CLINICAL STUDY PROTOCOL

Study Title:	Impact of hypoglycaemia in patients with DIAbetes mellitus type 2 on PLATElet activation
Short Title:	**DIAPLATE study**
Protocol Number:	HS-2017-04
Sponsor:	Medical University of Graz
Protocol Date:	13th February 2018
Protocol Version:	3.5
Ethics number:	30-012 ex 17/18

Medical University of Graz, Division of Endocrinology and Diabetology, Cardiovascular Diabetology Research Group, Austria, 8036 Graz, Auenbruggerplatz 15; Telephone: +43 (0)316 385 78038; Fax: +43 (0)316 385 14332

Financial support: Merck, NJ, USA

CONTACT LIST

Chief Investigator

Harald Sourij, M.D., Associate Professor
Division of Endocrinology and Diabetology
Department of Internal Medicine
Medical University of Graz
Auenbruggerplatz 15
8036 Graz, Austria
Tel: +43 316 385 81310
Fax: +43 316 385 13428
Center for Biomarker Research in Medicine, CBmed
Stiftingtalstraße 5
8036 Graz, Austria

Biometrician

Regina Riedl, DI Dr.
Institute for Medical Informatics, Statistics and Documentation
Auenbruggerplatz 2
8036 Graz

Sponsor

Medical University of Graz
Auenbruggerplatz 2-4
8036 Graz, Austria

Financial support

Merck, Kenilworth, NJ, USA

Clinical Lead

Felix Aberer, M.D.
Division of Endocrinology and Diabetology
Department of Internal Medicine
Medical University of Graz
Auenbruggerplatz 15
8036 Graz, Austria
Tel: +43 316 385 86113 Fax: +43 316 385 13428

Study Manager:

Peter Pferschy, BSc.
Division of Endocrinology and Diabetology
Department of Internal Medicine
Medical University of Graz
Auenbruggerplatz 15
8036 Graz, Austria
Tel: +43 316 385 78030
Fax: +43 316 385 13428

Signatures:

Principal Investigator:

Assoz.-Prof. Dr. Harald Sourij

Signature Date

Confidentiality Statement

This document contains confidential information that must not be disclosed to anyone other than the sponsor, the investigator team, regulatory authorities and members of the Research Ethics Committee.
Trials Sponsor: Medical University of Graz, Auenbruggerplatz 2-4, 8036 Graz

Table of contents

Abbreviations

AE	Adverse event
ACTH	Adrenocorticotropic hormone
BMI	Body mass index
CRF	Case report form
CRH	Counter regulatory hormones
EC	Ethical Committee
ECG	Electrocardiography
FPFV	First patient first visit
FPLV	First patient last visit
GIR	Glucose infusion rate
HbA1c	Glycated haemoglobin 1c
HSS	Hypoglycaemic symptoms score
ICAM	Intercellular adhesion molecule
IGF-1	Insulin-like-growth factor 1
IL-6	Interleukin-6
PAI-1	Plasminogen activator inhibitor
PAP	Platelet activity parameters
PTH	Parathyroid hormone
PG	Plasma glucose
SDF	Source data form
T2DM	Type 2 Diabetes Mellitus
TMF	Trial master file
VCAM	Vascular adhesion molecule
vWF	von Willebrand factor

Sponsor	Medical University of Graz Auenbruggerplatz 2-4 8036 Graz, Austria
Principal Investigators	Harald Sourij, MD, Associate Professor
Indication	Type 2 diabetes mellitus without history of cardiovascular disease
Study design and phase	Monocentric, single arm, open, mechanistic trial
Study Short Title	Platelet activity during hypoglycaemia
Keyword	Hypoglycaemia, platelet activity, type 2 diabetes mellitus
Aims of the trial	*Primary objective:* - The primary objective is to investigate platelet activation during different levels of hypoglycaemia induced by a stepwise hyperinsulinaemic, hypoglycaemic clamp experiment in patients with T2DM *Secondary objective:* To investigate - Platelet activation and recovery at one day and one week after the clamp experiment - Changes of proatherothrombotic markers during the hypoglycaemic clamp - Counter regulatory hormone response during the clamp
Outcome measures of the trial	*Primary outcome measures:* - Changes in platelet activation measured by light transmittance aggregometry based on ADP activation from baseline to the end of the hypoglycaemia phase (i.e. 45 mg/dl for 30 minutes) (visit 3) *Secondary outcomes measures:* - Changes in platelet activation measured by activation marker, such as CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from baseline to the end of the hypoglycaemia phase - Changes in platelet activation measured by activation marker, such as LTA, CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from the end of the hypoglycaemic clamp to one day after the clamp - Changes in platelet activation measured by activation marker, such as LTA, CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from the end of the hypoglycaemic clamp to one week after the clamp - Changes in platelet activation measured by PFA-100 from baseline to the end of the hypoglycaemia phase - Changes in platelet activation measured by PFA-100 from the end of the hypoglycaemic clamp to one day and one week after the clamp - Changes in interleukin-6 (IL-6), von Willebrand factor [vWF], plasminogen activator inhibitor (PAI-1), vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) from baseline to the end of the hypoglycaemia phase

	- Changes in interleukin-6 (IL-6), von Willebrand factor [vWF], plasminogen activator inhibitor (PAI-1), vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) from the end of the hypoglycaemic clamp to one week after the clamp - Difference of platelet activation parameters during different states of hypoglycaemia - Counter regulatory hormone response during hypoglycaemia
Number of patients	- 14
Time schedule	- EC Submission: SEP/2017 - Local Authority Submission.: SEP/2017 - First Patient In (FPFV): JAN/2018 - Last Patient In: APR/2018 - Last Patient Out (LPLV): MAY/2018 - Data Base Lock: JUL/2018 - First Results available: JUL/2018 - Clinical Study Report: DEC/2018
Main inclusion criteria	- Male or female aged 18-64 years (both inclusive) at the time of signing informed consent. - Subjects diagnosed with type 2 diabetes (defined by WHO-criteria) and on stable treatment for a period of 90 days prior to screening with metformin as monotherapy or diet only. Stable is defined as unchanged dose. - Body mass index (BMI) between 20.0 and 35.0 kg/m^2 (both inclusive). - HbA1c between 43 and 64 mmol/mol (6.0% – 8.0%) (both inclusive). - No use of platelet inhibiting therapy (e.g. aspirin, clopidogrel, ticagrelor, prasugrel)
Main exclusion criteria	- All other forms of diabetes (type 1 diabetes, gestational diabetes) than type 2 diabetes mellitus. - Treatment with any glucose lowering agent(s) other than metformin in a period of 60 days prior to screening. An exception is short-term treatment (≤ 7 days in total) with insulin due to intercurrent illness. - Clinically significant abnormal haematology, platelet count, biochemistry and coagulation. - Uncontrolled hypertension defined as resting blood pressure at screening (after resting for 5 min) outside the range of 90−160 mmHg for systolic or 50−100 mmHg for diastolic. - Chronic liver failure as assessed by the investigator. - ALT or AST levels > 3x ULN - Previously known cardiovascular disease and / or past cardiovascular events, or past episodes of a congestive heart failure syndrome (NYHA II - NYHA IV) - Females of child bearing potential without adequate contraceptive methods (i.e. sterilisation, intrauterine device, vasectomised partner; or medical history of hysterectomy) - eGFR <45 ml/min/1,73 m2

