Diplomarbeit

The impact of hypoglycemia on platelet function and coagulation parameters in subjects with type 2 diabetes mellitus

A review of existing literature and data of an experimental trial (the DIAPLATE study)

eingereicht von

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Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CHD	Coronary heart disease
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CT	Closure time
DIC	Disseminated intravascular coagulation
DPP-4	Dipeptidyl peptidase-4
DVT	Deep vein thrombosis
ET-1	Endothelin-1
F II	Factor II
F V	Factor V
F VII	Factor VII
F VIII	Factor VIII
F X	Factor X
F XI	Factor XI
FFA	Free fatty acids
GLP-1	Glucagon-like peptide-1
GLUT-4	Glucose transporter type 4
HAAF	Hypoglycemia associated autonomic failure
HDL	High-density lipoprotein
ICAM-1	Intercellular adhesion molecule-1
IGT	Impaired glucose tolerance
IL-1	Interleukin-1
IL-6	Interleukin-6

IL-8	Interleukin-8
INR	International Normalized Ratio
LTA	Light transmittance aggregometry
LDL	Low-density lipoprotein
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor 1
PGI_2	Prostaglandin I_2
PKC	Protein kinase C
PT	Prothrombin time
ROS	Reactive oxygen species
SGLT-2	Sodium glucose transporter-2
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
TF	Tissue factor
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
t-PA	Tissue-type plasminogen activator
TXA_2	Thromboxane A_2
u-PA	Urokinase-type plasminogen activator
VCAM	Vascular cell adhesion molecule
VEGF	Vascular epithelial growth factor
VTE	Venous thromboembolism
vWD	Von Willebrand disease

Zusammenfassung

Zielsetzung: Die kardiovaskulären Auswirkungen chronischer Hyperglykämien bei Typ 2 Diabetes wurden bisher intensiv erforscht. Rezente Theorien schreiben aber auch der Hypoglykämie eine wichtige Rolle in der Entstehung von kardiovaskulären Schäden bei T2DM zu. So zeigte sich beispielsweise in der ACCORD Studie, dass eine intensive Blutzuckersenkung bei T2DM sogar mit gehäuftem Auftreten von kardiovaskulären Erkrankungen sowie mit einer erhöhten Gesamtmortalität verbunden ist. In dieser Arbeit wird eine Zusammenfassung bestehender Literatur in Bezug auf die Auswirkungen von Hypoglykämien auf den kardiovaskulären Metabolismus und eine experimentelle Studie (DIAPLATE) präsentiert.
Methoden: 14 PatientInnen mit T2DM (10 Männer und 4 Frauen, Alter 55 ± 7 Jahre, $HbA1_c$ 51 ± 7 mmol/mol) und Metformin Therapie ohne bestätigte kardiovaskuläre Begleiterkrankung wurden in die monozentrische, offene, einarmige, mechanistische Studie eingeschlossen.
Es wurde der Einfluss einer stufenweise erreichten Hypoglykämie (1.Plateau 63 und 2. Plateau 45 mg/dl, jeweils für 30 min) auf Parameter der Plättchenaggregation, Gerinnungsaktivierung, Endothelfunktion sowie Inflammation im Rahmen eines hyperinsulinämisch-hypoglykämischen Clamp-Experiments untersucht. Des Weiteren wurden Veränderungen in den Zielparametern einen Tag sowie eine Woche nach der Clamp-Untersuchung erhoben.
Resultate: Ein signifikanter Anstieg ließ sich in $PAC1_{pos}CD62P_{pos}$, $PAC1_{pos}CD63_{pos}$, $PAC1_{pos}CD62P_{pos}CD63_{pos}$ positiven Plättchen, PAI-1, Fibrinogen, Faktor VIII, von Willebrand Faktor und D-Dimer sowie in den Entzündungsparametern VCAM und ICAM nachweisen. Dieser Effekt ließ sich zum Teil während des Experiments aber vor allem auch bis zu einer Woche danach nachweisen. Die Ergebnisse dieser Studie stellen einen weiteren Erklärungsansatz dafür, dass Hypoglykämien mit einem ungünstigeren Outcome assoziiert sind.

Abstract

Aims: Chronic hyperglycemia in type 2 diabetes mellitus (T2DM) causing cardiovascular disease has been an essential subject of research in diabetology in the last decades. Recent theories reveal that hypoglycemic events in diabetes is involved in the development and occurrence of cardiovascular disease. For instance, in the ACCORD study, an intensive glucose lowering therapy regimen in type 2 diabetes was associated with a higher risk of cardiovascular disease and mortality. In this thesis a review of existing literature regarding the influence of hypoglycemia on the cardiovascular metabolism and data of an experimental trial, DIAPLATE, will be presented.

Methods: In the depicted study, 14 subjects with T2DM (10 men and 4 women, age 55 ± 7 years, HbA1c 51 ± 7 mmol/mol) without established cardiovascular comorbidity and treated with metformin monotherapy were included to a monocentric, open, single arm and mechanistic study. The acute and prolonged (up to one week) effects of a stepwise hyperinsulinemic-hypoglycemic clamp experiment (63 mg/dL and 45 mg/dL, for 30 minutes respectively) on markers of platelet aggregation, coagulation, endothelial function as well as inflammation were investigated.

Results: A significant increase in $PAC1_{pos}CD62P_{pos}$, $PAC1_{pos}CD63_{pos}$ and $PAC1_{pos}CD62P_{pos}CD63_{pos}$ positive platelets, PAI-1, fibrinogen, factor VIII, von Willebrand factor and D-Dimer as well as in VCAM and ICAM was observed. These effects were observed during the experiment but especially one week after the investigation. Therefore, the DIAPLATE study supports and enhances existing literature, delivering evidence for the connection between hypoglycemic events and prothromboembolic and proinflammatory processes in T2DM.

1 Introduction

Type 2 diabetes mellitus is a chronic disease, defined for its late cardiovascular complications caused by chronic hyperglycemia promoting increased atherogenesis.[1] It is extensively investigated and proven that chronic hyperglycemia leads to vascular endothelial damage and ultimately to cardiovascular events.[2] Therefore, the core aim of antidiabetic therapy is to decrease plasma glucose levels and maintain a stable glucose range.[3] However, recent studies reveal that not only hyperglycemia, but also hypoglycemia leads to vascular damage such as atherosclerosis involving impairment of structure and function of platelets provoking thromboembolic complications. Trials investigating the effects of hypoglycemia on platelet function were primarily executed in healthy individuals or subjects with Type 1 diabetes mellitus.[4–7] These studies did not systemically evaluate the correlation between the degree of hypoglycemia and platelet activation in subjects with T2DM and the sustainability of platelet activity after hypoglycemic episodes. Therefore, the crucial question of the DIAPLATE study is if and of which dimension platelets, coagulation and inflammation are affected by a single episode of a stepwise clamp-induced hypoglycemic event.

1.1 Epidemiology of type 2 diabetes

In 2012 1.5 million deaths caused by diabetic complications were recorded and 2.2 million deaths were attributed to higher blood glucose level than normal (≥ 7mmol/L) increasing the risk of cardiovascular morbidity and other diseases as susceptibility to infection and cancer. 43% of these deaths happen before the age of 70. 422 million people with the diagnosis diabetes mellitus were appraised worldwide in 2014 resulting in a prevalence of 8,5% in adults.[8] The prevalence of diabetes is rising from approximately 30 million cases back in 1985 to a predicted number of 642 millions in the year 2040. Even though the prevalence of both T1DM and T2DM is growing worldwide, the prevalence of T2DM is remarkably higher reasonably due to the rise of obesity, decreased physical activity and higher age of population.[9]

1.2 Pathophysiology

Key features in the pathophysiological pathway of T2DM include damaged insulin secretion, insulin resistance, increased hepatic glucose production, altered fat metabolism and systemic inflammation. In addition to that, obesity and genetic predisposition also contributes to diabetic development.[9] Over 80% of patients with T2DM are obese[10]. Insulin resistance is defined as the decreased function of insulin enabling glucose to reach their target cells such as myocytes, hepatocytes or adipocytes. Although insulin resistance is already happening in early stages of T2DM, glucose tolerance is scarcely affected due to beta islet cells still being capable of compensating insulin resistance with increased insulin production. If insulin resistance and hyperinsulinemia proceeds, these pancreatic cells lose their ability to maintain the high secretion rate of insulin. Impaired glucose tolerance (IGT) occurs. Furthermore, as insulin secretion decreases, and beta cell failure continues, blood glucose rises to hyperglycemic levels, aggravated by the absent suppression of glucagon secretion and therefore increased hepatic glucose production. Nevertheless, the contribution, whether insulin resistance or diminished insulin secretion dominates the development of T2DM, varies from individual to individual.[9] DeFronzo summarizes key pathophysiologic pathways in T2DM as “the ominous octet”[11]. The ominous octet includes beta cell failure and alpha-cell activation, insulin resistance in muscle and liver, accelerated lipolysis, incretin resistance in the gastrointestinal system, intensified renal glucose reabsorption and impaired suppression of appetite via cerebral insulin resistance.[11]

1.3 Diabetes and cardiovascular complications

At the time T2DM is diagnosed, often accidentally by high blood or urine glucose at routine examinations, vascular structures in patients are already affected silently by the disease.[2] Typical diabetic vascular damages can be divided in microvascular and macrovascular complications. Microvascular impairment consists of retinopathy, neuropathy and nephropathy, which may result blindness, nontraumatic limb amputation and renal failure. Coronary heart disease (CHD), peripheral artery disease and cerebrovascular disease belong to the group of macrovascular diabetic

complications.[9] Changes in vascular homeostasis caused by deficiencies in endothelium and smooth muscle cells involving a proinflammatory and prothrombotic state are essential mechanisms leading to atherothrombosis in patients with T2DM. The inequality between decreased nitric oxide (NO) and increased reactive oxygen species (ROS) plays a major role in the development of endothelial dysfunction.[2] In physiologic endothelial cells, NO is a potent vasodilator, reduces platelet aggregation, lowers leukocytes and neutrophil adhesion, acts as a radical scavenger and inhibits the synthesis of cytokines.[12] Hyperglycemia generates superoxide anions, which deactivate NO, impair endothelial structures and suppress the activity of antioxidant enzymes. The hyperglycemic state activates Protein Kinase C (PKC), which plays a crucial mediating role in damaging endothelial cells. Once activated PKC alternates the permeability of the endothelium, stimulates inflammation, angiogenesis, cell growth, apoptosis and extracellular matrix expansion and also inhibits NO production. PKC-dependent ROS also induce transcription of pro-inflammatory genes promoting chemoattractant molecules, such as monocyte vascular adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1), which leads to leukodiapedesis and ultimately the formation of foam cells in atherogenesis. Furthermore, PKC acts as a mediator in the secretion of Endothelin-1 (ET-1), in the stimulation of thromboxane A2 (TXA2) via upregulating cyclooxygenase-2 (COX-2) and also as mediator in the inhibition of prostacyclin. These 3 pathways are responsible for increased platelet aggregation and vasoconstriction. Free fatty acids (FFA) bind Toll-like-receptors (TLR) which results in activating nuclear factor kappa beta (NF$\kappa\beta$), a pivotal factor in the development of inflammation in the vascular wall by promoting the transcription of Tumor Necrosis Factor Alpha (TNFα), Interleukin-1 (IL-1), Interleukin-6 (IL-6), Endothelin, VCAM-1, ICAM-1, E-selectin, thrombomodulin, tissue factor, and vascular endothelial growth factor (VEGF). Besides, TLR also intensifies insulin resistance through downregulating GLUT4-transporters (glucose transporter 4). Intracellular stored FFA oxidate and again, ROS are produced and cause vascular inflammation, AGE-Synthesis, a decrease of Prostaglandin I2 (PGI2) synthesis and PKC activation. High triglycerids, low high-density lipoproteins, high remnant lipoproteins, increased apolipoprotein B and small and dense low density lipoproteins (LDL) result in the atherogenic fat pathway.[2]

Hyperglycemia and insulin resistance also affect coagulation as well as platelet aggregation. Insulin resistance causes to a decrease of tissue plasminogen activator (t-PA), which allows tissue factor (TF) to transform fibrinogen to fibrin via stimulation of thrombin. PAI-1, which is essential for controlling fibrinolysis, vWF (von Willebrand factor), a key player of the intrinsic coagulation pathway, and fibrinogen levels are elevated in diabetic patients, which favors procoagulant events.[13–15]

Moreover, hyperglycemia in diabetes generates a change in the homeostasis of Ca^{2+} in platelets releasing even more pro-aggregation factors as well as stimulation of glycoproteins Ib + IIb/IIIa interacting with vWF, which results in platelet form alteration, degranulation and aggregation. Subjects with diabetes display a faster coagulation response and an intensified platelet aggregation due to Ca^{2+} accumulation in platelets.[2] Hyperglycemia, insulin resistance, obesity, dyslipidemia, increased systemic inflammation and oxidative stress are held accountable for an elevated baseline platelet activation and hyperreactive thrombocyte aggregation in T2DM.[16]

1.4 Hypoglycemia and diabetes

The pathophysiological pathways above mostly describe mechanisms caused by hyperglycemia, now the aim of this present work is to highlight the influence of the opposite, hypoglycemia, on the cardiovascular system under diabetic circumstances.

