Title: The effects of acute insulin-induced hypoglycaemia on endothelial microparticles in adults with and without type 2 diabetes

Short running title: Endothelial microparticles responses to hypoglycaemia

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Abstract

Aims: Endothelial microparticles (EMPs) are novel surrogate markers of endothelial injury and dysfunction that may be 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 (<2.2mmol/l: <40mg/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 following hypoglycaemia and analysed for $CD31^+$ (Platelet Endothelial Cell Adhesion Molecule-1 or PECAM-1), $CD54^+$ (Intercellular Adhesion Molecule 1 or ICAM-1), $CD62\text{-}E^+$ (E-selectin), $CD105^+$ (Endoglin), $CD106^+$ (Vascular Cell Adhesion Molecule 1 or VCAM-1) and $CD142^+$ (Tissue Factor) EMPs by flow cytometry. The peak elevations (% rise from baseline) in EMP within 240 minutes following induced hypoglycaemia were modelled using a regression model with adjustment for relevant covariates. All EMPs were expressed as percentage from baseline for each time point and total areas under the curve ($AUC_{BASE\text{-}24h}$) were calculated.

Results: Following insulin-induced hypoglycaemia, levels of circulating EMPs were maximal at 240 minutes ($p<0.001$) and returned to baseline values within 24 hours for both groups. The peak elevations (% rise from baseline) seen in $CD31^+$, $CD54^+$, $CD62\text{-}E^+$, $CD105^+$ and $CD142^+$ EMPs within 240min were associated with diabetes status after adjustments for all relevant covariates. Individuals with type 2 diabetes showed increased $CD31^+$ EMPs $AUC_{BASE\text{-}24h}$ ($p=0.015$) and $CD105^+$ EMPs $AUC_{BASE\text{-}24h}$ ($p=0.006$) compared to controls, but there were no differences for $CD54^+$ ($p=0.89$), $CD62\text{-}E^+$ ($p=0.13$), $CD106^+$ ($p=0.36$) or $CD142^+$ ($p=0.79$) EMPs $AUC_{BASE\text{-}24h}$.

Conclusions: The associations between peak elevations within 240min following insulin-induced hypoglycaemia for $CD31^+$, $CD54^+$, $CD62\text{-}E^+$, $CD105^+$ 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 microparticles, endothelial dysfunction, hypoglycaemia, insulin, type 2 diabetes mellitus

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 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 approximately 7% within 2 years of insulin therapy initiation and reach up to 31% following ≥10 years of insulin therapy initiation (7-9). The frequency of asymptomatic mild hypoglycaemia is also high; approximately 1 in 2 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 (SBP), 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, leucocytosis, 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 EMPs 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 characterising 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 this study was to explore the effects of acute insulin-induced hypoglycaemia on EMPs in adults with and without type 2 diabetes.

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 partaking. 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 Caucasian, aged between 40-70 years. Participants in the type 2 diabetes group were diagnosed with type 2 diabetes <10 years and all were on stable dose of medication (metformin, statin and/or angiotensin converting enzyme inhibitor/angiotensin receptor blocker) over the last 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 [HbA1c levels ≥10% (86 mmol/mol)] 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 >6% (42 mmol/mol). The following exclusion criteria were applied for both groups; current smokers, body mass index (BMI) <18 or >50 kg/m^2, excessive alcohol consumption, 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 six months were excluded. Women who were pregnant, breastfeeding or intended 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 (Visits 1-3). During Visit 1, participants were screened against inclusion and exclusion criteria by medical history, clinical examination, routine blood tests and an electrocardiogram. 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 >20min) >24h and individuals with type 2 diabetes on medication withheld their oral hypoglycemic agents in the morning of the visit. Participants were weighed (Marsden Weighing Machine Group Ltd, UK) and height was taken barefoot using a wall-mounted stadiometer. Blood pressure was measured using a sphygmomanometer (Datascope Duo Masimo Set, Mindray Ltd, UK) and a blood sample was collected in the fasted state before insulin infusion and used as baseline. A continuous insulin infusion was performed to induce hypoglycaemia. Blood samples were taken at 0, 30, 60, 120 and 240min after hypoglycaemia. After 240min participants were provided lunch and were allowed their (morning) diabetes medications. Patients 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.