	- Treatment with beta-blockers, antiarrhythmic agents or neuroleptic drugs - Active smoker or intake of illicit substances. - Regular NSAR/NSAID intake

1. INTRODUCTION AND RATIONALE

Increased platelet activation is significantly involved in the pathophysiology of micro- and macrovascular diabetic complications. Previously performed studies suggested platelet activation in both, hyper- and hypoglycaemic states, leading to a potentially increased risk for thromboembolic complications. Hypoglycaemia, in particular severe hypoglycaemic episodes, has been associated with increased cardiovascular or overall mortality in previous studies [6, 7]. Potential mechanisms include arrhythmias or increased risk for thrombembolism, based on platelet activation and/or hypercoagulability.

However, studies investigating the effect of hypoglycaemia on platelet activity were mainly performed in healthy individuals or subjects with type 1 diabetes ([3],4,5]). In addition these studies did not systemically evaluate the correlation between the degree of hypoglycaemia and platelet activation in subjects with type 2 diabetes and the sustainability of platelet activation after the hypoglycaemic event.

Data on the impact of hypoglycaemia on platelet activation and markers of pro-atherothrombotic biomarkers in subjects with T2DM are scarce. Therefore we aim to explore platelet activation and atherothrombotic biomarkers during a stepwise hypoglycaemic clamp in subjects with T2DM and without established cardiovascular disease. In addition we aim to investigate for how long these potential effects sustain after the hypoglycaemic event.

Secondary objectives are to explore associations of the extent of hypoglycaemia and counter regulatory response and platelet activation.

1.1 AIM OF THE STUDY

The aim of this experimental study is to investigate the impact of hypoglycaemia on platelet activation parameters (PAP) during a hyperinsulinaemic hypoglycaemic clamp study.

1.2 STUDY HYPOTHESIS

Hypoglycaemia in patients with T2DM leads to increased platelet activation.

2. OBJECTIVES AND OUTCOMES

2.1 PRIMARY OBJECTIVE

The primary objective is to investigate platelet activation during different levels of hypoglycaemia induced by a stepwise hyperinsulinaemic, hypoglycemic clamp experiment in patients with T2DM.

2.2 SECONDARY OBJECTIVES

To investigate

- Platelet activation and recovery at one week after the clamp experiment
- Changes of pro-atherothrombotic markers during the hypoglycaemic clamp
- Counter regulatory hormone response during the clamp

2.3 PRIMARY OUTCOME

- Changes in platelet activation measured by light transmittance aggregometry based on ADP activation from baseline to the end of the hypoglycaemia phase (i.e. 45 mg/dl for 30 minutes) (visit 3)

2.4 SECONDARY OUTCOMES

- Changes in platelet activation measured by activation marker, such as CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from baseline to the end of the hypoglycaemia phase
- Changes in platelet activation measured by activation marker, such as LTA, CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from the end of the hypoglycaemic clamp to one day after the clamp
- Changes in platelet activation measured by activation marker, such as LTA, CD62P, CD63 and PAC1, on $CD41^{pos}$ or $CD42b^{pos}$ platelets from the end of the hypoglycaemic clamp to one week after the clamp
- Changes in platelet activation measured by PFA-100 from baseline to the end of the hypoglycaemia phase
- Changes in platelet activation measured by PFA-100 from the end of the hypoglycaemic clamp to one day and one week after the clamp
- Changes in interleukin-6 (IL-6), von Willebrand factor [vWF], plasminogen activator inhibitor (PAI-1), vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) from baseline to the end of the hypoglycaemia phase
- Changes in interleukin-6 (IL-6), von Willebrand factor [vWF], plasminogen activator inhibitor (PAI-1), vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) from the end of the hypoglycaemic clamp to one week after the clamp
- Difference of platelet activation parameters during different states of hypoglycaemia
- Counter regulatory hormone response during hypoglycaemia

3. STUDY DESCRIPTION

3.1 DESIGN

Monocentric, single arm, open, mechanistic trial to evaluate the effect of hypoglycaemia on PAP.

3.2 DURATION OF STUDY

Active study duration will be 5 days for each study participant.

4. STUDY POPULATION

We will study 14 subjects with T2DM without history of cardiovascular events or manifest atherosclerosis.

4.1 INCLUSION CRITERIA

- Male or female aged 18-64 years (both inclusive) at the time of signing informed consent
- Subjects diagnosed with type 2 diabetes (diagnosed regarding WHO criteria) and on stable treatment for a period of 90 days prior to screening with metformin as monotherapy or diet only. Stable is defined as unchanged dose
- Body mass index (BMI) between 20.0 and 35.0 kg/m^2 (both inclusive)
- HbA1c between 43 and 64 mmol/mol (6.0% – 8.0%) (both inclusive)
- No use of platelet inhibiting therapy (e.g. aspirin, clopidogrel, ticagrelor, prasugrel)

4.2 EXCLUSION CRITERIA

- All other forms of diabetes (type 1 diabetes, gestational diabetes) than type 2 diabetes mellitus
- Treatment with any glucose lowering agent(s) other than metformin in a period of 60 days before screening. An exception is short-term treatment (≤ 7 days in total) with insulin due to intercurrent illness
- Impaired hypoglycaemic awareness determined at the discretion of the investigator
- Medical history of arrhythmia as atrial fibrillation, atrial flutter, atrioventricular dissociation disorders or ventricular arrhythmias
- Previously known cardiovascular disease and / or past cardiovascular events, or past episodes of a congestive heart failure syndrome (NYHA II - NYHA IV)
- Severe hypoglycaemic event requiring third party help in the last 6 months
- Known allergy to human insulin or dextrose solution
- Clinically significant abnormal haematology, biochemistry, lipids, hormones, coagulation or urinalysis

- Uncontrolled hypertension defined as resting blood pressure at screening (after resting for 5 min, measured in sitting position) outside the range of 90–160 mmHg for systolic or 50–100 mmHg for diastolic
- Chronic liver failure with severe liver dysfunction as assessed by the investigator
- ALT or AST levels > 3x ULN
- eGFR <45 ml/min/1,73 m^2
- Any musculoskeletal disorders holding back from stay in bed in a lying position during the time of the clamp experiments
- Treatment with beta-blockers, antiarrhythmic agents or neuroleptic drugs
- Active smoker or intake of illicit substances
- Regular intake of NSAR/NSAIDs
- Any mental disorders or psychiatric conditions which may interfere with understanding or conduction of study related procedures
- Females of child bearing potential without adequate contraceptive methods (i.e. sterilisation, intrauterine device, vasectomised partner; or medical history of hysterectomy)

Exclusion from clamp investigation:

- Treatment with NSAR/NSAID or metamizole within 10 days prior to clamp visits
- Fasting plasma glucose >9.0 mmol/l (>162 mg/dl) on the day of clamp investigation

If one of these exclusion criterial for the clamp visits is met, the clamp visit will be postponed for 10-15 days.