1.4.1 Epidemiology of hypoglycemia and diabetes

Driving a patient into hypoglycemia is a severe concern in treating diabetes and often limits an optimal antidiabetic therapy due to its impact to the health consequences or the patient´s anxiety of following hypoglycemic episodes.[17] Glucose-lowering agents causing hypoglycemia include for example insulin, sulfonylureas or glinides.[18] In the United States 100.000 visits of emergency departments and 30.000 hospitalizations due to insulin-derived hypoglycemia were recorded in the years 2007-2011.[19]

The incidence of hypoglycemia in patients with insulin-treated T2DM is estimated to be about a third of the incidence of hypoglycemia in T1DM, in which severe hypoglycemic events occur about 3 times per year per patient.[18] The incidence of severe hypoglycemic events in insulin-treated patients with diabetes is revealed to be 0,35 episodes per patient-year in T2DM and 1,15 events per patient per year in T1DM, demonstrated by Donnelly et. al..[20] The prevalence of hypoglycemia in patients with T2DM is estimated to be around 20 times lower the prevalence of hypoglycemia in T1DM, according to Cryer et al. This prevalence is attributed to the fact that patients with T2DM are mainly treated with antidiabetic agents with low or negligible hypoglycemic potential.[21] Hence, the prevalence of hypoglycemic events in patients receiving metformin is 8,6%; with insulin 30,5% and with metformin, insulin and sulfonylureas 61,5%.[22] According to a novel german study, 460 episodes of severe hypoglycemia per 100 000 patients with T2DM were recorded in 2006 compared to an event rate of 360 per 100 000 patients in 2016. In this study a decline in the occurrence of severe hypoglycemia in T2DM over 10 years is displayed due to a decreased usage of sulfonylureas and human insulin in standard antidiabetic treatment regimens.[23] Nevertheless, avoiding hypoglycemia in diabetes treatment is still a challenging task to achieve and requires a lot of effort to prevent serious systemic complications.

1.4.2 Definition and classification of hypoglycemia

Hypoglycemia is defined as a low plasma glucose concentration which will ultimately lead to an impaired target organ function. In 1938 3 key findings were observed in diagnosing hypoglycemia, which is valid until today, known as Whipple's triad.
This triad includes: 1) signs and symptoms compatible with low blood glucose, 2) low blood glucose concentration and 3) resolution of signs and symptoms when glucose increases.[24] Blood glucose levels are highly suspicious for diabetes if fasting glucose is >126 mg/dL, 2h plasma glucose during an oral glucose tolerance test (OGTT) is >200 mg/dL or randomly assessed glucose is >200 mg/dL.[25] Glucose levels below the threshold of 70 mg/dL (3,9 mmol/L) are defined to be hypoglycemic levels.[18]

Classification	Plasma glucose	Symptoms
Severe (major)	≤70 mg/dL or improvement after therapy	Symptoms of hypoglycemia; patient requires assistance to initiate therapy
Documented symptomatic (minor)	≤70 mg/dL	Symptoms of hypoglycemia
Asymptomatic	≤70 mg/dL	No symptoms, but plasma glucose indicates hypoglycemia
Probable symptomatic	No measurement	Symptoms, but plasma glucose was not determined
Relative	>70 mg/dL	Patient reports symptoms, but plasma glucose does not indicate hypoglycemia

Table 1: 2005 Classification of hypoglycemia, derived from reference [26]

According to the guidelines provided by the American Diabetes Association classification of hypoglycemia includes 5 groups:

First, a severe hypoglycemic event which heavily affects the central nervous system is capable of triggering neuroglycopenic symptoms like seizures or coma. Therefore, the patient needs assistance to initiate glucose increasing therapy. Plasma glucose may not be measured due to the urgent character of the event but resolving symptoms after glucose restoration confirm an antecedent hypoglycemic episode.

Second, documented symptomatic hypoglycemia includes a plasma glucose level below 70 mg/dL, but the patient is aware of his symptoms and therefore able to initiate self-treatment.

Third, asymptomatic hypoglycemia consists of a glucose level of 70 mg/dL or below but doesn’t go along with corresponding symptoms.

Fourth, in the type of probable symptomatic hypoglycemia subjects show symptoms and reacts properly but plasma glucose is not assessed.

Fifth, the last type of this listing is relative hypoglycemia, in which patients experience hypoglycemic manifestations without a corresponding low level of plasma blood glucose. In fact, plasma glucose levels are above the lower threshold of 70 mg/dL. This is often seen in insulin treated patients with chronically inadequate glycemic control.[26] More easily, the American Diabetes Association has newly defined a classification for hypoglycemia by reducing the types of hypoglycemia mentioned above to 3 essential

levels of hypoglycemia.[27]

Level	Explanation
Level 1	Glucose <70 mg/dL and glucose ≥54 mg/dL
Level 2	Glucose <54 mg/dL
Level 3	A severe manifestation of hypoglycemia leading to mental or physical alteration, Patient needs assistance

Table 2: Updated 2019 classification of hypoglycemia, derived from reference[27]

In this updated table level 3 hypoglycemia is classified independently from blood glucose levels. This was concluded due to the fact that some individuals are able to act appropriately at blood glucose levels below 54 mg/dL without the need of third-party involvement. Therefore level 3 was established to separate individuals with and without requirement of third-party help.[28]

1.4.3 Symptoms of hypoglycemia

Neuroglycopenic symptoms describe the direct effect of low blood glucose in the brain and include weakness, fatigue, sensations of warmth, thought disturbances, confusion, behavioral alterations and in severe cases seizures, coma and functional brain death.[21] On the other hand, autonomic symptoms can be split up into adrenergic and cholinergic manifestations. Adrenergic symptoms are caused by norepinephrine and epinephrine secretions and include palpitations, anxiety and tremor. Hunger, paresthesia and diaphoresis are manifestations of the cholinergic system, stimulated by acetylcholine.[18] In table 2, corresponding symptoms to specific blood glucose values are displayed.

Glucose level	Consequences
83 mg/dL	Endogenous insulin secretion stops
68 mg/dL	adrenergic symptoms
58-50 mg/dL	Neuroglycopenic symptoms; Autonomic symptoms
54-43 mg/dL	Neurophysiologic dysfunction; EEG changes
50 mg/dL	Cognitive dysfunction
<27 mg/dL	Severe neuroglycopenic symptoms, reduced consciousness, seizures and coma

Table 2: Glucose range and corresponding effects and symptoms; derived from reference[18]

1.4.4 Physiology of hypoglycemic counter regulation mechanisms

In physiologic individuals, glucose levels are balanced by oral intake of glucose and hepatic or renal glucose production and glucose uptake and metabolism of target organs. If glucose levels drop, insulin secretion decreases, hepatic and renal glucose production increases, hepatic glycogenolysis is stimulated and glucose utilization of target cells is stopped. Furthermore, a rise of glucagon, produced by pancreatic alpha cells and epinephrine is observed. Low blood glucose, circulating amino acids and ß-adrenergic stimulation by epinephrine and norepinephrine all lead to an increase in glucagon production. Glucagon then promotes hepatic glucose secretion by intensifying glycogenolysis. In a hypoglycemic episode, glucagon stimulation and insulin suppression occur within minutes. This counter regulation happens without signal transmission by the central nervous system (CNS). Still, low blood glucose levels reaching the CNS activates the autonomic nervous system by stimulating the sympathoadrenal system to release adrenaline and noradrenaline. These hormones contribute to rising blood glucose by stimulating hepatic glycogenolysis, hepatic and renal gluconeogenesis as well as by suppressing insulin secretion and glucose uptake of insulin-sensitive cells. Prolonged counter regulatory mechanisms include secretion of cortisol and growth hormone, which cause an increase in glucose production and a decrease in glucose clearance.[18]

1.4.5 Glucose counter mechanisms in T2DM

Physiologic counter mechanisms to hypoglycemia include firstly a decrease in the secretion of insulin, secondly an increase of glucagon and thirdly, if the first two mechanisms are not sufficient enough to raise blood glucose, the stimulation of adrenomedullary hormones (catecholamines, cortisol). In advanced stages of T2DM pancreatic ß-cells lose their ability to produce adequate levels of insulin. In order to that, lowering glucose is dependant of exogenous insulin and endogenous responses to hypoglycemia cannot be replaced appropriately. Therefore the glucagon response does not react properly to hypoglycemia. Furthermore, a reduction of sympathoadrenal reaction is observed in patients with T2DM during hypoglycemia, caused by a diminished epinephrine release leading to deficient attempt to rise blood glucose. In recurrent hypoglycemic episodes, the threshold of stimulating epinephrin descends to even lower glucose levels. An impaired response of the sympathetic nervous system leads to the patient's malperception of hypoglycemic symptoms. Hypoglycemic unawareness is a challenging issue in detecting hypoglycemia. This lack of perception occurs, when alerting mechanisms, such as behavioral reactions or oral glucose intake, are delayed, reduced or even missing.[18] The concept of hypoglycemia-associated autonomic failure (HAAF) in diabetes describes deficiencies in glucose counter regulation and impaired hypoglycemia awareness to a prior hypoglycemic event or sleep after extensive physical activity, which causes a vicious cycle by triggering even more hypoglycemic episodes.[29] The prevalence of unawareness of hypoglycemia in insulin-treated subjects with T2DM is 10%. Moreover, hypoglycemia unawareness shows a 17 times higher risk of severe hypoglycemia than non-awareness in subjects with insulin-treated individuals with T2DM.[30] Furthermore, a link between hypoglycemia and ageing has been shown. In aged people, hypoglycemic manifestations are less intense than in younger people, which ends in unawareness of hypoglycemia. Young individuals already react in a state where blood glucose is still higher than the glucose level of cognitive changes, which grants the patient or observer time to intervene in time.[31] Risk factors favouring hypoglycemia in T2DM include increased age, deficits in diabetes knowledge, intense physical activity, nocturnal hypoglycemic episodes, malapplicated insulin or oral antihyperglycemic agents,

delayed or missed food intake, renal or liver damages and consumption of alcohol without adequate amount food intake.[18]

1.4.6 Antidiabetic drugs and hypoglycemia

Keeping the patient in a well controlled glycemic target range can be a tough task to achieve. Therefore it is essential to know which antidiabetic agent holds the potential of lowering blood glucose below physiologic ranges.