Insulin Infusion

Following an overnight fast, bilateral ante-cubital fossa indwelling cannulas were inserted 30-60min prior to the commencement of the test (0830h). To induce hypoglycaemia, soluble intravenous insulin (Humulin S, Lilly, UK) was given in a pump starting at a dose of 2.5mU/kg body weight/min with an increment of 2.5mU/kg body weight/min every 15min by hypoglycaemic clamp (27), until two readings of venous blood glucose measured by a glucose analyser (HemoCue® glucose 201+) ≤2.2 mmol/L (<40mg/dl) or a reading of ≤2.0 mmol/L (36mg/dl) (27). The blood sample schedule was timed subsequently in respect to the time point that hypoglycaemia occurred. Following the identification of hypoglycaemia, intravenous glucose was given in form of 150 ml of 10% dextrose and a repeat blood glucose check was performed after 5min if blood glucose was still <4.0 mmol/L. All patient achieved a blood glucose of ≤2.0 mmol/L (36mg/dl), though the median duration to severe hypoglycaemia was significantly greater in patients with type 2 diabetes compared to controls (54 *vs.* 30 min; supplementary Table 1); however, the duration of hypoglycemia was the same in both groups.

Blood samples preparation and biochemical analyses

Venous blood samples were analysed for serum insulin, total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), high sensitivity C-reactive protein (hs-CRP) and HbA1c. Samples were placed in sodium citrate anticoagulant at a 3.2% (0.109 M) (BD, UK) for EMPs analysis.

Serum blood samples were centrifuged at 3,500×G for 15min at 5°C. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK), with a coefficient of variation (CV) 6%, and no stated cross-reactivity with proinsulin. Total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and high sensitivity C-reactive protein (hs-CRP) levels were measured enzymatically on a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, UK) with CVs of <4.9, 0.9, 1.6 and 8.4%. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation. Plasma blood samples were analysed under the same conditions and analysed for HbA1c on a Menarini Diagnostics HB9210 premier (A.Menarini Diagnostics Ltd., Winnersh-Wokingham, UK).

EMP assessment and characterisation

Platelet-free plasma was prepared within 2 hours of blood sample collection using an initial centrifugation at 1,000xG for 10min followed by a second centrifugation of the supernatant at 12,000xG for 10min. All assays were performed on a BD Accuri™ C6 Plus flow cytometer (BD Biosciences). The EMPs 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, Diagnostica Stago, France) and set between 0.3 and 0.8 μm. The platelet-free plasma samples (25 μl) were directly incubated for 30min in the dark with 5 μl of fluorescin isothyocynate conjugated monoclonal antibodies against cell-type specific antigens; EMPs were identified using CD31 (Platelet Endothelial Cell Adhesion Molecule-1; PECAM-1) (BD Biosciences, UK); CD54 (Intercellular Adhesion Molecule 1; ICAM-1) (Bio-Rad, UK), CD62-E (E-selectin) (Bio-Rad, UK); CD105 (Endoglin) (BD Biosciences, UK), CD106 (Vascular cell adhesion molecule 1; VCAM-1) (BD Biosciences, UK) and CD142 (Tissue Factor) (Bio-Rad, UK). Following 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™, Nottingham, UK). A total of 25 μl of counting beads with an established concentration (AccuCheck

Counting Beads, Life Technologies Corporation, USA) were added to each sample to calculate EMPs as absolute numbers per microliter.