4.3 WITHDRAWAL CRITERIA

Subjects may be withdrawn from the study at the discretion of the Investigator or Sponsor due to a safety concern or if judged non-compliant with trial procedures. A subject must be withdrawn from treatment if one of the following applies:

- Subject chooses to withdraw from the study at any time
- Pregnancy or intention of becoming pregnant during study duration
- Intolerable adverse effects
- Major violation of the study protocol
- Other circumstances that would endanger the health of the subject if he/she were to continue his/her participation in the trial

Reasons for withdrawals and discontinuation of any subject from the protocol have to be recorded.

4.4 TERMINATION OF THE ENTIRE TRIAL

Premature termination of the clinical trial will be considered if the sponsor believes it is necessary to terminate the clinical trial for safety reasons, or when the clinical trial proves to be impracticable.

5. VISIT PROCEDURES, MEASUREMENTS AND ASSESSMENTS

5.1 RECRUITMENT AND SCREENING

The site will recruit potential subjects by using an available local recruitment registry. No study-related procedures are undertaken before obtaining informed consent. The study team will explain in detail the study procedures and asks the participant about his/her willingness to participate in this research study. After informed consent is signed and obtained, participants are given a signed copy of the informed consent document and are assigned a screening identification number in ascending order starting with “01”.

After obtaining informed consent at the screening visit, subject´s eligibility will be further assessed and documented by using a source data form (SDF) with a list of inclusion and exclusion criteria, medical history (including all medications past and current) will be acquired and the following measurements will be performed: body weight, height, blood pressure, electrocardiography, pregnancy test, blood parameters (see Table1, including Hepatitis B, C and HIV-Test).

Subjects unable to provide written informed consent will not be included in the study.

A summary of all visits and procedures has been outlined below (table 1). A more detailed description of each visit and accompanying procedures can be found under section 5.2

5.2 VISIT SCHEDULE

	Visit 1 (V1) *Screening / Baseline*	**Visit 2 (V2)** *(2-10 days after V1)*	**Visit 3 (V3)** *(10-24 days after V2)*	**Visit 4 (V4)** *(1 day after V3)*	**Visit 5 (V5)** (6±1 days after V4)
Informed Consent	X				
Check Inclusion/exclusion criteria	X				
Demographics, medical history	X				
Concomitant medication	X				
Vital signs	X	X	X	X	X
Height	X				
Weight	X	X			X
Physical examination	X		X		X
ECG	X		X		
Blood sampling for platelet activity parameters		X	X	X	X
Blood sampling for hormones		X	X	X	X
Blood cell count	X				X
HbA1c	X				
Lipids	X	X			X
Liver function parameters (AST, ALT, GGT)	X				X
Renal function parameters (creatinine, eGFR)	X				X
Serology for Hepatitis B, C and HIV	X				
Pregnancy test (in female)	X		X		
Adverse Events		X	X	X	X
Hyperinsulinemic-Euglycemic Clamp investigation		X			
Hyperinsulinemic-Hypoglycemic Clamp investigation			X		

Table 1. Study related investigations

5.2.1 Visit 1

- Obtain informed consent of potential participant verified by signature on the study informed consent form
- Collect blood to check for inclusion criteria (Blood count, HbA1c, glucose, lipids, renal and liver function parameters)
- Collect and prepare blood samples for biobank
- Obtain demographic information, medical history, medication history
- Record vital signs

- Perform ECG
- Measure body weight and body height, calculate BMI
- Perform physical examination
- Verify inclusion/exclusion criteria

5.2.2 Visit 2 – Hyperinsulinemic-euglycemic clamp (2-10 days after Visit 1)

The hyperinsulinemic – euglycemic clamp will be performed at visit 2. Patients will be advised to attend to the study center (Clinical Research Center [CRC], Stiftingtalstraße 24, 8010 GRAZ) at 06:30 am after an overnight 10 hours fasting period. In case of antidiabetic treatment with Metformin, the morning dose should not be administered. Vital signs will be determined and possible Adverse Events will be documented. Patients will be advised to place in the study bed in which they will remain during the whole clamp experiment.

At approximately 07:00 in the morning, two intravenous cannulas (catheters) will be inserted: one cannula will be inserted in an antecubital vein of a forearm for sampling of serial measurements of plasma glucose (approximately every 5-30 min, depending on the clamp procedure and safety). The hand will be placed in a heated environment to enable drawing of arterialised-venous blood. For controlled variable infusion of glucose 20% and infusion of human soluble insulin (40 IU Actrapid®, 100 IU/ml in 99.6 mL saline), an antecubital vein of the contralateral arm will be cannulated. Both cannulas will be kept open by a slow continuous 0.9% saline infusion. After the baseline blood samples for blood glucose, insulin and platelet activity have been taken (timepoint 0 min), the hyperinsulinemic – euglycemic clamp will be started. A variable i.v. infusion of glucose 20% or infusion of human soluble insulin will be initiated in order to obtain a plasma glucose target of 5.5 mmol/L (100 mg/dL) ± 10%. The insulin infusion should be stopped completely if glucose has to be infused and vice versa. When the plasma glucose target of 5.5 mmol/L (100 mg/dL) ± 10% is reached, a continuous insulin infusion (2.5 mU/kg/min) of human soluble insulin will be applied for 120 min to acutely raise and maintain serum insulin concentrations. Blood glucose will be clamped at the concentration of 5.5 mmol/L by a variable infusion of glucose 20%. Blood samples for measurement of plasma glucose concentrations will be obtained at 5 min interval, for measurement of insulin concentrations and platelet activity every 30 min (timepoints 0, 60, 90, 120 min.) throughout the clamp. For safety reasons, serum potassium will be measured at start of the clamp procedure and after that at the discretion of the investigator. Potassium will be substituted at discretion of the investigator. After 120 min, the clamp experiment is completed. Subjects will receive a meal and will remain at the study center until stabilisation of PG. When patients are in a normoglycemic state and in a good clinical condition, determined by the investigator, they will be discharged equipped with carbohydrates for hypoglycemia prevention.