The highest hypoglycemic risk of all antidiabetic drugs is held by insulin preparations due to their potential to increase glucose uptake in target cells and therefore decrease blood glucose directly. However, the incidence of hypoglycemia varies whether the insulin is human or an insulin analogon. Basal insulin analogues (such as detemir/levemir or glargine/lantus) and short acing insulin analogues (such as Aspart/Novolog, Glulisine/Apidra or Lispro/Humalog) showed a reduction of the incidence of hypoglycemia compared to human basal insulins (NPH) or human short-acting insulins (Humulin R or Novolin R). This reduction can be attributable to the fact that insulin analogues have a more physiologic effect, lower inter- and intrapatient variability and in case of basal insulin analogues, are capable of an improved protraction and steadiness of absorption.[18]

Following insulin, sulfonylureas and their derivates also bear a hypoglycemic risk as they boost insulin secretion in pancreatic β-cells in a glucose independent manner. The rise in circulating insulin then exceeds insulin resistance, therefore blood glucose can be decreased. Sulfonylureas are now used in diabetic treatment over 60 years. Nowadays, sulfonylureas are used less frequent due to its side effects, which consist of β-cell failure, weight gain and hypoglycemic risk profile.[32] The usage of sulfonylureas is associated with an increased risk for cardiovascular disease (CVD), especially when glibenclamid/glyburid is applied.[33] Due to the latest guidelines of the American Diabetes Association, the use of sulfonylurea for the treatment of T2DM is attributed to be used as second-line therapy after metformin in countries where costs play a major role.[34]

Furthermore, meglitinides attach to the same receptor as sulfonylureas, but with a faster onset and a shorter duration.[32] Therefore, theoretically, meglitinides should

bear a minor risk compared to sulfonylureas, but studies showed a similar risk in both groups.[35]

Other antidiabetics such as metformin and thiazolidinediones (as pioglitazone) have a very low risk of hypoglycemia. Additionally incretines like glucagon like peptide-1 (GLP-1) and dipeptidyl peptidase-4 (DPP-4) inhibitors also carry a minimal rate due to their glucose-dependent effects.[18] Furthemore, the novel substance class of sodium glucose cotransporters-2 (SGLT-2) inhibitors also show no association with a higher rate of hypoglycemic side effects. SGLT-2 inhibitors improve insulin resistance and therefore diminished insulin secretion, increase glucose reabsorption in proximal renal tubuli mediated by SGLT-1 and stimulate glucagon secretion. These pathways explain the steadiness of blood glucose during the use of SGLT-2 inhibitors. Nevertheless, the combination of SGLT-2 inhibitors and sulfonylureas show a higher risk of hypoglycemia.[36]

1.5 The cardiovascular impact of hypoglycemia in diabetes

CVD as a consequence of hypoglycemia is the main cause of death of individuals with either T1DM and T2DM.[37] As described above, the micro- and macrovascular complications of hyperglycemia have been widely examinated, but the influence of hypoglycemia on the cardiovascular system in diabetes is a recent subject of interest based on the evidence of an increased cardiovascular risk and all-cause mortality.[38]

Interestingly, the cardiovascular effects of intensive glucose control are a subject of recent interest and provides an ambivalent character. While studies have shown beneficial effects of intensified glucose control[39], others were not able to reproduce these findings or even display adverse effects of intensive glucose lowering strategies by increased CVD and mortality.[40–43] In the Action To Control Cardiovascular Outcome in Diabetes (ACCORD) trial participants with advanced T2DM (known cardiovascular disease or present cardiovascular risk factors) were randomized two groups, one received intensive glucose lowering therapy with a target HbA_{1c} below 6.0% and the other received a standard glucose lowering strategy with a target HbA_{1c} of 7.0-7.9%. After 3.5 years of a 5-year follow-up phase intensified glycemic therapy already displayed an unexpected increase in total

mortality and CVD deaths. These findings even persisted although target glycated hemoglobin levels for the intensive treatment group were adjusted to 7.0-7.9% as a reaction to the first results after 3.5 years. As a consequence, an intensified glucose decreasing strategy should not be carried out in clinical practice especially when antidiabetic agents which bear hypoglycemic potential are used.[44] Furthermore, in 2019 the Veterans Affair Diabetes Trial (VADT) published their 15-year follow up results of intensive glucose control in military veterans with T2DM. In this trial, 5.6 years of an intensified glycemic regimen did not lead to a decreased risk of cardiovascular events compared to standard therapy.[45]

Sympathetic nervous system activation, dysrhythmias, increased cardiac workload, hypercoagulation, inflammatory processes, platelet hyperreactivity and endothelial dysfunction contribute to hypoglycemia-induced impact to the cardiovascular system.[4,46]

The release of catecholamines in reaction to hypoglycemia causes a remarkable change in hemodynamics as they increase not only the contractility of the myocardium, but also the stroke volume and cardiac output and therefore the cardiac workload. These mechanisms result in an aggravation of an already compromised diabetic heart. As counter regulatory mechanism to hypoglycemia, catecholamines lead to an increase of blood glucose levels, which also worsens cardiovascular stress. Especially adrenaline is capable of triggering hypokalemia resulting in cardiac arrhythmias and a lengthened QTc interval, which affects cardiac repolarization and might lead to sudden cardiac death.[46]

In previous studies, during hypoglycemia, a rise of CD40 expression on monocytes and an increase of plasma sCD40L was observed in in healthy patients and patients with T1DM, both are considered as markers of inflammation. In addition to that higher levels of C-reactive protein (CRP), IL-6, IL-8 and TNF-alpha were seen during acute hypoglycemic levels.[6,47]

Furthermore, hypercoagulation has been observed in T2DM, which contributes to thrombotic events.[48,49] Following hypoglycemia, an increase of coagulation markers and procoagulant alterations in fibrin diameter, density and clot lysis time have been demonstrated in diabetes.[13,50–52]

A rise in proatheromatous factors such as Intracellular Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM), E-Selectin and Vascular Epithelial Growth Factor (VEGF) during hypoglycemia was observed in patients with T1DM

and T2DM, which ultimately leads to endothelial and vascular dysfunction and proatherogenic mechanisms.[46] It has also been discovered, that repeated episodes of endothelial dysfunction thicken intima and media wall of the carotid and femoral arteries.[53] Moreover, hypoglycemia does not only decrease the vasodilatory capability of arterial wall smooth muscles via endogenous NO pathway but also impairs the exogenous NO-donor activating vasodilation.[6,46,52] According to the findings of Ceriello et al. rebound hyperglycemia following a phase of hypoglycemia promotes endothelial damage and prothrombotic changes even more.[54]

Another trial investigated the effects of a single hypoglycemic (50 mg/dL) episode on platelet reactivity in ten subjects with T2DM. Results show reduced platelet sensitivity to prostacyclin, which normally inhibits platelet aggregation, 24 hours after the hypoglycemic stimulus.[55]

Markers of platelet aggregation have been shown to be hyperreactive after hypoglycemia in diabetes.[7,47]

In this trial, procoagulant changes in PAI-1, fibrinogen, F VIII and vWF as well as ADP induced platelet activation and changes in (CD62/CD63) triggered by hypoglycemia are examined in patients with T2DM. Recent findings concerning these parameters are summarized below.

1.5.1 Adenosin diphosphate (ADP)

ADP was the first discovered activator for platelet aggregation. It plays a crucial role in mediating hemostasis and arterial thrombotic events. Therefore, it is known as a major key factor in the pathophysiology of coronary heart disease, myocardial infarct, unstable angina pectoris and stroke. Adenosine diphosphate leads to a platelet activation via ADP-receptors on thrombocytes and is secreted by granules via auto-stimulation in thrombocytes. Activated platelets then alter their shape, release more granules, change their calcium homeostasis and release thromboxane A_2 (TXA_2), which physiologically prevents bleeding, but can be fatal in patients with already impaired blood vessels.[56] It has been previously shown that an episode of hypoglycemia increases levels of ADP in healthy subjects and T1DM.[6,7]

1.5.2 CD62P, CD63

The glycoprotein CD62P (also known as p-selectin) is saved in granules in thrombocytes and endothelial cells. Once it is activated, it is shed to the plasma in its soluble form. P-selectin is responsible for thrombus formation and stabilization and is also held accountable to promote plaque development and therefore atherosclerosis. CD63 is a lysosomal membrane protein on the surface of platelets following release reaction. CD63 is involved in the spreading of platelets and phosphorylation of immobilized fibrinogen. CD62P and CD63 are solid markers of in vivo platelet activation. An increase of CD62P/CD63 expression is linked to Diabetes mellitus, acute coronary syndrome (ACS), peripheral vascular disease and acute cerebrovascular ischemia.[57,58]
A couple of trials noticed an increase of P-selectin during as well as at the end of a hypoglycemic clamp in healthy individuals.[6,50] One study recognized elevated levels of P-selectin after a rebound hyperglycemia following a hypoglycemic episode in subjects with T1DM.[54] Moreover, an instant rise of P-selectin was observed after a hypoglycemic clamp experiment in participants with T2DM.[47]

1.5.3 PFA-200 (platelet function analyzer 200)

The platelet function analyzer 200 is a tool to screen platelet function and therefore primary hemostasis. In clinical practice, the PFA-200 is used to screen for von Willebrand disease (VWD), platelet disorders and monitor treatment with desmopressin. It is based on the principle of the simulation of blood coagulation during dynamics of impaired microcirculation and shear stress. A whole blood sample is aspirated and passes through a microscopic aperture of a cut membrane, which contains collagen and either epinephrine or adenosin 5'-diphosphate (ADP). These platelet agonists together with high shear forces due to simulated hemodynamics lead to platelet attachment, activation and aggregation, which ultimately occlude the cleft in the membrane. This occlusion is represented as closure time (CT).[59,60]

1.5.4 Fibrinogen

Fibrinogen is a crucial key factor in hemostasis. Besides its prothrombotic effects, fibrinogen also contributes to wound healing, inflammation and angiogenesis. Endothelial wall damage, foreign surfaces and activated blood cells trigger the coagulation cascade, which ends with thrombin converting soluble fibrinogen to insoluble fibrin. This process is essential for forming a mechanically stable clot, to prevent any loss of blood. In contrast, the fibrinolytic system avoids extensive clotting and thrombus formation. Moreover, fibrinogen is held accountable for cross-linking activated platelets in primary hemostasis. The imbalance between fibrinolysis and thrombosis either favors hemorrhages or, on the other hand, contributes to obliterating blood vessels, events known as myocardial infarction, deep vein thrombosis or ischemic stroke, common complications in diabetic patients.[61] In patients with T2DM fibrinogen levels are elevated, which is well investigated in various studies.[14,62,63] Recent studies discovered a hypoglycemia-induced alteration of fibrin clot structures in patients with T2DM. An increase in fibrin fiber diameter as well as fibrin density network was observed by Chow et al. Furthermore, clot lysis time was prolonged at the end of the hypoglycemic clamp as well as the day after and 7 days after the experiment. These alterations lead to denser clots in diabetic subjects, which ultimately favors cardiovascular disease.[47]

1.5.5 Von Willebrand factor (vWF) and factor VIII (FVIII)

Von Willebrand factor as well as factor VIII are considered as major key players in the intrinsic path of hemostasis as they trigger the clot formation cascade. In already impaired blood vessels, stimulation of coagulation and platelet adhesion critically affect the occurrence of cardiovascular events. Von Willebrand factor is a large multimeric glycoprotein which is mostly produced in endothelial cells but also in granules of platelets and megacaryocytes. If vascular damage occurs, vWF enables the adhesion of circulating platelets to the site of injury and also releases FVIII which plays an essential role in clotting by activating FXI, which ultimately leads to the transformation of fibrinogen to fibrin via Thrombin. FVIII cannot survive alone in blood plasma without vWF, therefore both circulate as a molecular complex.[64–67]

Novel findings highlight a correlation between high FVIII levels and an increased risk of CHD and stroke.[68]

Moreover, recent studies reveal a strong association between thrombosis and inflammation and consider vWF as a link in this thromboinflammatory connection. As an acute phase reactant, vWF is also secreted in response to inflammation. Besides supporting clot formation and platelet adhesion, vWF also attracts leukocytes in inflamed tissue via adhesion and extravasation. Therefore, vWF ultimately contributes to the development of atherosclerotic plaques.[69]

Supporting this theory, in 1999, Jager et al. already showed increased levels vWF in subjects with diabetes.[13] However, data concerning hypoglycemia, diabetes and vWF levels remain rare. Fisher et al. discovered a significant rise in vWF after hypoglycemia in insulin-dependent patients compared to healthy individuals. In the trial of Wright et al., plasma vWF decreased in individuals with T1DM during an euglycemic clamp and did not drop during the hypoglycemic clamp.[50]

1.5.6 D-Dimer

D-Dimer is a remnant product of fibrin degradation produced by a plasmin-mediated cleavage of clots. Epitopes of these fragment molecules can be targeted by antibodies in testing assays and therefore, D-Dimer serves as a marker of hypercoagulability and is associated with thrombosis-related conditions. Its clinical importance lays in the exclusion of deep vein thrombosis (DVT) and pulmonary embolism (PE) due to its high sensitivity and low specificity. D-Dimer is also used for diagnosing disseminated intravascular coagulation (DIC) and risk stratification for venous thromboembolism (VTE).[70,71] Elevated D-Dimer is seen as a useful indicator for macrovascular complications of T2DM such as coronary heart disease or diabetic nephropathy.[72,73] Data dealing with hypoglycemia and correlating D-Dimer levels are poor investigated.