Statistical analysis

All variables were checked for extreme outliers [> 3 times interquartile range (IQR) above the third quartile or < 3 times IQR below the first quartile] graphically. Participants indicated as extreme outliers for >3 EMPs (out of 6 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 ANOVA 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 baseline for each time point, total and partial areas under the curve ($AUC_{BASE\text{-}24h}$ and $AUC_{BASE\text{-}240min}$) 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, SBP, diastolic blood pressure (DBP), HbA1c, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, insulin levels, *hs*-CRP. Statistical significance was set at $p \leq 0.05$. We performed additional statistical analyses to examine the clinical utility of EMPs in predicting hypoglycaemia. Our data showed that following 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 following acute hypoglycaemia, hence, this should be useful in detecting hypoglycaemic episodes among these patients. We used the highest elevation in each EMPs within 240 min following insulin-induced hypoglycaemia and calculated the percentage rise from baseline 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, IL) and R version 3.4.1.

Results

Demographic and clinical characteristics

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

EMP responses to 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 120min and baseline (p=0.008), 0min (p=0.006), 30min (p=0.005) and 240min (p=0.001) following hypoglycaemia. $CD31^+$ EMPs at 240min were increased compared to all other time points (all p-values <0.001). No differences were shown between groups at any time point (time x group interaction effect, p=0.081) (Figure 1). $CD31^+$ EMPs $AUC_{BASE\text{-}240min}$ (p=0.028) and $AUC_{BASE\text{-}24h}$ (p=0.015) were higher in the type 2 diabetes group compared to the control group (Figure 2). Stepwise regression analysis showed that only diabetes status and HbA1c significantly predicted $CD31^+$ EMPs $AUC_{BASE\text{-}240min}$ (R^2=0.192, p=0011) and $AUC_{BASE\text{-}24h}$ (R^2=0.296, p=0.001). The percent rise in $CD31^+$ EMPs (p=0.03) was significantly higher in those with diabetes compared to controls (Table 2).

There was an increase in $CD54^+$ EMPs at 120min compared to baseline (p=0.009), 0 min (p=0.002) and 240 min (p=0.001) following hypoglycaemia. A higher number of $CD54^+$ EMPs was shown at 240min after hypoglycaemia compared to all other time points (all p-values <0.0001). $CD54^+$ MPs responses following hypoglycaemia were indifferent between groups for all time points (time x group interaction effect, p=0.75). $CD54^+$ EMPs $AUC_{BASE\text{-}240min}$ (p=0.57) or $AUC_{BASE\text{-}24h}$ (p=0.89) were not different between groups (Figure 2). The percent rise in $CD54^+$ EMPs (p=0.04) was significantly higher in those with diabetes compared to controls (Table 2).

Elevations were seen for $CD62\text{-}E^+$ EMPs between 120min and baseline (p=0.005), 0min (p <0.001), 240min (p=0.019) and 24h (p<0.001) following hypoglycaemia. $CD62\text{-}E^+$ EMPs at 240min were greater compared to all other time points (BASE, p<0.0001; 0min, p<0.0001; 30min, p<0.0001; 60min, p<0.0001; 120min, p=0.002 and 24h, p<0.0001). There were no differences between groups at any time point (time x 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 $AUC_{BASE\text{-}240min}$, p=0.25 or $AUC_{BASE\text{-}24h}$, p=0.13) (Figure 2). The percent rise in CD62$^+$ EMPs (p=0.03) was significantly higher in those with diabetes compared to controls (Table 2).

CD105$^+$ EMPs at 240min were higher compared to those at baseline, 0min, 30min, 60min and 24h after hypoglycaemia (all p-values <0.0001). There was a significant time x 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 $AUC_{BASE\text{-}240min}$ (p=0.046) and $AUC_{BASE\text{-}24h}$ (p=0.006) were higher in the type 2 diabetes group compared to controls (Figure 2). Stepwise regression analyses did not reveal any variable that can significantly predict CD105$^+$ EMPs $AUC_{BASE\text{-}240min}$ and $AUC_{BASE\text{-}24h.}$ The percent rise in CD105$^+$ EMPs (p=0.006) was significantly higher in those with diabetes compared to controls (Table 2).