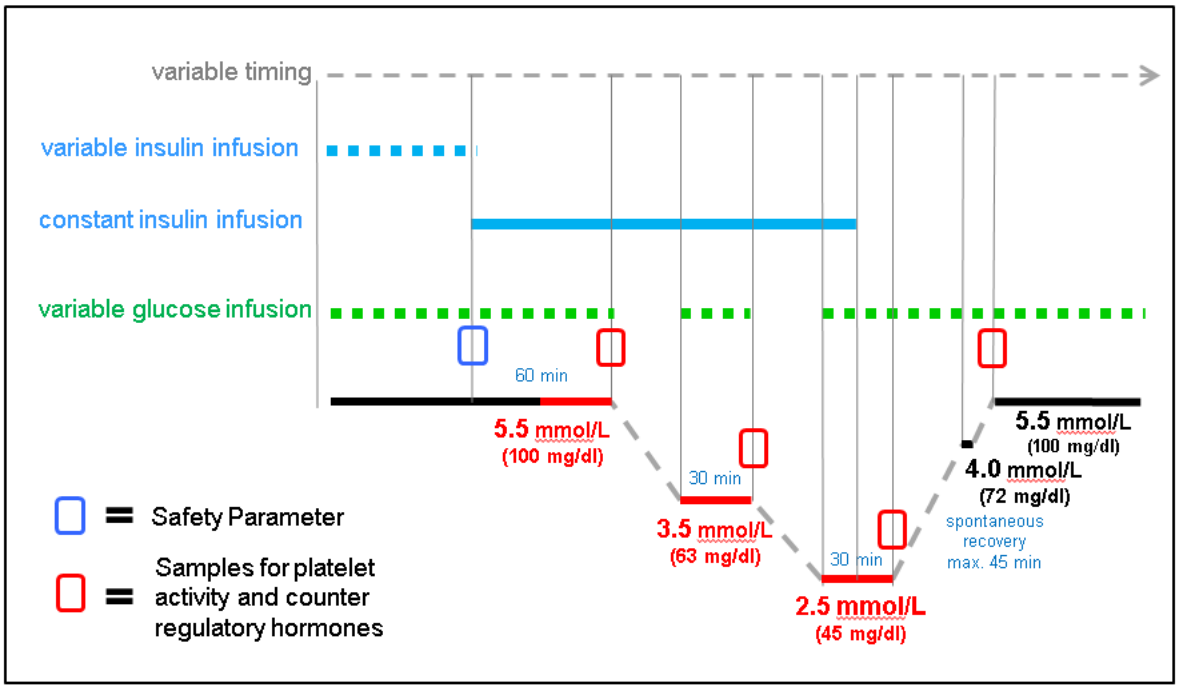
5.2.3. VISIT 3 – HYPERINSULINEMIC-HYPOGLYCEMIC CLAMP (10-24 DAYS AFTER VISIT 1)

The hyperinsulinemic-hypoglycemic clamp will be performed at Visit 3. Patients will be advised to attend to the study center (Clinical Research Center [CRC], Stiftingtalstraße 24, 8010 GRAZ) at 06:30 am after an overnight 10 hours fasting period. In case of antidiabetic treatment with Metformin, the morning dose should not be administered. Vital signs will be determined and documented, females with child bearing potential will do a pregnancy test and a physical status will be performed by the physician in charge, and possible Adverse Events will be documented. Patients will be advised to place in the study bed in which they will remain during the whole clamp experiment. Then, pulse and blood pressure monitoring will start.

At approximately 07:00, two intravenous cannulas (catheters) will be inserted: one cannula will be inserted in an antecubital vein of a forearm for sampling of serial measurements of plasma glucose (approximately every 5-30 min, depending on the clamp procedure and safety). The hand will be placed in a heated environment to enable drawing of arterialised-venous blood. For controlled variable infusion of glucose 20% and infusion of human soluble insulin (40 IU Actrapid®, 100 IU/ml in 99.6 mL saline), an antecubital vein of the contralateral arm will be cannulated. Both cannulas will be kept open by a slow continuous 0.9% saline infusion. After the baseline blood samples for blood glucose, insulin, counter regulatory hormone parameters and platelet activity have been taken, the hyperinsulinemic hypoglycemic clamp will be started. A variable i.v. infusion of glucose 20% or infusion of human soluble insulin will be initiated in order to obtain a plasma glucose target of 5.5 mmol/L (100 mg/dL) ± 10%. When the plasma glucose target of 5.5 mmol/L (100mg/dL) ± 10% is reached the high constant insulin infusion (2.5 mU/kg/min) will be initiated. After 30 minutes the plateau 5.5mmol/L will be started. After 30 min on the 5.5 mmol/L plateau the glucose infusion will be interrupted, the PG will be allowed to decline to 3.5 mmol/L and by restarting the variable glucose infusion, kept stable at 3.5 mmol/L. Thereafter the glucose infusion will be interrupted and the PG will be allowed to decline to 2.5 mmol/L or a nadir at a higher concentration in case 2.5 mmol/L cannot be reached or symptoms of hypoglycaemia are unacceptable. By restarting the variable glucose infusion the PG will be kept stable at 2.5 mmol/L or nadir and assessments on the plateau will be performed. After 15 min on the 2.5 mmol/L or nadir plateau the constant high insulin infusion will be terminated. Starting at time point 25 min on the 2.5 mmol/L or nadir plateau the glucose infusion will be tapered off, if possible, to allow a spontaneous recovery from hypoglycaemia. PG will not be allowed to fall below 2.2 mmol/L. If PG has not reached 4.0 mmol/L 45 min after termination of the constant high insulin infusion, a constant high glucose infusion (5.5 mg/kg/min) will be initiated to increase PG to a level of 4.0 mmol/L. When reaching the PG level of 4.0 mmol/L assessments will be performed and blood samples will be taken. The PG will then be increased to 5.5 mmol/L for safety reasons and the clamp terminated, if deemed safe by the investigator. PAP and CRH samples will be obtained at the end of each plateau phase as well as after recovery from hypoglycaemia at PG 100mg/dl (see figure 1).