1.5.7 Prothrombin time (PT)

Prothrombin time is used in clinical practice as a daily routine to assess the extrinsic and common pathway of the coagulation cascade. Therefore, deficiencies in factors II, V, VII, X and low fibrinogen levels can be detected via PT. Prothrombin time measures the timespan between adding thromboplastin (a mixture of tissue factor, calcium and phospholipids) to a patient's blood plasma until a blood clot formation. The World Health Organization introduced a standardized format for PT, known as International Normalized Ratio (INR), in order to unify differences in preparations of thromboplastin and make results comparable.[74] In patients with T2DM significant decreases of PT have been displayed, supporting the theory of diabetic hypercoagulability.[49,51] Additionally, elevated tissue factor levels have been found in T2DM, which represents a pathologic plasma tissue factor activity.[75]

1.5.8 Plasminogen activator inhibitor-1 (PAI-1)

Plasminogen activators, such as tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA) are essential players in the defense against vascular thrombosis by stimulating fibrinolysis. Plasminogen activator inhibitor (PAI-1) is secreted in endothelium, adipose tissue and the liver, stored in thrombocytes and acts as the main suppressor of t-PA und u-PA by inhibiting the cleavage of plasminogen to plasmin. This blockage ultimately alters blood coagulation balance by restricting fibrinolysis. PAI-1 is stimulated by vascular inflammation and atherosclerosis. Hypertension, homocysteine, triglycerides and an inversed relation to high density lipoprotein (HDL) cholesterol all are displayed to be linked with increased PAI-1 levels. Therefore, PAI-1 plays an essential role in developing CVD in T2DM. Furthermore, a correlation between PAI-1 levels and insulin resistance and therefore pathogenesis of T2DM has been found.[76–78]

Additionally, in the Framingham Heart Study elevated PAI-1 levels were revealed to be a potent parameter in the prediction of cardiovascular events even after adjusting for established risk factors.[78]

Several studies observed the changes of PAI-1 in healthy subjects or individuals with T1DM. In 2010, Joy et al. measured an increase of PAI-1 after a hypoglycemic clamp in healthy individuals, but not in patients with T1DM. However, during hypoglycemia individual peak values of PAI-1 were increased in both healthy and diabetic subjects. This finding could support the theory of acute hypoglycemia

stimulating the secretion of PAI-1.[7] A couple of other studies measured a rise of PAI-1 during and at the end of hypoglycemia in healthy people or no alterations of PAI-1 levels between insulin-dependent patients and healthy controls.[6,79] Interestingly, a hyperglycemic episode following hypoglycemia may lead to an increase of PAI-1 in subjects with T1DM, according to Ceriello et al..[54] Another trial showed a decrease of PAI-1 concentrations during euglycemic circumstances in patients with T1DM but a serious suppression of this PAI-1 fall in T2DM.[52]

1.5.9 Vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM)

Vascular cell adhesion molecules (VCAMs) and intracellular adhesion molecules (ICAMs) are essential markers of inflammation as they allow leucocytes, mostly monocytes, to adhere to the luminal endothelial surface and on leukocytes per se. This process results in the migration of circulating leukocytes to the site of inflammation. It has been displayed that the level of these adhesion molecules is elevated in an inflammatory environment, mediated by IL1, TNF-α, IL-4 and IF-γ. Needless to say, this increase of adhesion molecules contributes to the formation of atherosclerotic plaques and therefore promotes cardiovascular complications. According to several studies, a rise in these adhesion molecules is associated with the occurrence of diabetes, atherosclerosis, ischemic stroke, retinopathy and ACS, although these findings are not uniform for the different adhesion molecules. [80–85]
A couple of studies concerning VCAM and ICAM levels in association with hypoglycemia in diabetic subjects have been carried out by Joy et. al. The results show an increase of these molecules during hypoglycemic clamp experiments not only in healthy individuals, but also in patients with T1DM and T2DM. In healthy individuals, VCAM and ICAM rose during the hypoglycemic phase compared to euglycemia. In T1DM, these molecules were also shown to be elevated from baseline during hypoglycemia and decreased during euglycemia. In subjects with T2DM levels of VCAMs and ICAMs were observed to be increased during hypoglycemia compared to healthy individuals.[6,7,52]

1.5.10 Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic and complex cytokine associated with a major role in inflammatory processes, such as stimulation of B-cell differentiation and attraction and activation of thymocytes, T-cells, macrophages, natural killer cells and acute phase reactants in liver cells. However, IL-6 also possesses anti-inflammatory functions as it is suggested to induce a curative effect in replication settings by triggering immunoregulatory cell activation.[86] Despite of that, other properties of IL-6 include maintenance of bone hemostasis, regulation of metabolism and various neural functions.[87] IL-6 expression is provoked by viral or bacterial stimuli, proinflammatory other cytokines like TNF-α or IL-1, oxidative stress, physical exercise and hyperglycemia. Irregular activation of IL-6 and increased duration of IL-6 exposure to tissues leads to chronic low-grade inflammation, hence insulin resistance and ultimately T2DM. [86,88,89] Moreover, increases in IL-6 have been revealed to represent markers of metabolic disorder and cardiovascular disease.[90,91] Hypoglycemia is not only associated with rises of IL-6 in healthy individuals[92], but also in subjects with T1DM and T2DM.[6,7,47,50]

2 Methods

2.1 Objectives

The primary aim of the DIAPLATE trial was to investigate platelet activation, coagulation properties, endothelial function as well as inflammation during different levels of hypoglycemia in subjects with T2DM. These stages of hypoglycemia were induced by a stepwise hyperinsulinemic, hypoglycemic clamp experiment.
Furthermore, secondary objectives consisted of platelet activation, proatherothrombotic and proinflammatory markers and recovery at one week after the clamp.

2.2 Primary Outcome

The changes in the activation of platelets were measured by light transmittance aggregometry (LTA) based on ADP activation from the baseline to the end of the hypoglycemic phase, which was set at a plasma glucose level of 45 mg/dL for 30 minutes.

Secondary outcomes were examined as follows:

- From baseline to the end of the hypoglycemic phase (visit 3)
    - Changes in platelet activation measured by activation marker, such as CD62P, CD63 and PAC1, on $CD41_{pos}$ or $CD42b_{pos}$ platelets measured by PFA-200
    - Changes in IL-6, vWF, factor VIII, PT, D-Dimer, fibrinogen, PAI-1, VCAM and ICAM

- From the end of the hypoglycemic clamp to one day after the clamp and one week after the clamp (visit 3 vs. visit 4/visit 5):

- Changes in platelet activation measured by activation marker, such as LTA, CD62P, CD63 and PAC1, on $CD41_{pos}$ or $CD42b_{pos}$ platelets measured by PFA-200
- Changes in IL-6, vWF, factor VIII, PT, D-Dimer, fibrinogen, PAI-1, VCAM and ICAM

2.3 Study description

This trial was selected to be monocentric, open, single arm, and mechanistic.

2.4 Study population

In- and exclusion criteria of the trial consisted of the following criteria:

2.4.1 Inclusion criteria

- Male or female aged 18-64 years (both inclusive) at the time of signing informed consent
- Subjects diagnosed with T2DM (according to American Diabetes Association criteria[25]) and on a stable treatment, i.e. unchanged in dosage, for a period of 90 days prior to screening with metformin as monotherapy or diet only.
- Body mass index (BMI) between 20.0 and 35.0 kg/m2 (both inclusive)
- Glycated hemoglobin A1c (HbA_{1c}) levels between 43 and 64 mmoL/moL/6.0% – 8.0% (both inclusive)

2.4.2 Exclusion criteria

- All other forms of diabetes mellitus than T2DM
- Patients using any kind of platelet aggregation inhibitors

- Therapy with any glucose lowering agents 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
- Defective awareness of hypoglycemia
- Subjects with confirmed CVD and/or previous cardiovascular incidents or prior manifestations of congestive heart failure (New York Heart Association stage II–IV)
- Subjects with cardiac arrhythmia such as atrial fibrillation, atrial flutter, atrioventricular dissociation disorders or ventricular arrhythmias
- Patients with unstable 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
- Severe hypoglycemic event requiring third party assistance within the last 6 months
- Known allergy to human insulin or dextrose solution
- Abnormal findings in hematology, biochemistry, lipids, hormones or coagulation
- Chronic liver failure with severe liver dysfunction; alanine transaminase (ALT) or aspartate aminotransferase (AST) levels > 3x upper limits of normal (ULN)
- eGFR <45 ml/min/1,73 m_2
- Any musculoskeletal disorder preventing a lying position during the time of the clamp experiments
- Regular smoking or usage of illicit substances
- Regular intake of non-steroidal anti-inflammatory drugs (NSAIDs), beta-blockers, antiarrhythmic agents or neuroleptic drugs
- Any mental disorders or psychiatric conditions which may interfere with understanding or conduction of study related procedures
- Women of child bearing potential without adequate contraceptive methods (i.e. sterilisation, intrauterine device, vasectomised partner; or medical history of hysterectomy)

Postponement of the clamp investigation:

- NSAID or metamizole treatment within 10 days before the clamp visits
- Plasma glucose >162 mg/dL in a fasting state on the day of the clamp investigation

If one of these points was met, the clamp experiment was performed with a delay of 10-15 days.

2.5 Visit procedures, measurements and assessment

2.5.1 Visit 1

At visit 1 the patient obtained the informed consent of potential participant confirmed by signature on the study informed consent form. Furthermore, blood samples were collected to check inclusion/exclusion parameters and at the same time the samples were prepared for biobanking. Additionally, demographic information, medical history and medication history as well as vital signs, ECG, body weight and height, BMI and physical examination were recorded.

2.5.2 Visit 2: Hyperinsulinemic-euglycemic clamp

At this visit the hyperinsulinemic – euglycemic clamp was performed after a nightly 10-hour fasting period and 2-10 days after V1. In case of antidiabetic treatment with Metformin, the morning dosage was not applied. Vital signs were recorded and possible adverse events documented. The patients were advised to stay the whole duration of the clamp procedure in the study bed. Approximately at 7 a.m. two intravenous catheters were inserted, one positioned in an antecubital vein for sampling of serial measurements of plasma glucose (roughly every 5 minutes, depending on the clamp procedure and patient safety). Moreover, the arm was placed in a heated environment enabling proper collection of arterialized venous blood. On the contralateral arm, another intravenous cannula was installed to allow controlled changing infusion of glucose 20% and infusion of human soluble insulin (40 IU ActrapidR, 100 IU/ml in 99,6 mL saline). Both cannulas were kept open by a slow continuous flow of 0,9% saline. After the baseline blood samples for blood

glucose, insulin and platelet activity had been taken (timepoint 0 min), the hyperinsulinemic – euglycemic clamp was initiated. To achieve a plasma glucose level of 100 mg/dL ± 10%, a variable infusion of glucose 20% or infusion of human soluble insulin was induced. The infusion of insulin was stopped once glucose was applicated and vice versa. As the glucose target level of 100 mg/dL was reached, an insulin infusion of human soluble insulin (2.5 mU/kg/min) was applied for 120 minutes raising and maintaining serum insulin concentrations continuously. In order to clamp plasma glucose at 100 mg/dL, a variable infusion of glucose 20% was induced. Every 5 minutes, blood samples for measuring plasma glucose concentrations were collected. After the time periods of 0,60,90 and 120 minutes following clamp initiation, blood samples to investigate platelet activation and coagulation were gathered. Serum potassium levels were measured at the start, during and after the clamp and eventually corrected if significant and potentially harmful changes were detected. At the timepoint of 120 minutes, the experiment ended.

After analysis of the results the trial was enhanced and another euglycemic clamp experiment in 6 subjects was carried out in order to investigate whether the observed results were more insulin and not hypoglycemia related.