CD106$^+$ EMPs increased at 120 min from 0min (p=0.011) after hypoglycaemia. CD106$^+$ EMPs were also higher at 240min compared to all time points (baseline, p<0.0001; 0min, p<0.0001; 30min, p<0.0001; 60min, p=0.002; 120min, p=1.0 and 24h, p=0.001). No significant differences were shown between groups at any time point for CD106 EMPs (time x group interaction effect p=0.32) (Figure 1). CD106$^+$ EMPs $AUC_{BASE\text{-}240min}$ (p=0.79) or $AUC_{BASE\text{-}24h}$ (p=0.36) were not different between groups (Figure 2).

CD142$^+$ EMPs were higher at 120min compared to baseline (p=0.004), 0min (p=0.006) and 240min (p=0.035) following hypoglycaemia. CD142$^+$ EMPs at 240min appeared to be increased compared to all other time points (BASE, p<0.0001; 0min, p<0.0001; 30min, p<0.0001; 60min, p<0.0001; 120min, p=0.035 and 24h, p<0.0001). No differences were shown between groups at any time point (time x group interaction effect, p=0.40) (Figure 1). CD142$^+$ EMPs $AUC_{BASE\text{-}240min}$ (p=0.68) or $AUC_{BASE\text{-}24h}$ (p=0.79) 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 to controls (Table 2).

Discussion

This study characterised 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; EMPs levels were increased at 240min following hypoglycaemia and returned to their baseline values within 24h. The elevations (% rise from baseline) seen in CD31$^+$, CD54$^+$, CD62$^+$, CD105$^+$ and CD142$^+$ EMPs within 240min

were associated with diabetes status after adjustments for covariables, indicating that their assessment within this timeframe would identify a hypoglycaemic event in this clinically relevant population. Furthermore, overall responses to hypoglycaemia over time (AUCs) were greater for $CD31^+$ and $CD105^+$ EMPs in individuals with type 2 diabetes compared to 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 120min following 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 to the endothelial cells (i.e., hypoglycaemia, hyperglycaemia or hypoxia) (28, 29). The greatest elevations in all EMPs occurred at 240min following hypoglycaemia. Due to blood sampling schedule of this study, we are 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 (16). Data from 24 hours following 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 (240min) and subsequent recovery within a similar timeframe (24h). With the goal to assess the clinical usefulness of EMPs and their potential for detecting hypoglycaemic episodes among these patients, we expressed the peak elevation for each EMP within 240min as percentage rises from baseline and modelled 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 baseline for $CD31^+$, $CD54^+$, $CD62^+$, $CD105^+$ and $CD142^+$ EMPs were associated with diabetes status after adjustments for these co-variables. These results have important clinical implications and suggest EMPs have the potential to be utilised 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 their increased risk for hypoglycaemia (30). As such, the identification and standardisation 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 to allow more objective optimisation of glycaemic control such as in patients with impaired hypoglycaemic awareness.

When data were expressed as AUC, overall responses for $CD31^+$ and $CD105^+$ EMPs to hypoglycaemia were more marked in patients with type 2 diabetes compared to healthy controls, perhaps a sign of increased apoptosis of endothelial cells and atherosclerosis in this group (16). 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 (1-6). 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 (31) and $CD31^+$ EMPs have been demonstrated to contribute to atherosclerotic lesion formation in regions of disturbed blood flow (32).

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 (12, 14). 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), vascular endothelial growth factor, in response to hypoglycaemia relative to euglycaemia (14). In another study by Wright et al (12), 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 (12, 14), glucose clamps were used to equate glucose at hypoglycemic levels of 2.5 mmol/l for 60min and at 2.9 mmol/l for 120min, respectively. Notably, greater increases in proinflammatory factors were reported by Joy et al (14) compared to those by (12), confirming that the duration of hypoglycaemia is an important characteristic of a hypoglycaemic stimulus. The effects of hypoglycaemia in our 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 (13).