After initation of the high constant insulin infusion potassium concentration will be measured at the discretion of the investigator and potassium replaced if necessary. ECG will be continuously monitored during the clamp procedure using patient cardiac monitoring system "Dräger". One 12-lead ECG will be recorded during run-in phase (before starting constant high insulin infusion) and at hypoglycaemia or nadir of 2.5 mmol/L at approximately 15 min. In case a subjects PG does not decrease from 5.5 mmol/L to 3.5 mmol/L within 30 minutes, the 3.5 mmol/L assessment plateau will be initiated in any case after 30 minutes after the end of assessment plateau 5.5 mmol/L high insulin. In case a subjects PG does not decrease from 3.5 mmol/L to 2.5 mmol/L within 30 minutes, the 2.5 mmol/L assessment plateau will be initiated in any case after 30 minutes after the end of assessment plateau 3.5 mmol/L. In the above cases, the glucose infusion should not be initiated at the start of the assessment plateaus to allow a further decrease in PG levels to the scheduled target PG levels. Subjects will receive a meal and will remain at the study center until stabilisation of PG. When patients are in a normoglycemic state and in a good clinical condition, determined by the investigator, patients will be discharged home.

Figure 1. Hyperinsulinemic-Hypoglycemic Clamp with time points of sampling collection



5.2.3 VISIT 4 – FOLLOW-UP VISIT (ONE DAY AFTER THE CLAMP EXPERIMENT)

Visit 4 will be performed for a follow up one day after the clamp investigation. Vital signs will be recorded and possible adverse events will be raised and documented. Then venous blood sampling will be performed for PAP and CRH.

Visit procedures:

- Record vital signs
- Collect blood for platelet activation parameters
- Collect blood for counter regulatory hormones
- Collect and prepare blood samples for biobank
- Record adverse events as reported by participant or observed by investigator

5.2.4 Visit 5 - Follow-up Visit (6±1 days after Visit 4 - Close out Visit)

This visit will take place 6±1 days after the clamp experiment. Vital signs will be recorded, a physical examination will be performed and possible adverse events will be raised and documented. Additionally to the measurements at Visit 4 blood for blood count and chemistry will be sampled, as well as body weight will be determined.

Visit procedures:

- Record adverse events as reported by participant or observed by the investigator
- Record vital signs
- Collect blood for routine biochemistry
- Collect blood for platelet activity parameters
- Collect blood for counter regulatory hormones
- Collect and prepare blood samples for biobank
- Perform physical examination
- Determination of body weight

5.3 Description of procedures and measurements

5.3.1 Medical history and physical examination

Determination of medical history will be performed at the screening visit to record illnesses, health disorders and concomitant medication. Physical examination will be performed at the Screening visit 1, visit 3 as well as at the closing visit according to local procedure. The physician will perform the physical examinations with the focus on cardiac, lung and abdominal examination. Any abnormal, clinical significant findings must be recorded in the SDF. Any adverse events will be recorded. Any changes in medications will be recorded in the SDF.

5.3.2 Electrocardiography

An ECG will be performed at the screening visit. The ECG will be interpreted, signed and dated by the investigator. ECG will be continuously monitored during visit 3.

5.3.3 Vital signs

Pulse should be recorded at all visits after resting for five minutes in a sitting position. Systolic and diastolic blood pressure will be measured in sitting position at all visits.

5.3.4 Body weight and height

Weight should be measured at the screening and closing visit, as well as at visit 2. The same and calibrated pair of scales should preferably be used throughout the trial.

Height will be recorded at the screening visit. BMI (body mass index) will be calculated as follows: BMI = weight (kg)/height^2 (m^2).

5.3.5 Routine biochemistry

Blood samples will be obtained at all visits in the fasting state and processed by the local laboratory using standard methods for routine tests. Patients can take their regular morning medications but are asked that they do not take metformin in case of a concomitant metformin therapy. Patients should bring their regular medication along to their study visits to be further advised by the doctor. See table below for a detailed description of blood collection at each visit.

5.3.6 Blood sample collection and plasma extraction for biobanking

Blood will be collected via venous puncture into 8 ml serum and 6 ml EDTA vacutainers and centrifuged within 30 min of collection. Plasma should be transferred into Eppendorf tubes and stored at -80 °C locally.

5.3.7. Parameters of special interest

Venous blood for determination of following parameters will be drawn during the clamp investigation as well as one day and one week after the clamp. Time points of sampling collection during the clamp are indicated in Figure 1.

Platelet activity parameters (PAP):

PAP are determined in the course of blood sampling at visits 2, 3, 4 and 5. In both Clamp tests, PAP are determined at five time points. The sodium citrate blood samples will be kept at ambient temperature after collection and transferred immediately to the Central Laboratory of the University

Hospital Graz for analyses. Detailed information about sampling will be summarized in the dedicated SOP.

Light transmission aggregometry

Measured on a Chronolog 700 Lumi-Aggregometer (Chronolog Corp, Havertown, PA) using standard agonists of platelet aggregation (Collagen, ADP, arachidonic acid, TRAP) to stimulate platelet aggregation in platelet-rich plasma (Gurbel PA et al. Circulation 2009)

Platelet Function Analyser-200

Measured on a PFA-200 (Siemens Healthineers, Marburg) using Collagen/ADP and Collagen/Epinephrine cartridges to monitor closure-time in-vitro.

Quantification of platelet function and activation by flow cytometry (FACS)

Blood samples will be processed for multicolour FACS analysis within 60min of collection. Fluorochrome-conjugated monoclonal antibodies will be used to stain platelet surface antigens, such as CD41 (platelet integrin αIIbβ3), CD42b (platelet glycoprotein Ib), CD62P (platelet α-granule membrane protein), CD63 (platelet dense granule and lysosomal membrane protein) and PAC-1 (activation induced neoepitope on integrin αIIbβ3). Single platelets will be identified according to the size and granularity parameters and the expression of CD41 and CD42b. Platelet activation markers (CD62P, CD63 and binding of PAC-1) will be quantified within $CD41^{pos}$ and $CD42b^{pos}$ platelets as well as $CD45^{pos}CD42b^{pos}$ platelet-leukocyte complexes using a BD LSRFortessa flow cytometer (Becton Dickinson, US).

Additional parameters:

- Interleukin-6 (IL6): IL 6 is a proinflammatory cytokine which is produced during acute inflammation and stimulates secretion of hepatic acute-phase-proteins[10, 11]. Higher serum levels of IL6 showed to be a risk factor for cardiovascular disease with and without diabetes mellitus[12-14]. Measurement is concucted by electroluminescence immunoassay (ECLIA)
- Von Willebrand factor (vWF): vWF is an essential glycoprotein initiating adhesion of thrombocytes to impaired endothelial wall. Especially in DM2 vWF showed to be associated with risk of cardiovascular disease [15, 16]. We will measure vWF concentrations by ELISA.
- Plasminogen Activator inhibitor (PAI-1): PAI-1 is secreted by thrombocytes during vessel regeneration. Patients with obesity and T2DM show higher basal values of PAI-1 which revealed to be related to a higher risk of atherothrombotic complications [17, 18]. Measurements of PAI-1 are performed by and ELISA method.

