., L.A.M., E.S.K., S.L.A. and T.S. participated in study conception and design. A.A. performed the acquisition of data. A.A., M.P., H.D., L.A.M., E.S.K., S.L.A. and T.S. participated in analysis and/or interpretation of data. M.P. drafted the paper; all authors reviewed and approved the final manuscript. T.S. is the guarantor of the study.

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

How to cite this article: Al-Qaissi A, Papageorgiou M, Deshmukh H, et al. Effects of acute insulin-induced hypoglycaemia on endothelial microparticles in adults with and without type 2 diabetes. Diabetes Obes Metab. 2019;21:533-540. https://doi.org/10.1111/dom.13548