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 (16, 33-35). Indeed, insulin and counter-regulatory hormones trigger increases in pro-inflammatory mediators including tumour necrosis factor

(TNF-a), interleukins (IL-6, IL-8) (13, 14), plasminogen activator inhibitor type 1 (PAL-1) (14), which have been shown to provoke the release of MPs *in vitro* (34). Further actions of these hormones involve enhanced lipolysis and elevated levels of triglycerides and non-esterified fatty acids (NEFA) (15); which may also explain a rise in EMP release (14). Other mechanisms which have been implicated in the regulation of EMPs include the activated sympathetic nervous system, which though haemodynamic alterations, exerts shear stress on blood vessels (11, 35), disruptions in the redox balance of cells and oxidative stress (36).

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 (37). CD54, an adhesion molecule, enables leukocytes rolling within vasculature and leukocyte-endothelial cells interactions for the regulation of vascular permeability (38). CD62-E originates exclusively by endothelial cells and allows the binding of neutrophils, monocytes, and T cell subpopulations at sites of inflammation (39). CD105 regulates TGF-β signalling in endothelial cells and it is involved in haematopoiesis, angiogenesis and nitric oxide-dependent vasodilatation (40). 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 (40). CD106 is a major regulator of leukocytes transmigration and a modulator of endothelial signalling through NADPH oxidase-generated reactive oxygen species (41). 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 (42). Taken together, the EMPs which were elevated in response to hypoglycaemia in our 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 EMPs-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 (16, 22, 23, 25).

Conclusions

Acute hypoglycaemia increased EMPs indicating the induction of endothelial stress and their appearance was maximal at 240min 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.

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Authors' contributions

AA, LAM, ESK, SLA and TS participated in study conception and design. AA performed the acquisition of data. AA, MP, HD, LAM, ESK, SLA and TS participated in analysis and/or interpretation of data. MP drafted the paper; all authors reviewed and approved the final manuscript. TS is the guarantor of the study.

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Figure Legends

Figure 1. Effect of hypoglycaemia on $CD31^+$ (A), $CD54^+$ (B), $CD62\text{-}E^+$ (C), $CD105^+$ (D), $CD106^+$ (E) and $CD142^+$ (F) EMP count in the type 2 diabetes (solid line, black circle) and control (dashed line, white circle) groups at baseline and at 0, 30, 60, 120, 240 min and 24h following induced hypoglycaemia. The grey frame indicates the short-term EMP response to hypoglycaemia (up to 240 min). The double line in the x axis indicate that the time points between 240 min and 24h are not presented. Values are expressed as mean±1SD. Two-way repeated-measures ANOVA revealed the following: a significant main effect of time for all EMPs (*p*-values≤0.001). There was a time x group interaction effect for CD105 EMPs (*p*=0.023) only, but post-hoc analysis revealed no further statistical differences between groups.

CD31: Platelet Endothelial Cell Adhesion Molecule-1 or PECAM-1, CD54: Intercellular Adhesion Molecule 1 or ICAM-1, CD62-E: E-selectin, CD105: Endoglin, CD106: Vascular cell adhesion molecule 1 or VCAM-1, CD142: Tissue Factor.

Figure 2. $AUC_{BASE–240min}$ (A) and $AUC_{BASE\text{-}24h}$ (B) analyses of $CD31^+$, $CD54^+$, $CD62\text{-}E^+$, $CD105^+$, $CD106^+$ and $CD142^+$ EMPs in the type 2 diabetes group (white bars) and control group (black bars). Data were expressed as %BASE and used to calculate AUCs. Values are expressed as mean±1 SD. *, significantly different from the control group ($p<0.05$).

AUC: Area under the curve, CD31: Platelet Endothelial Cell Adhesion Molecule-1 or PECAM-1, CD54: Intercellular Adhesion Molecule 1 or ICAM-1, CD62E: E-selectin, CD105: Endoglin, CD106: Vascular cell adhesion molecule 1 or VCAM-1, CD142: Tissue factor.

Table 1. Demographic and clinical characteristics of the study participants.