2.5.3 Visit 3: Hyperinsulinemic-hypoglycemic clamp

At this visit a hyperinsulinemic-hypoglycemic clamp was performed after an overnight 10 hours fasting period 10-24 days after V2. Preparations and placements of intravenous catheters were executed in the same way as in the hyperinsulinemic-euglycemic clamp experiment, described above. After the baseline blood samples for blood glucose, insulin and platelet activity had been taken (timepoint 0 min), the hyperinsulinemic-hypoglycemic clamp was initiated. To maintain a plasma glucose target of 100 mg/dL ± 10% for 30 minutes, a variable infusion of glucose 20% or infusion of human soluble insulin was induced. Once this specific plasma glucose target was reached, high constant insulin infusion (2.5 mU/kg/min) was started. Following 30 minutes of the plateau at 100 mg/dL the glucose infusion was interrupted, enabling a fall of plasma glucose to 63 mg/dL. By reinfusing glucose, the concentration was maintained at 63 mg/dL for a plateau of 30 minutes. Thereafter glucose infusion was stopped again allowing the concentration drop to

the nadir of 45 mg/dL or a higher nadir in case 45 mg/dL could not be reached or the symptoms of hypoglycemia were unacceptable. Following the first 15 minutes on the 30-minute plateau at the nadir, high insulin infusion was terminated. After 25 minutes on the nadir plateau the glucose infusion was cut off, if possible, in order to recover spontaneously from hypoglycemia. Plasma glucose was not allowed to drop lower than 40 mg/dL. If the plasma glucose hadn't been recovered up to 72 mg/dL after termination of high insulin, a constant high glucose infusion (5.5 mg/kg/min) was initiated to raise plasma glucose rapidly. Once the level of 72 mg/dL was reached, assessments were performed, and blood samples were taken. Thereafter the plasma glucose again was elevated to 100 mg/dl for safety reasons and the clamp experiment terminated. At the end of every plateau phase during the clamp and as well after recovery of hypoglycemia platelets blood samples were collected in order to investigate platelet activity, coagulation, endothelium integrity and inflammation in different stages of hypoglycemia. (Fig.1)

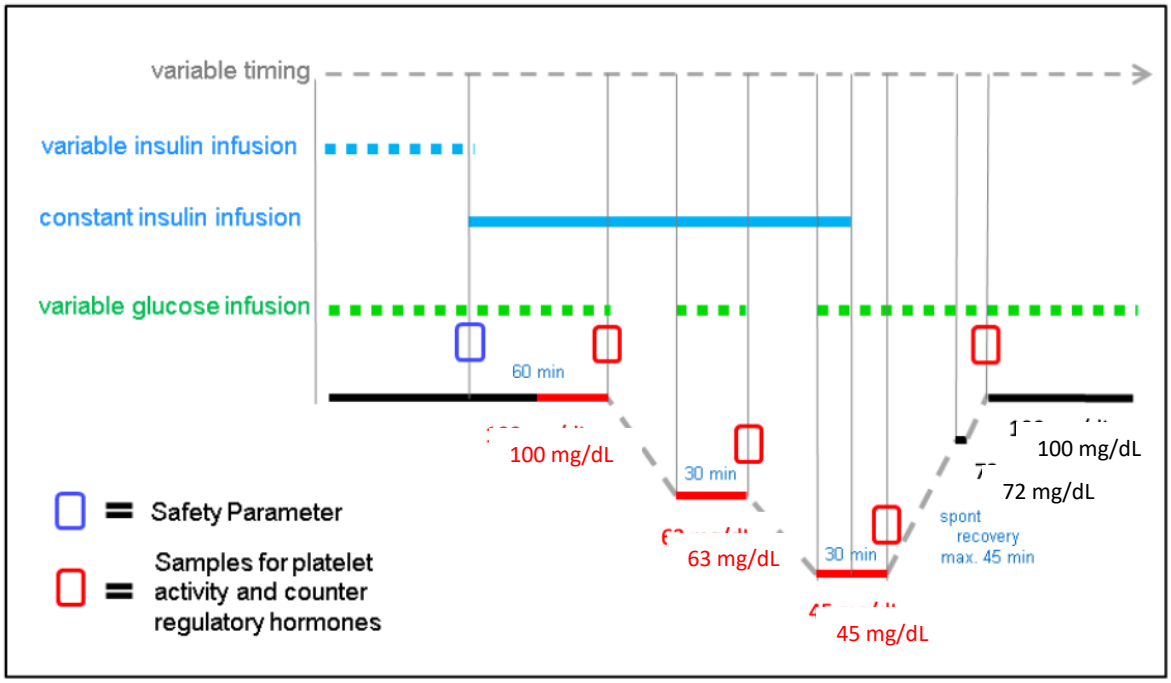

Figure 1: Hyperinsulinemic-hypoglycemic clamp with timepoints of sample collection

2.5.4 Visit 4 – Follow-up visit

Visit 4 was performed one day after the clamp investigation at V3. Vital signs were recorded, blood samples were collected and also prepared for biobank and any adverse event documented.

2.5.5 Visit 5 – Follow-up visit (close-out visit)

Visit 5 took place 6 ± 1 days after V4. Vital signs were recorded again, a physical examination was performed and any adverse events documented. In addition to the same blood measurements at V4, blood count, blood chemistry and body weight were gathered.

An image graphically summarizing the study procedure is shown in Figure 2.

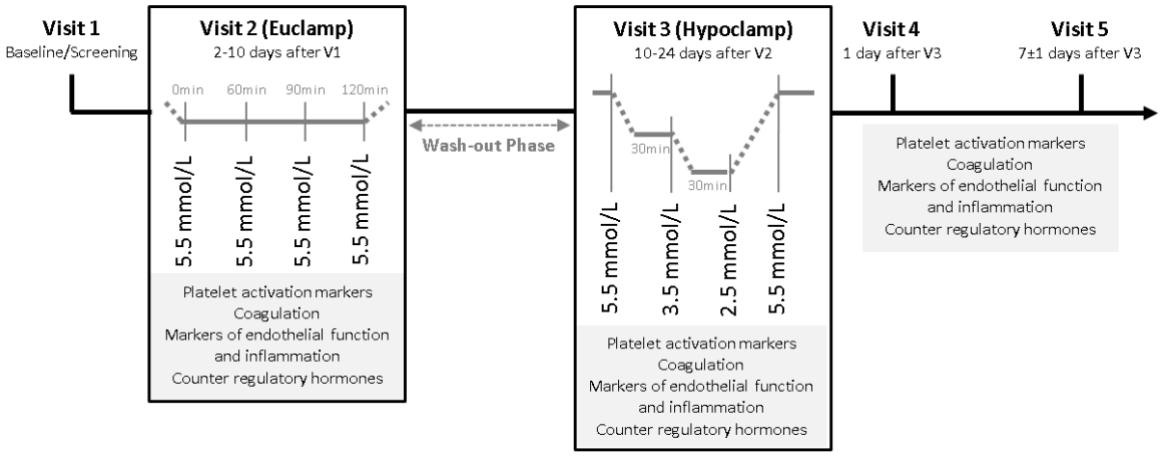

Figure 2: Study procedure (5,5 mmol/L = 100 mg/dL; 3,5 mmol/L = 63 mg/dL; 2,5mmol/L = 45 mg/dL)[93]

2.5.6 Addendum: Euglycemic clamp with follow-up

Someone might consider that the effects on the platelet and coagulation parameters are rather present due to the insulin application than the antecedent hypoglycemic event. Therefore, after analysis of the results the trial was enhanced by another euglycemic clamp experiment with a follow up in 6 subjects willing to participate again. This time a follow-up examination on day one and day 7 after the euglycemic

clamp was performed in order to investigate whether the observed are hypoglycemia related and not cause of exogenous insulin administration.

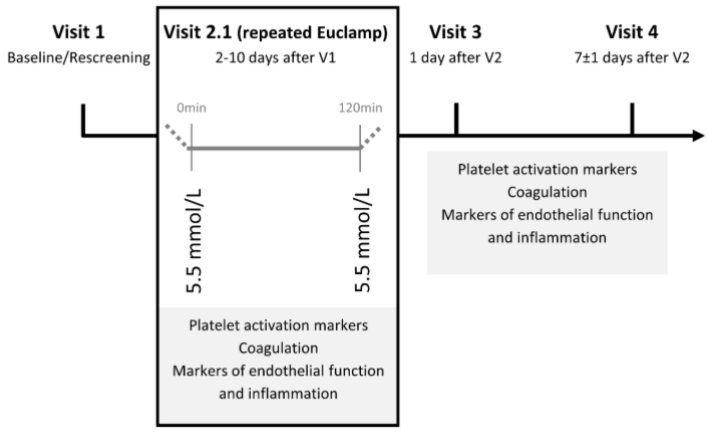

Figure 3: Study procedure euglycemic clamp follow-up (5,5 mmol/L = 100 mg/dL) [93]

2.6 Description of procedures and measurements

2.6.1 Medical history and physical examination

Determination of medical history was performed at the screening visit to record acute illnesses, health disorders and concomitant medication. Physical examination was performed at the screening visit 1, visit 3 as well as at the closing visit according to local procedure. The physician raised the physical examinations with the focus on cardiac, lung and abdominal examination. Any abnormal, clinically significant finding, adverse event or change in medication was recorded.

2.6.2 Electrocardiogram

An electrocardiogram was performed at the screening visit and continuously during the clamp in V3. The ECG was interpreted, signed and dated by the investigator.

2.6.3 Vital signs

Heart rate and blood pressure were recorded at all visits after resting for five minutes in a sitting position. Systolic and diastolic blood pressure were measured in a sitting position at all visits.

2.6.4 Body weight and height

The body weight was raised at the screening and visit 2, as well as at the closing visit. The same and calibrated pair of scales were used throughout the trial.
Height was recorded at the screening visit. BMI (body mass index) was calculated as follows: BMI = weight (kg)/height$_2$(m).

2.6.5 Routine Biochemistry

Blood samples were obtained at all visits in a fasting state and processed by the local laboratory using standard methods for routine tests. Participants were allowed to take their morning medication and in case of metformin therapy, the morning dose was not applied. The subjects brought their regular medication along to their study visits to be further advised by the doctor.

2.6.6 Blood sample collection and plasma extraction for biobanking

Blood samples were gathered via venous puncture into 8 ml serum and 6 ml EDTA vacutainers and centrifuged within 30 min of collection. Blood plasma was transferred into Eppendorf tubes and stored at -80 °C locally.

2.6.7 Parameters of special interest

To examine parameters of platelet aggregation, coagulation, endothelial function and inflammation, venous blood was drawn 5 times during both clamp investigations as well as one day and one week after the clamp (visits 2,3,4 and 5). Time points of sampling collection during the clamp are shown above in Figure 1. Additionally, blood samples were kept in sodium citrate and at ambient temperature. After collecting, the samples were instantly delivered to the Clinical Institute for Medical

and Chemical Laboratory Diagnostics (CIMCL) at the university hospital of Graz and the Center for
Biomarker Research in Medicine (CBmed).
In the following table performed methods of measurements are listed.

Parameter	Reagent	Method of measurement
Number of platelets		Sysmex XE-2100TM Automated Hematology System (Sysmex Austria GmbH, Vienna, Austria)
Prothrombin time	Thromborel® S reagent (Siemens Healthcare Diagnostics GmbH, Vienna, Austria)	Behring BCS XP Analyzer (Siemens Healthcare Diagnostics GmbH, Vienna, Austria)
Fibrinogen	Multifibren® U reagent (Siemens Healthcare Diagnostics GmbH, Vienna, Austria)	Behring BCS XP Analyzer, (Siemens Healthcare Diagnostics GmbH, Vienna, Austria)
D-Dimer	Innovance® D-Dimer (Siemens Austria GmbH, Vienna, Austria)	Coagulation analyzer BCS XP (Siemens Austria, Vienna)
Von Willebrand factor antigen	Sta Liatest® VWF:AG Stago	Coagulation analyzer BCS XP (Siemens Austria, Vienna)
Factor VIII	Coagulation Factor VIII Deficient Plasma, Siemens	Coagulation analyzer BCS XP (Siemens Austria, Vienna)
Light transmittance aggregometry (LTA)	• Collagen (Chronolog Corporation, Havertown, Pennsylvania, USA)	Chronolog 700 Lumi-Aggregometer (Chronolog Corp, Havertown, PA)

	• ADP (Sigma-Aldrich Handels GmbH, Vienna, Austria) • arachidonic acid (Chronolog Corporation, Havertown, Pennsylvania, USA) • thrombin receptor-activated peptide-6 (TRAP-6) (Bachem Distribution Services GmbH, Weil/Rhein, Germany)	
PFA-200	Collagen/ADP and Collagen/epinephrine	Platelet Function Analyser-200® (Siemens Healthineers, Marburg)

Platelet function and activation (flow cytometry)	• half of the aliquots were treated with ADP sodium salt (Sigma-Aldrich, Germany)
	• Antibodies to stain samples:
	  • anti-CD41 PE (Thermo Fisher Scientific, USA)
	  • anti-CD42b PE-CF594 (BD Biosciences, USA)
	  • anti-CD62P APC (Thermo Fisher Scientific, USA)
	  • anti-CD63 PE-Cyanine 7 (Thermo Fisher Scientific, USA)
		BD LSRFortessa flow cytometer (Becton Dickinson, US)
		Standadization and calibration: cytometer tracking and performance beads (CS&T IVD beads, BD Biosciences, USA) and BDOneFlowTM setup beads (BD Biosciences, USA)

	• anti-PAC-1 FITC (BD Bioscience, USA)	
IL-6		Electroluminescence immunoassay (ECLIA)
PAI-1		ELISA (Biomedica, Vienna, Austria)
VCAM and ICAM		ELISA (Biomedica, Vienna, Austria)

Table 3: Methods of collecting parameters of interest

2.7 Statistical analyses

2.7.1 Sample size and power considerations

One study already discovered a value of 70 ± 12% of LTA based on ADP stimulation in T2DM.[94] In order to achieve a clinically significant difference of 10% in LTA compared to baseline and taking a standard deviation of 12% into consideration, 14 participants were required to reach a power of 80% including a paired t-test with a 0.05 two-sided significance level. One subject was excluded from the study and a substitute subject was included to maintain a power value of 80%.