- Vascular adhesion molecule (VCAM): VCAMs are a group of cell adhesion molecules which promote adhesion of lymphocytes, monocytes and eosinophils to vascular endothelium. However it also may contribute to neointima formation after arterial injury and so on promotes vascular damage after acute vessel injury [19, 20]. Measurements are performed by an ELISA method.
- Intercellular adhesion molecule (ICAM): As VCAMs ICAMS are part of the immunoglobulin superfamily which take part in the leukocyte adhesion to endothelium and so contribute to augmentation of atherosclerotic plaque formation [21, 22]. Measurements will be performed by an ELISA method.

Counter regulatory hormones (CRH), C- peptide, renin, aldosterone, parathyroid hormone will be measured for a secondary objective to find out more about possible correlations with PAP and variations of counter regulatory hormones during the clamp, one day and one week after the trial.

- ACTH: Adrenocorticotropic hormone (ACTH) is measured in EDTA-plasma by an Immulite ® 2000 ACTH chemiluminescent immunometric assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, U.S.A.)
- Cortisol: Serum Cortisol concentrations will be determined by the ADVIA Centaur cortisol assay which is a competitive immunoassay based on chemiluminescence technology assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, U.S.A.)
- Aldosterone and Renin: They will be measured by the IDS-iSYS Aldosterone Renin assay which is based on chemiluminescence technology. (Immunodiagnosticsystems, Boldon, United Kingdom).
- C-Peptide: Serum C-peptide will be measured with the ADVIA Centaur which is a sandwich immunoassay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, U.S.A.)
- Epinephrine/ Norepinephrine: Normetanephrine and Metanephrine are measured in EDTA-plasma by a MetCombi Plasma ELISA (DRG Instruments GmBh, Marburg, Germany)
- IGF1: Insulin-like-growth factor1 (IGF1) will be measured with the IDS-iSYS assay which is based on chemiluminescence technology (Immunodiagnosticsystems, Boldon, United Kingdom)
- Glucagon will be analysed by using ELISA kits (Mercodia AB, Uppsala, Sweden)
- Parathyroid hormone (PTH): Intact PTH was determined in plasma by electrochemiluminescence immunoassay “ECLIA” on an Elecsys 2010 (ECLIA) (Roche Diagnostics, Mannheim, Germany)

6. ADVERSE EVENTS (AE)

An AE is any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporarily associated with the use of the trial device/procedure, whether or not considered related to the treatment.

All adverse events that occur during this study will be recorded on the adverse event case report forms.

6.1. Adverse Event description

For the purposes of the study, AEs will be followed up according to local practice until the event has stabilised or resolved, or the Follow-up Visit, whichever is the sooner. SAEs will be recorded throughout the study.

6.2. Severity of Adverse Events

Mild: Awareness of event but easily tolerated

Moderate: Discomfort enough to cause some interference with usual activity

Severe: Inability to carry out usual activity

6.3. Causality of Adverse Events

Unrelated:	No evidence of any causal relationship
Unlikely:	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after device implant). There is another reasonable explanation for the event (e.g. the patient's clinical condition, other concomitant treatment).
Possible:	There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after device implant). However, the influence of other factors may have contributed to the event (e.g. the patient's clinical condition, other concomitant treatments).
Probable:	There is evidence to suggest a causal relationship and the influence of other factors is unlikely
Definite:	There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out

6.4. Abnormal Laboratory Test Results

All clinically significant abnormal laboratory test results except increased fasting glucose and elevated HbA1c will be recorded as adverse events.

6.6. Adverse events related to the clamp experiment

The trial will include a hypoglycaemic clamp procedure during Visit 3. Within the clamp experiment hypoglycaemia is intentionally induced. Depending on potency of hypoglycaemia patients might feel discomfort, sweating, lack of concentration, dizziness, palpitations, or general discomfort. Hypoglycaemia will

be controlled by frequent venous glucose measurements. In case of unacceptable or unbearable hypoglycaemia during the clamp procedure, glucose will be administered and clamp investigation will be terminated if deemed necessary for the investigator or the subject wants to break up. During the entire clamp experiment, patients will be continuously monitored for glucose, heart rate, blood pressure and potassium. Medical staff is highly experienced and qualified for performing clamp investigations. The clamp may be interrupted or terminated for safety reasons at any time at the investigators discretion.

Independently from hypoglycaemia related adverse event, some discomfort may occur as a result from vein puncture or venous line issues. In some cases local haematoma can occur, very seldom installed venous lines might lead to local infection and phlebitis of the punctured vein which may require local and/or systemic treatment.

6.7. Reporting of SAEs

Rapid reporting of all SAEs, occurring during the study or within 30 days following the completion of the study by the subject, must be performed as detailed in SAE reporting instructions. SAE reporting instructions will be filed in the Investigator Site File. The sponsor will report the SAE to the ethics committee and local authorities.

A suspected unexpected serious adverse reaction (SUSAR) is designated as such according to Guideline 2001/20/EG. A serious adverse reaction is deemed unexpected when it is not listed in the corresponding basic document (Summary of Product Characteristics, IB, IMPD).

7. STATISTICAL ANALYSES

7.1 Sample size and power considerations

We have shown previously that light transmittance aggregometry (LTA) based on ADP activation in a population of subjects with type 2 diabetes is 70±12% (Bethel MA et al. Diabetic Medicine 2016). For a clinically relevant difference in LTA from baseline to hypoglycaemia of 10% and assuming a standard deviation of 12%, 14 subjects are needed to achieve a power of 80% using a paired t-test with a 0.05 two-sided significance level. To account for potential study drop-outs, a maximum of 16 subjects will be included in this trial if required.

7.2 Data analysis

The analysis of the collected data in the study will be performed with SAS v9.4. Demographic and baseline characteristics will be summarized descriptively. Continuous variables will be presented as means, standard deviation, median, minimum and maximum, for categorical data frequencies and relative frequencies will be used.

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 (i.e. 45 mg/dl for 30 minutes) at visit 3 will

be analysed using a paired t-test. Data will be adjusted for platelet activation variability captured at the euglycemic clamp visit. (Details will be outlaid in the statistical analysis plan). A two-sided p-value of <0.05 is considered to indicate statistical significance.

The secondary endpoints will be presented descriptively and changes over the time points (from baseline to the end of the hypoglycaemia phase at visit 3) will be explored using parametric or non-parametric tests for dependent data. If applicable, linear models, accounting for the repeated measurements will be considered to investigate changes from the end of the hypoglycaemic clamp (last measurement of visit 3) to one day (visit 4) and 6±1 days after the clamp (visit 5) and changes during different states of hypoglycaemia at visit 3.