Baseline	**Type 2 Diabetes (n=23)**	**Controls (n=22)**	**p-value**
Age (years)	62±7	55±10	<0.0001
Sex (M/F)	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/m^2)	32±4	28±3	<0.0001
Systolic BP (mmHg)	131±8	122±8	0.001
Diastolic BP (mmHg)	81±7	75±6	0.003
Duration of diabetes (years)	4.5±2.9	N/A	
Insulin (uIU/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
Triglyceride (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

Data are presented as mean±1SD.

BMI: Body mass index, BP: Blood pressure, HDL-cholesterol: High density lipoprotein cholesterol, LDL-cholesterol: Low density lipoprotein cholesterol, CRP: C-reactive protein. HbA1c: Haemoglobin A1c

Table 2. Associations of diabetes status with peak elevations (% rise from baseline) in EMPs within 240 min following insulin-induced hypoglycaemia.

Peak elevations within 240 min (% rise from baseline)	Beta	Standard Error	p-value
$CD31^+$ 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

The regression models accounted for age, diabetes status, age, sex, BMI, baseline HbA1c, insulin and total cholesterol levels as covariates.

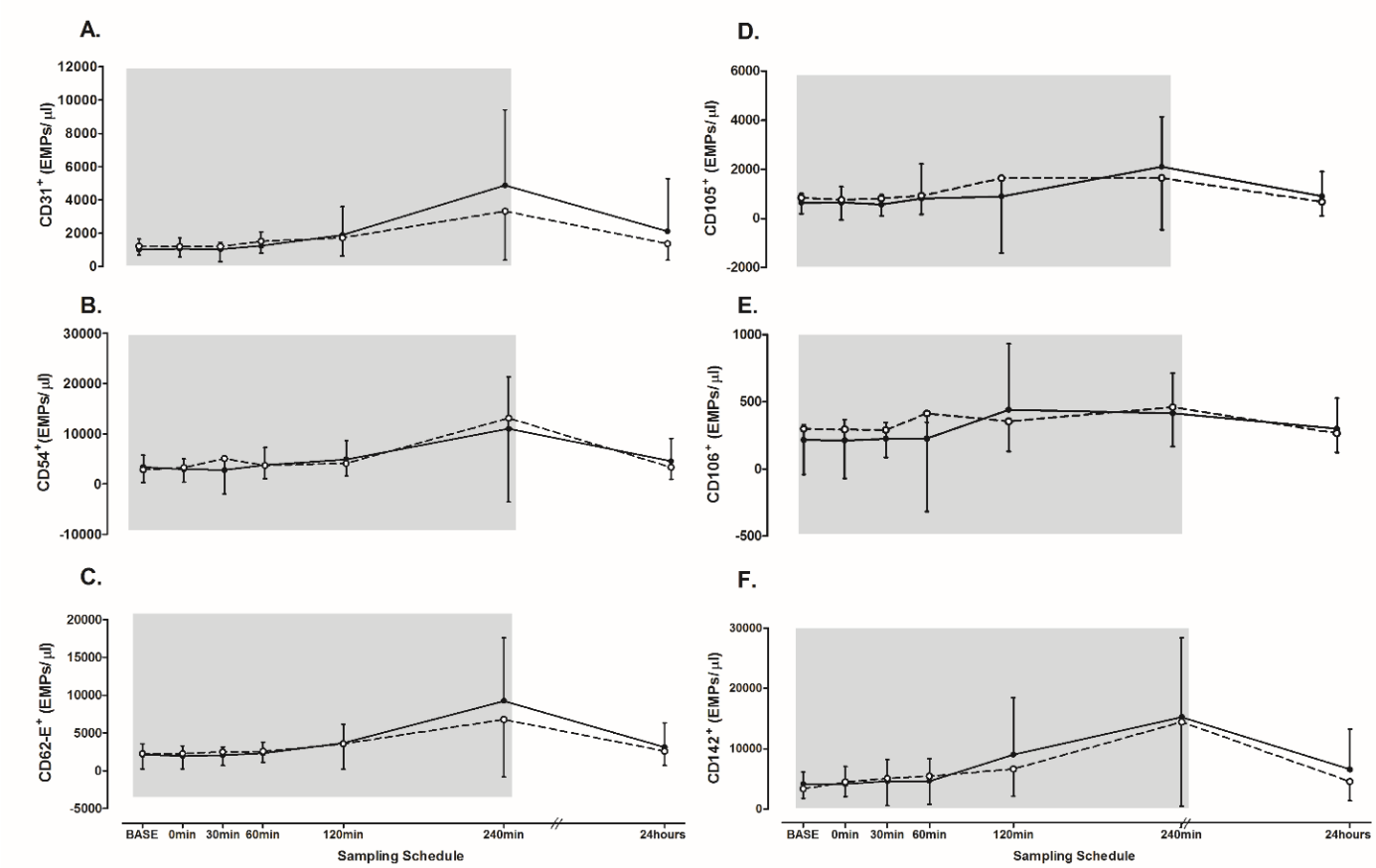
A.
CD31+ (EMPs/ µl)
12000
10000
8000
6000
4000
2000
0
D.
CD105+ (EMPs/ µl)
6000
4000
2000
0
-2000
B.
CD54+ (EMPs/ µl)
30000
20000
10000
0
-10000
E.
CD106+ (EMPs/ µl)
1000
500
0
-500
C.
CD62-E+ (EMPs/ µl)
20000
15000
10000
5000
0
-5000
F.
CD142+ (EMPs/ µl)
30000
20000
10000
0
BASE 0min 30min 60min 120min 240min 24hours
Sampling Schedule
BASE 0min 30min 60min 120min 240min 24hours
Sampling Schedule