2.7.2 Data analysis

Using SAS® software version 9.4 (SAS Institute, Cary, NC, USA) the statistical analysis of the collected data was executed. For baseline and demographic values, means ± standard deviation, median, minimum and maximum were used to express quantitative variables and frequencies and percentages were used to represent categorical variables. The analysis of the primary endpoint was done via a paired t-test on baseline values compared to data at the end of the hypoglycemic nadir measured by LTA. Linear models for multiple assessments over time were

performed for primary and secondary parameters of interest to scrutinize alterations in these values comparing baseline to timepoints 60, 90 and 120 minutes in the euglycemic clamp as well as comparing baseline to the glucose level of 63 mg/dL, 45 mg/dL, glucose normalization, one day and one week (7 days) after the clamp experiment. Descriptive statistics (i.e. mean ± standard error) for analyzed data over time are demonstrated as graphics below. In order to achieve statistical significance, a two-sided p-value of <0.05 was determined. Taking possible occurring errors of multiple testing into consideration, p-values <0.01 (OR <0.001) were defined to realize statistical significance in secondary outcome parameters.

3 RESULTS

Overall 19 subjects were selected for the study, of which 4 did not match inclusion criteria. One participant was not included due to concomitant steroid replacement therapy and three were excluded because of inadequate levels of HbA_{1c}. Following visit 3, another participant dropped out and was not included in the concluding evaluation due to a delayed recognition of irregularly insulin therapy.
In total, the trial involved 14 subjects with T2DM. In table 4, baseline characteristics and demographic data is presented. No adverse event was recorded throughout all of the clamp experiments.

Number of subjects	14
Age, years	55 ± 7
Male, n (%)	10 (71.4)
Female, n (%)	4 (28,6)
Body mass index (kg/m²)	28.9 ± 3.3
Weight (kg)	86.4 ± 15.1
Systolic blood pressure (mmHg)	133 ± 13
Diastolic blood pressure (mmHg)	83 ± 8

Fasting plasma glucose (mg/dL)	129 ± 17
Triglycerides (mg/dL)	179 ± 118
Cholesterol (mg/dL)	202 ± 46
HDL-C (mg/dL)	46 ± 14
LDL-C (mg/dL)	121 ± 39
Diabetes duration (years)	5 ± 4
Daily metformin dose (mg)	1336 ± 599
HbA_{1c}, mmol/mol (%)	51 ± 7 (6.8 ± 2.8)
ACE-Inhibitors, n (%)	4 (28.6)
AT-II receptor antagonists n (%)	5 (35.7)
Calcium antagonists n (%)	3 (21.4)
Diuretics n (%)	1 (7.1)
Statins n (%)	4 (28.6)

Table 4: Baseline Characteristics: Data are n (%) or mean ± SD if not otherwise indicated; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; ACE, angiotensin-converting-enzyme; AT: angiotensin [93]

3.1 Blood glucose

Baseline glucose levels were 128 ± 18 mg/dL prior to the euglycemic clamp and 128 ± 21 mg/dL before initiation of the hypoglycemic investigation. All subjects reached the predetermined glucose plateaus and glucose levels were maintained throughout the defined time period during both clamp experiments. Details concerning clamp quality are indicated in the following table.

Pat.ID	Before Clamp	Start 100 mg/dL	Stop 100 mg/dL	Start 63 mg/dL	Stop 63 mg/dL	Start 45mg/dL	Stop 45 mg/dL	Re-covery
2	158,92	98,92	103,96	63,06	61,98	43,96	47,93	107,93
3	96,94	90,99	92,97	61,98	61,08	45,05	50,09	101,98
4	101,98	92,07	103,06	60,00	63,06	43,06	49,01	103,06
5	103,96	92,97	98,02	63,06	61,98	45,05	47,03	110,09
6	123,06	96,04	96,94	61,98	61,98	45,05	41,98	110,99

7	127,03	100,00	92,07	63,06	61,98	41,08	47,93	101,08
8	152,97	100,00	96,04	61,98	61,08	45,05	47,03	107,93
9	123,06	103,96	101,08	58,92	63,06	41,98	45,95	101,08
10	150,09	107,03	94,95	61,98	63,96	47,93	50,09	105,04
11	132,97	101,08	109,01	63,06	65,04	45,05	40,00	110,09
12	160,00	103,96	100,00	61,98	63,06	45,05	47,03	96,04
13	125,04	90,09	103,06	61,08	61,08	41,98	47,03	105,04
14	134,05	98,02	96,94	67,93	63,06	47,93	45,95	120,00
15	105,95	90,09	100,00	63,06	61,98	45,05	47,93	107,93
MEAN	**128,29**	**97,52**	**99,15**	**62,37**	**62,46**	**44,52**	**46,78**	**106,31**
SD	**21,33**	**5,59**	**4,66**	**2,02**	**1,15**	**2,01**	**2,79**	**5,81**
MIN	**96,94**	**90,09**	**92,07**	**58,92**	**61,08**	**41,08**	**40,00**	**96,04**
MAX	**160,00**	**107,03**	**109,01**	**67,93**	**65,04**	**47,93**	**50,09**	**120,00**

Table 5: Glucose values during hyperinsulinemic-hypoglycemic clamp experiment at visit 3 (mg/dL)

3.2 Platelet activation markers

No alterations in thrombocyte activation determined by LTA based on ADP activation were measured throughout hypoglycemia, recovery and follow up at day 1 and 7 as well as during the euglycemic clamp compared to baseline levels. (Fig.4 A)

A significant increase $PAC1_{pos}CD62P_{pos}$, $PAC1_{pos}CD63_{pos}$ and $PAC1_{pos}CD62P_{pos}CD63_{pos}$ positive platelets assessed via flow cytometry was found including a persistent elevation on day 1 as well as day 7 after the hypoglycemic clamp investigation. (Fig.4 B-E)

A significant decrease in closure time via PFA-200 was observed at the glucose plateau of 45 mg/dL including a sustained effect 24 hours later. (Fig. 4F)

Neither in the euglycemic clamp nor in the follow-up supplemental examination significant changes were observed in platelet activation markers. (Fig.5 A-F, Fig.10 A-I)

Fig. 4A

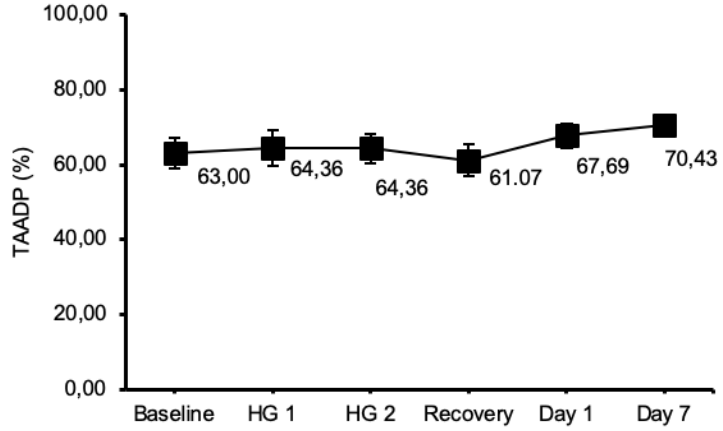

Fig. 4B

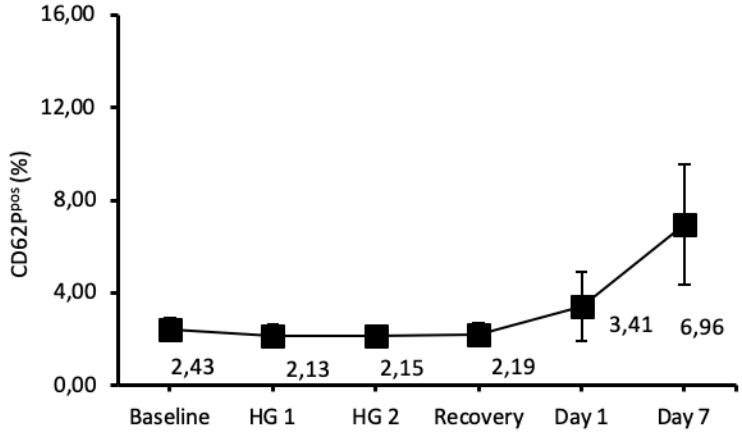

Fig. 4C

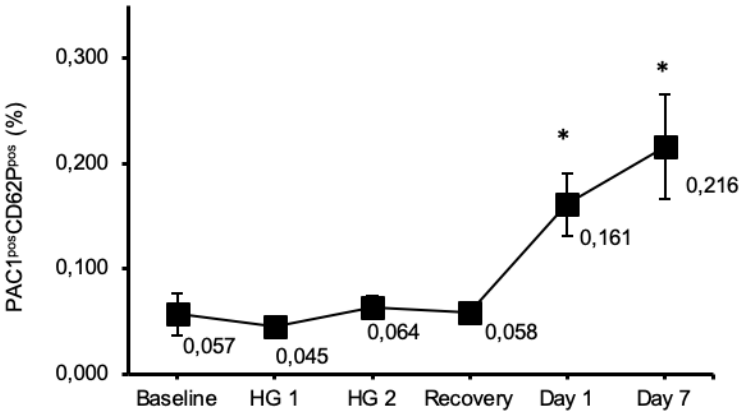

Figure 4: *Effects of hypoglycemia on platelet function (1): (A) platelet activation adenosine diphosphate (TAADP), (B) $CD62P_{pos}$ and (C) $PAC1_{pos}CD62P_{pos}$*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL)[93]

Fig. 4D

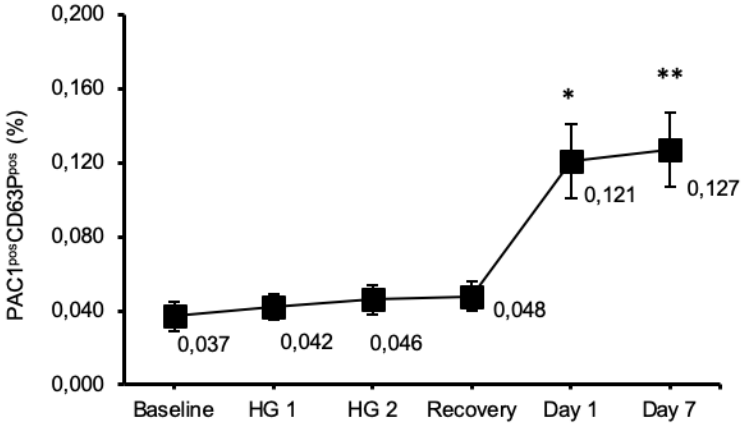

Fig. 4E

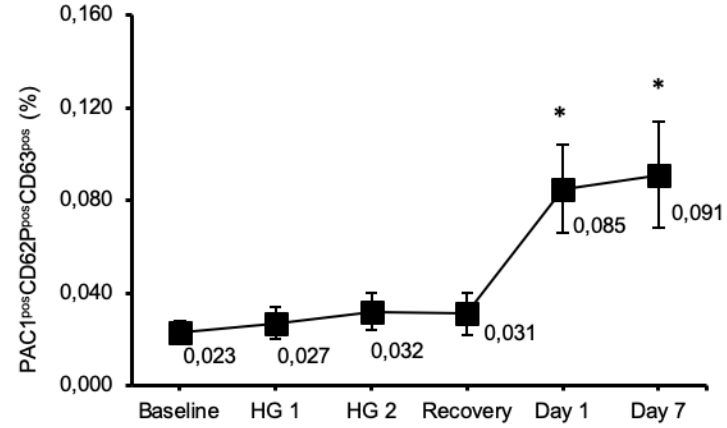

Fig.4F

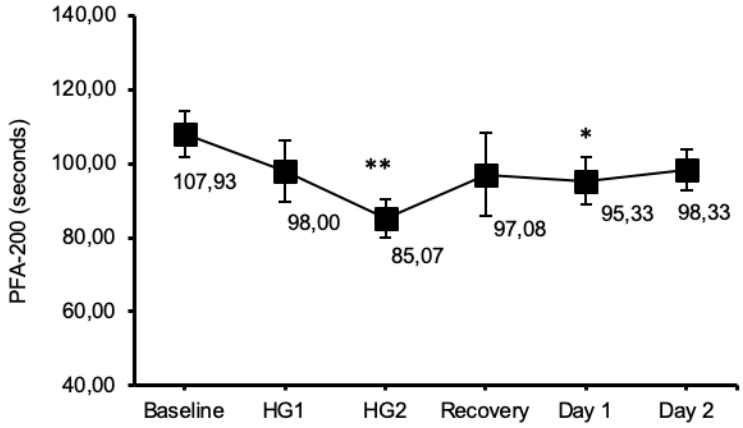

Figure 4: *Effects of hypoglycemia on platelet function (2): (D) PAC1$_{pos}$CD63P$_{pos}$, (E) PAC1$_{pos}$CD62P$_{pos}$CD63$_{pos}$ and (F) PFA-200*