8. DATA MANAGEMENT

Data Management is the responsibility of the Medical University of Graz, Department of Endocrinology and Diabetology and the study Monitor. The subject and the biological material obtained from the subject will be identified by subject number, trial site and trial identification number. Appropriate measures such as encryption or deletion will be enforced to protect the identity of human subjects in all presentations and publications as required by local/ regional and national requirements.

Appropriate measures such as encryption of data files will be used to assure confidentiality of subject data when it's transmitted over open networks.

The electronic laboratory data will be considered source data. In cases where laboratory data is transferred via non-secure electronic networks, data will be encrypted during transfer.

This study will capture and process data using an electronic Case Report Form (eCRF) which will be built by the Institute for Medical Informatics and Documentation, Medical University of Graz, which is a fully validated high quality electronic data capture system, which has a full audit trail and controlled level of access. Data and reports will be extracted from the database throughout the study to monitor progress and training will be provided to all study staff on use of the database.

9. REGULATORY, ETHICAL AND LEGAL ISSUES

9.1. Declaration of Helsinki

The investigator will ensure that this study is conducted in full conformity with the 1964 Declaration of Helsinki and all subsequent revisions.

9.2. Good Clinical Practice

The study will be conducted in accordance with the guidelines laid down by the International Conference on Harmonisation for Good Clinical Practice (ICH GCP E6 guidelines).

9.3. INDEPENDENT ETHICS COMMITTEE/ COMPETENT AUTHORITY

9.3.1. INITIAL APPROVAL

Prior to the enrolment of subjects, the Ethics Committee at the Medical University of Graz must provide written approval of the conduct of the study at named sites, the protocol and any amendments, the Patient Information Sheet and Consent Form, any other written information that will be provided to the subjects, any advertisements that will be used and details of any subject compensation.

9.3.2. APPROVAL OF AMENDMENTS

Proposed amendments to the protocol and aforementioned documents must be submitted to the Ethics Committee for approval as instructed by the Sponsor. Amendments requiring approval may be implemented only after a copy of the approval letter has been obtained.

Amendments that are intended to eliminate an apparent immediate hazard to subjects may be implemented prior to receiving Sponsor or the Ethics Committee approval. However, in this case, approval must be obtained as soon as possible after implementation.

9.4. INSURANCE

Participant insurance according to legal requirements will be contracted.

9.5. INFORMED CONSENT

The participation of a subject in this clinical trial is voluntary. The investigator or a member of the research team will approach the patient to obtain informed consent. The background of the proposed study, the procedure, the follow-up schedule and all potential benefits and risks will be carefully explained to each subject. The person obtaining the informed consent shall:

- Avoid any coercion or undue influence of subjects to participate
- Not waive or appear to waive subject's legal rights
- Use language that is non-technical and understandable to the subject
- Clarify the subject that his/her data are confidential and are encoded with a subject ID number during the investigation
- Provide plenty of time for the subject to consider his/her participation
- Include dated signatures of the subject and of the clinical investigator
- Ask whether the subject has any questions about the study

After a subject has received and read the patient information sheet and agrees to participate in the study, the informed consent form approved by the Ethics Committee must be signed by the subject prior to any study

specific tests being performed. It will also be signed by the person responsible for collecting the informed consent. The original will be kept in the subjects study research notes (source documents), a copy will be given to the subject and a copy kept in their hospital notes.

9.6. Subject Confidentiality

The investigator must ensure that the subject's privacy is maintained. On the SDF or other documents submitted to the Sponsors, subjects will be identified by a subject ID number only. Documents that are not submitted to the Sponsor (e.g., signed informed consent form) should be kept in a strictly confidential file by the investigator. The investigator shall permit direct access to subjects' records and source document for the purposes of monitoring, auditing, or inspection by the Sponsor, authorised representatives of the Sponsor, Regulatory Authorities and Ethic Committees.

9.7. End of Trial

The trial will end after the last subject has been seen for their last study visit. All patients will be reviewed by a clinician at their last study visit in order to arrange return to appropriate routine clinical care pathways.

9.8. Study Documentation and Data Storage

The investigator must retain essential documents until notified by the Sponsor, and at least for 15 years after study completion, as per the Sponsor's requirements. Subject files and other source data (including copies of protocols, SDFs, original reports of test results, correspondence, records of informed consent, and other documents pertaining to the conduct of the study) must be kept for the maximum period of time permitted by the institution. Documents should be stored in such a way that they can be accessed/data retrieved at a later date, either in paper or electronically. Consideration should be given to security and environmental risks. No study document will be destroyed without prior written agreement between the Sponsor and the investigator.

10. ADMINISTRATIVE MATTERS

10.1. Source Data

Source documents comprise the SDF and hospital records, laboratory records and correspondence. All documents will be stored safely in a confidential manner at the site. The subject will be referred to by a unique study subject number/code, their initials and date of birth on all study-specific documentations. Source data will be made available for internal and external audits or inspections by regulatory authorities to authorised personnel only.

As a minimum requirement the following data must be source data verifiable in source documentation other than the eCRF:

- Existence of subject (subject identifier, subject number and date of birth)

- Confirmation of participation in the trial (subject identification number (ID), trial ID and signed and dated informed consent forms)
- Diagnosis/ indication under investigation
- Visit dates
- Data from AEs, safety information form and pregnancy forms
- Relevant medical history, concomitant illness
- Reason for exclusion or withdrawal

10.2 LANGUAGE

SDFs will be in German. Generic names for concomitant medications should be recorded in the SDF wherever possible. All written material to be used by subjects must use vocabulary that is clearly understood, and be in the language appropriate for the study site.

10.3. DATA COLLECTION

All data collected will be documented in the source documents (SDF) and will be transferred into an eCRF. Data collection for clamp performance will be done on a paper document.

10.6. MONITORING

A monitoring plan will be developed based on risk analysis and described in detail in the monitoring manual. During the course of the trial the Monitor will visit the trial sites to ensure that the protocol is adhered to, that all issues have been recorded and to perform source data verification. The study will be monitored periodically by a Clinical Trial Monitor responsible for this study.