Figure 1.

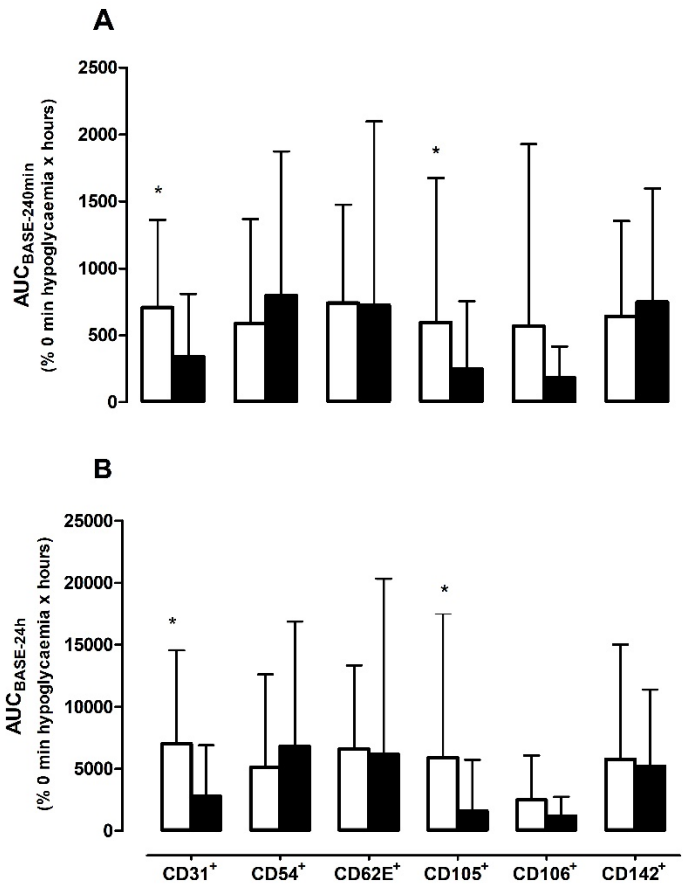
A
2500
2000
1500
1000
500
0
AUC BASE-240min
(% 0 min hypoglycaemia x hours)
*
*
B
25000
20000
15000
10000
5000
0
AUC BASE-24h
(% 0 min hypoglycaemia x hours)
*
*
CD31+
CD54+
CD62E+
CD105+
CD106+
CD142+

Figure 2.