** p<0.01; ** p<0,001,Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL)*[93]

Fig. 5A

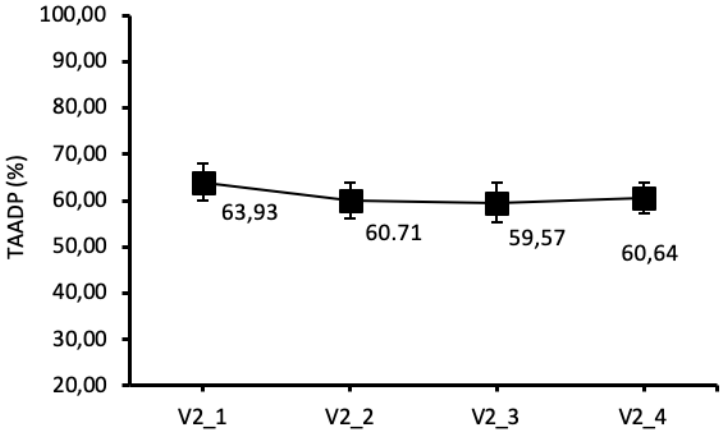

Fig. 5B

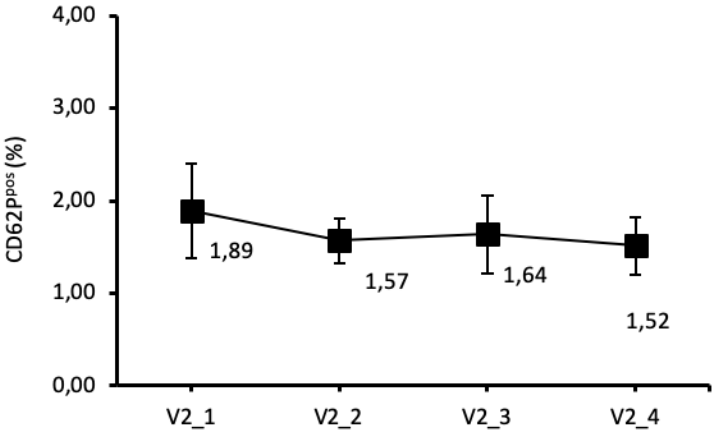

Fig. 5C

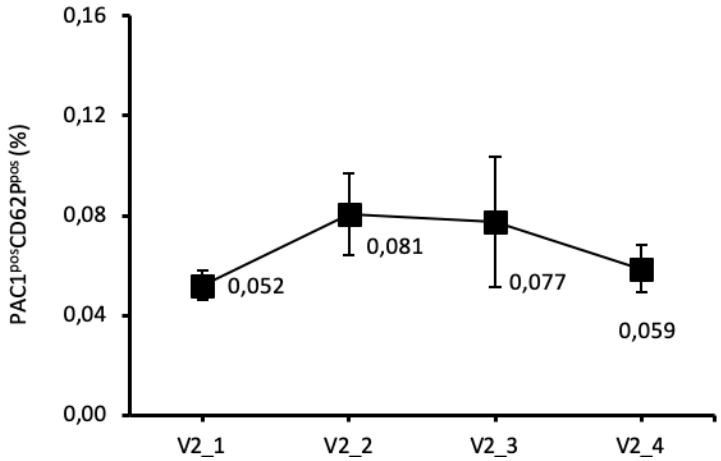

Figure 5: *Effect of the hyperinsulinemic-euglycemic clamp on platelet function (1): (A) platelet activation adenosine diphosphate (TAADP), (B) $CD62P_{pos}$ and (C) $PAC1_{pos}CD62P_{pos}$*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 60 min; V2_3: glucose 100 mg/dL at 90 min; V2_4: glucose 100 mg/dL at 120 min[93]

Fig. 5D

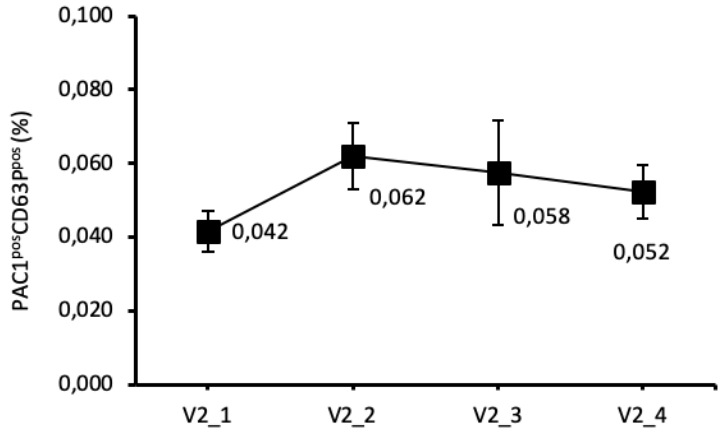

Fig. 5E

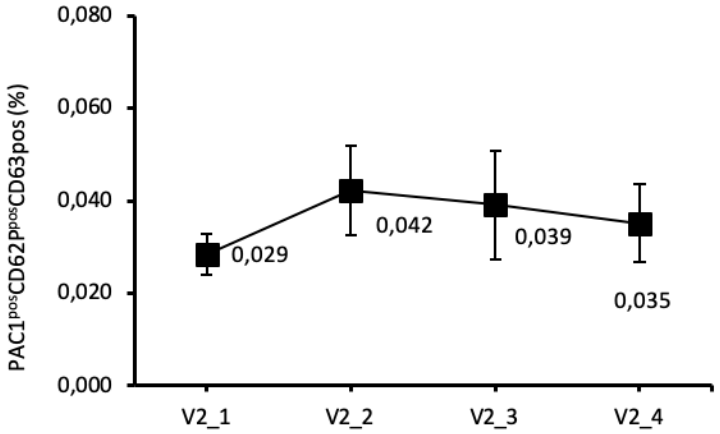

Fig. 5F

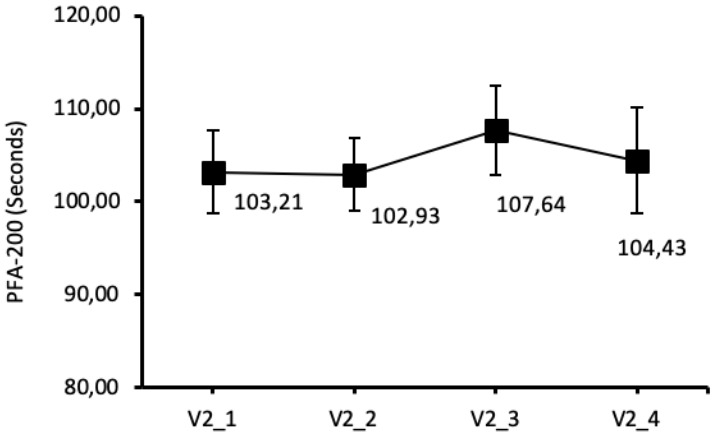

Figure 5: *Effect of the hyperinsulinemic-euglycemic clamp on platelet function (2): (D) PAC1$_{pos}$CD63P$_{pos}$, (E) PAC1$_{pos}$CD62P$_{pos}$CD63$_{pos}$ and (F) PFA-200*

** p<0.01; ** p<0,001; Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 60 min; V2_3: glucose 100 mg/dL at 90 min; V2_4: glucose 100 mg/dL at 120 min*[93]

3.3 Parameters of coagulation

Prothrombin time (PT) was significantly elevated one day after the hypoglycemic clamp with no sustained elevation 7 days after the hypoglycemic clamp experiment. (Fig.6 A)

Examined coagulation parameters (PAI-1, Fibrinogen and factor VIII) showed a significant rise not only 24 hours, but also a maintained elevation up to one week after the hypoglycemic experiment. However, values for D-Dimer and von Willebrand factor are represented by an increase one day after the hypoglycemic clamp, which did not last up to day 7.(Fig.6 B-F)

While some coagulation parameters (D-Dimer, von Willebrand factor activity, factor VIII) displayed a steady character throughout euglycemia, other indicators (fibrinogen, PAI-1) showed a non-significant numerical decline. (Fig.7 D-F) No significant changes were detected in the hyperinsulinemic-euglycemic follow-up visits. (Fig.10 A-I)

Fig. 6A

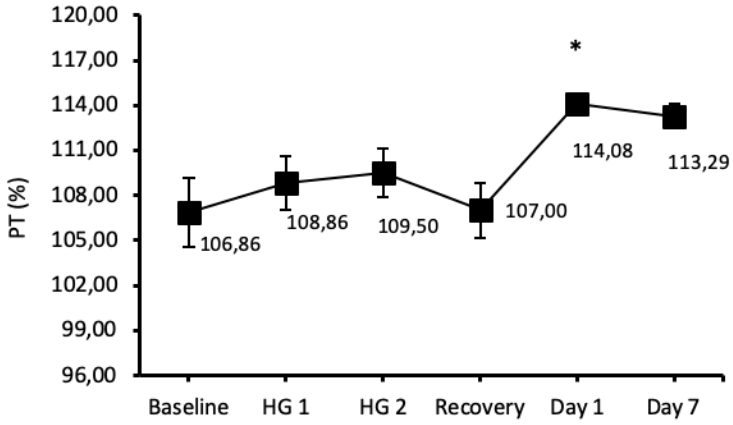

Fig. 6B

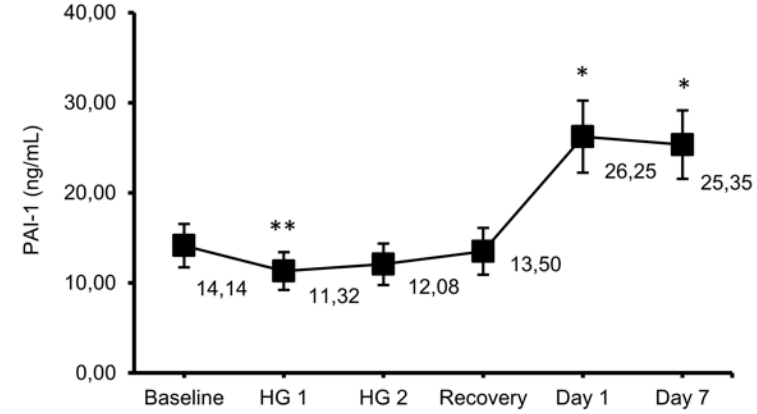

Fig. 6C

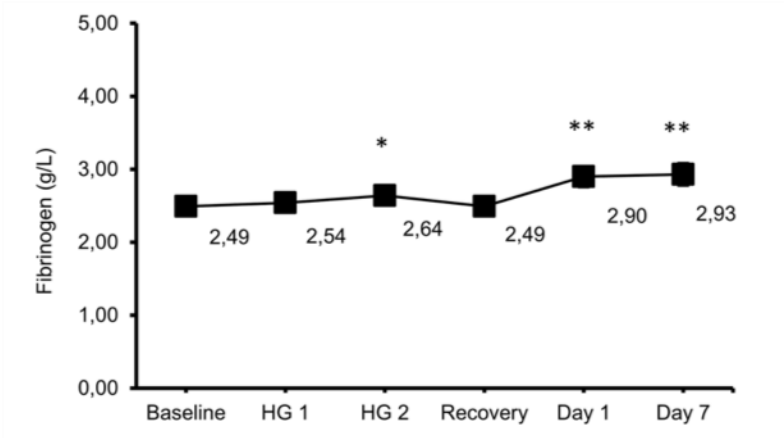

Figure 6: *Effects of hypoglycemia on coagulation parameters (1): (A) PT, (B) PAI-1, (C) Fibrinogen*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL).[93]