Initiation visit will be completed at all trial centre prior to the recruitment of participants, and will consist of review of protocol and trial documents, training with respect to trial procedures (informed consent, SAE reporting, inclusion and exclusion criteria) and review of GCP principles. Copies of the trial specific procedure manuals and related documents will be given to the investigators. The approved version of the protocol should be followed at all times, and any significant protocol deviations will be documented in a Protocol Deviation Form and any significant deviations will be recorded on a Protocol Violation Form submitted to the study coordination centre and Sponsor as soon as possible. The investigators will allow the monitors to:

- inspect the site, the facilities, device management and materials used for the trial
- meet all members of the team involved in the trial, and ensure all staff working on the trial are experienced and appropriately trained, and have access to review all of the documents relevant to the trial

- have access to the electronic case record forms and source data
- discuss with the investigator and site staff trial progress and any issues on a regular basis

The monitor will ensure that:

- A percentage of records will be inspected for confirmation of existence, eligibility based on the results of the Risk Assessment
- 100 % of consent forms will be reviewed along with all SAE's
- there is adherence to the protocol, including consistency with inclusion/exclusion criteria
- there is GCP and regulatory compliance
- trial documentation is complete and up to date (e.g. correct versions of documents being used, source data captured) and relevant documents are collected for the Trial Master File (TMF)
- the monitored eCRFs have been completed correctly and accurately, and all entries correspond to data captured in source documents

The Monitor must be given direct access to the source documents (original documents, data and records). Direct access includes permission to examine, analyse, verify and reproduce and record reports that are important to evaluation of the clinical trial.

All information dealt with during such visits will be treated as strictly confidential. At the end of the trial, close out visits will be performed by the monitor after the final participant visit has been completed and prior to database lock. During this visit the monitor will verify that all trial close out activities are completed – all queries resolved, missing data completed, monitoring completed, archiving arrangements in place, ISF completed and TMF documents collected, and end of trial notification. Each investigator will also be notified that an audit or inspection may be carried out - by the sponsor, sponsor's representatives or the host institution, or regulatory authorities - at any time, before, during or after the end of the trial. The investigator must allow the representatives of the audit or inspection team:

- to inspect the site, facilities and material used for the trial,
- to meet all members of his/her team involved in the trial,
- to have direct access to trial data and source documents, to consult all of the documents relevant to the trial. If an Investigator is informed of an impending audit or inspection, the trial coordination centre should be notified immediately.

11. DISCLUSURE OF DATA AND PUBLICATION

Information concerning the study, patent applications, processes, scientific data or other pertinent information is confidential and remains the property of the Sponsor.

12. REFERENCES

1. Jialal I, Dhindsa S. Hypoglycemia and the predisposition to cardiovascular disease: Is the pro-inflammatory-pro-coagulant diathesis a plausible explanation? *Atherosclerosis* 2016; **251**:504-506.
2. Chopra S, Kewal A. Does hypoglycemia cause cardiovascular events? *Indian J Endocrinol Metab* 2012; **16**:102-104.
3. Gogitidze Joy N, Hedrington MS, Briscoe VJ, Tate DB, Ertl AC, Davis SN. Effects of acute hypoglycemia on inflammatory and pro-atherothrombotic biomarkers in individuals with type 1 diabetes and healthy individuals. *Diabetes Care* 2010; **33**:1529-1535.
4. Dalsgaard-Nielsen J, Madsbad S, Hilsted J. Changes in platelet function, blood coagulation and fibrinolysis during insulin-induced hypoglycaemia in juvenile diabetics and normal subjects. *Thromb Haemost* 1982; **47**:254-258.
5. Trovati M, Anfossi G, Cavalot F, Vitali S, Massucco P, Mularoni E*, et al.* Studies on mechanisms involved in hypoglycemia-induced platelet activation. *Diabetes* 1986; **35**:818-825.
6. Ferrannini E, DeFronzo RA. Impact of glucose-lowering drugs on cardiovascular disease in type 2 diabetes. *Eur Heart J* 2015; **36**:2288-2296.
7. Seaquist ER, Miller ME, Bonds DE, Feinglos M, Goff DC, Jr., Peterson K*, et al.* The impact of frequent and unrecognized hypoglycemia on mortality in the ACCORD study. *Diabetes Care* 2012; **35**:409-414.
8. Aref S, Sakrana M, Hafez AA, Hamdy M. Soluble P-selectin levels in diabetes mellitus patients with coronary artery disease. *Hematology* 2005; **10**:183-187.
9. El-Mesallamy H, Hamdy N, Suwailem S, Mostafa S. Oxidative stress and platelet activation: markers of myocardial infarction in type 2 diabetes mellitus. *Angiology* 2010; **61**:14-18.
10. Gabay C. Interleukin-6 and chronic inflammation. *Arthritis Res Ther* 2006; **8 Suppl 2**:S3.
11. Kushner I. Regulation of the acute phase response by cytokines. *Perspect Biol Med* 1993; **36**:611-622.
12. Huber SA, Sakkinen P, Conze D, Hardin N, Tracy R. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 1999; **19**:2364-2367.
13. Saremi A, Anderson RJ, Luo P, Moritz TE, Schwenke DC, Allison M*, et al.* Association between IL-6 and the extent of coronary atherosclerosis in the veterans affairs diabetes trial (VADT). *Atherosclerosis* 2009; **203**:610-614.
14. Souza JR, Oliveira RT, Blotta MH, Coelho OR. Serum levels of interleukin-6 (Il-6), interleukin-18 (Il-18) and C-reactive protein (CRP) in patients with type-2 diabetes and acute coronary syndrome without ST-segment elevation. *Arq Bras Cardiol* 2008; **90**:86-90.
15. Frankel DS, Meigs JB, Massaro JM, Wilson PW, O'Donnell CJ, D'Agostino RB*, et al.* Von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease: the framingham offspring study. *Circulation* 2008; **118**:2533-2539.
16. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. *J Thromb Haemost* 2006; **4**:1186-1193.
17. Akkus MN, Polat G, Yurtdas M, Akcay B, Ercetin N, Cicek D*, et al.* Admission levels of C-reactive protein and plasminogen activator inhibitor-1 in patients with acute myocardial infarction with and without cardiogenic shock or heart failure on admission. *Int Heart J* 2009; **50**:33-45.
18. Thogersen AM, Jansson JH, Boman K, Nilsson TK, Weinehall L, Huhtasaari F*, et al.* High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. *Circulation* 1998; **98**:2241-2247.
19. Ley K, Huo Y. VCAM-1 is critical in atherosclerosis. *J Clin Invest* 2001; **107**:1209-1210.

20. O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D*, et al.* Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. Implications for the mode of progression of advanced coronary atherosclerosis. *J Clin Invest* 1993; **92**:945-951.
21. Fotis L, Agrogiannis G, Vlachos IS, Pantopoulou A, Margoni A, Kostaki M*, et al.* Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 at the early stages of atherosclerosis in a rat model. *In Vivo* 2012; **26**:243-250.
22. Lawson C, Wolf S. ICAM-1 signaling in endothelial cells. *Pharmacol Rep* 2009; **61**:22-32.