Fig. 6D

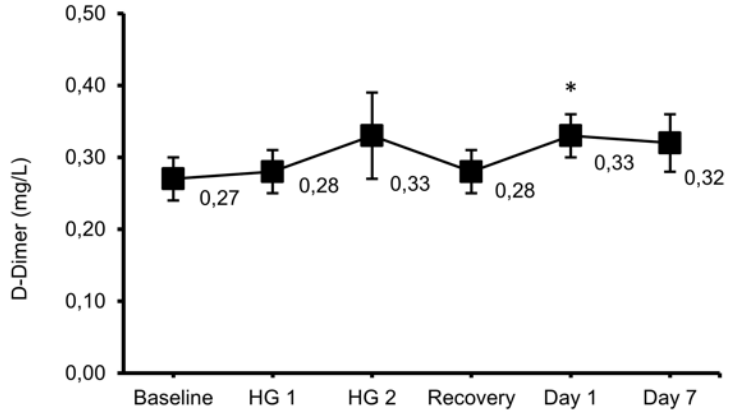

Fig. 6E

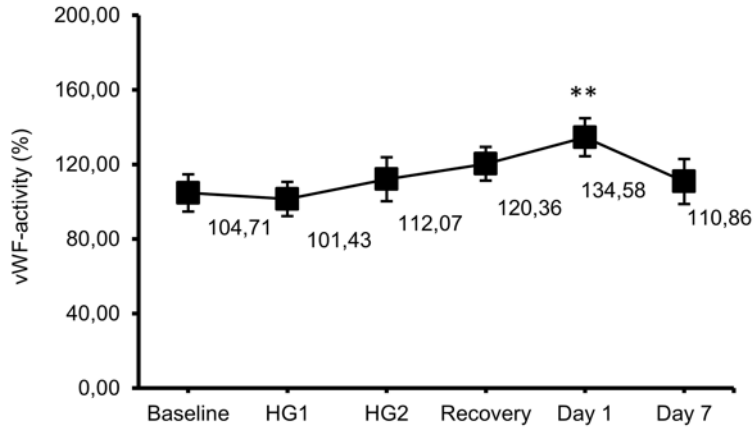

Fig. 6F

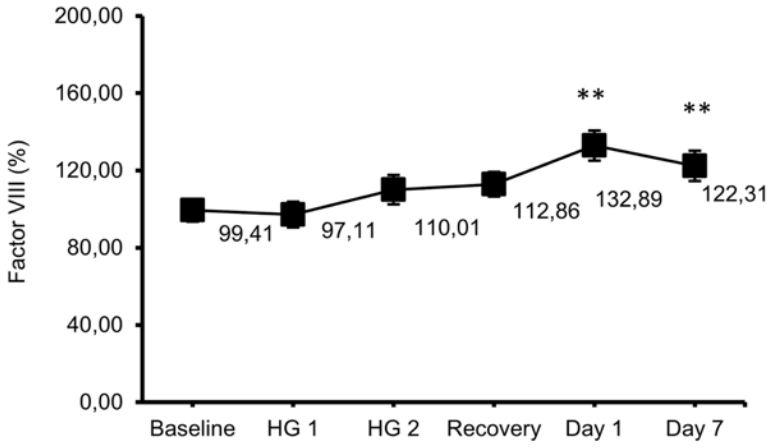

***Figure 6**: Effects of hypoglycemia on coagulation parameters (2): (D) D-Dimer, (E) vWF, (F) Factor VIII*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL)[93]

Fig. 7A

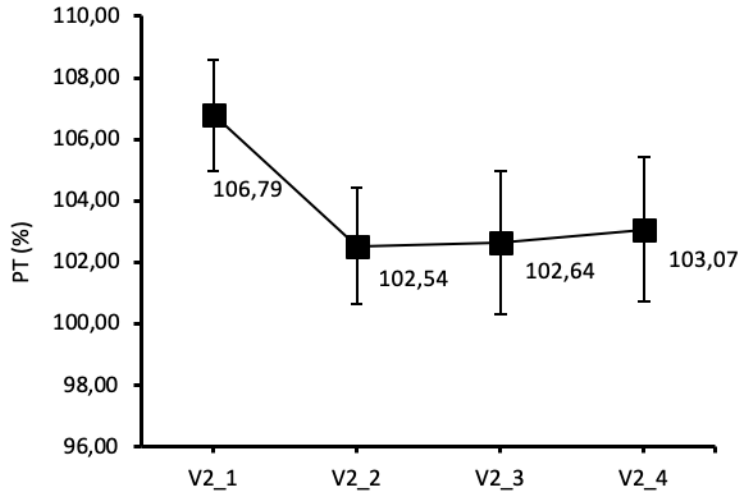

Fig. 7B

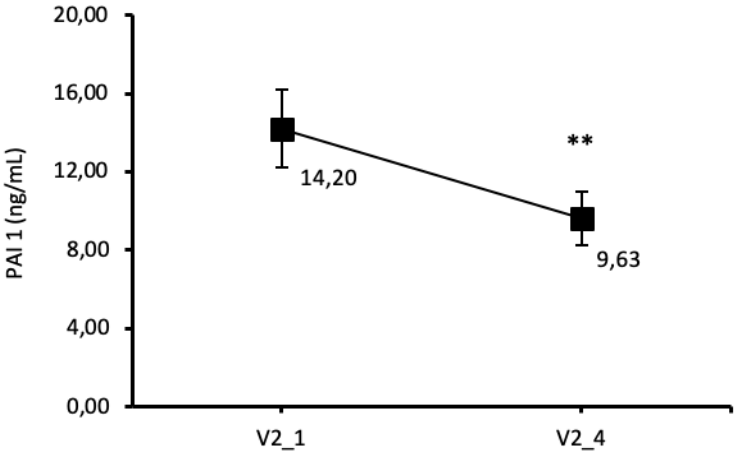

Fig. 7C

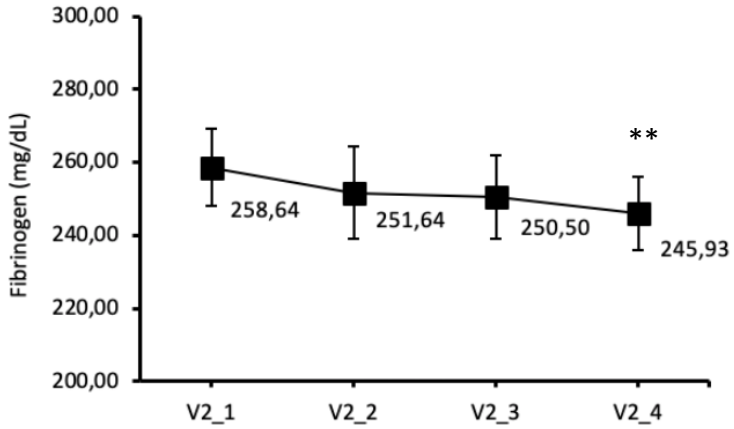

***Figure 7**: Effect of the hyperinsulinemic-euglycemic clamp on coagulation parameters (1): (A) PT, (B) PAI-1 and (C) Fibrinogen*

** p<0.01; ** p<0,001; Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 60 min; V2_3: glucose 100 mg/dL at 90 min; V2_4: glucose 100 mg/dL at 120 min*[93]

Fig. 7D

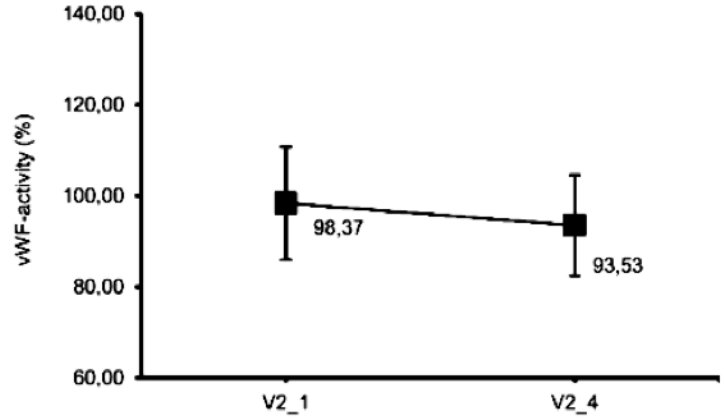

Fig. 7E

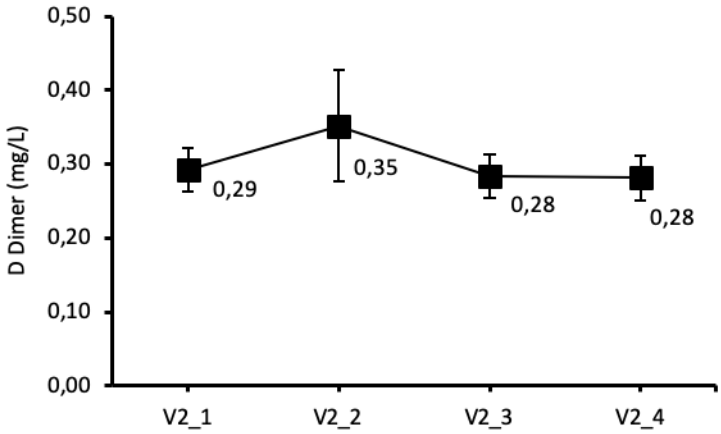

Fig. 7F

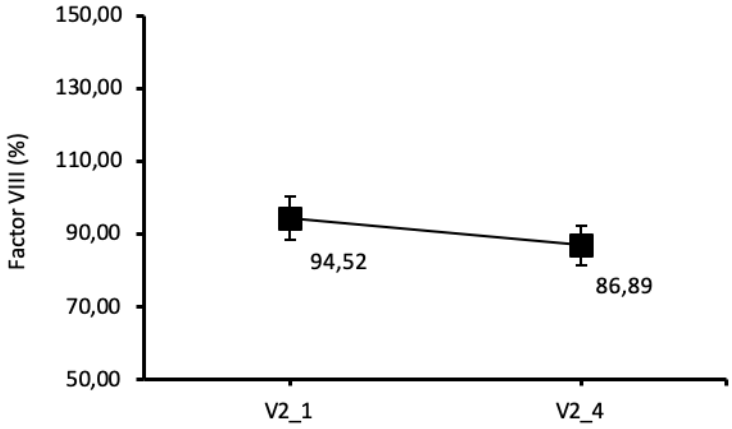

Figure 7: *Effect of the hyperinsulinemic-euglycemic clamp on coagulation parameters (2): (D) vWF, (E) D-Dimer and (F) Factor VIII*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 60 min; V2_3: glucose 100 mg/dL at 90 min; V2_4: glucose 100 mg/dL at 120 min[93]

3.4 Markers of inflammation and endothelial function

No alterations of vascular and intercellular adhesion molecules VCAM and ICAM levels have been observed during the hypoglycemic clamp, but levels reached a maximum after one week. Statistical significance has been reached for VCAM. (Fig.8 B; C)

Interleukin-6 (IL-6) was significantly increased represented by a zenith towards the ending of the hypoglycemic clamp without a sustained elevation at follow-up investigations. (Fig.8 A)

During the euglycemic-hyperinsulinemic clamp, IL-6 did not change, while VCAM and ICAM steadily decreased over time (ICAM showed a significantly lower level at the end of the clamp, $p<0.001$). (Fig.9 A-C) No alterations were found in the follow-up visits after hyperinsulinemia in the supplemental investigation.(Fig.10 A-I)

Fig. 8A

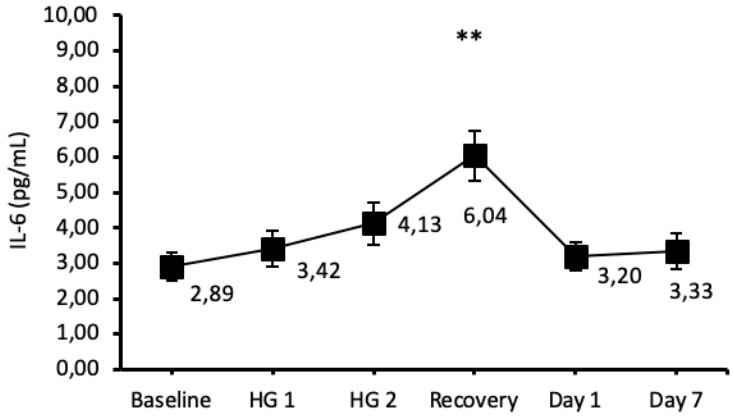

Fig. 8B

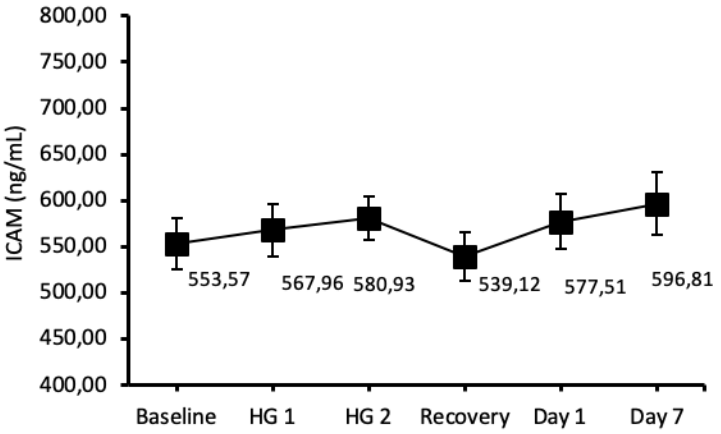

Fig. 8C

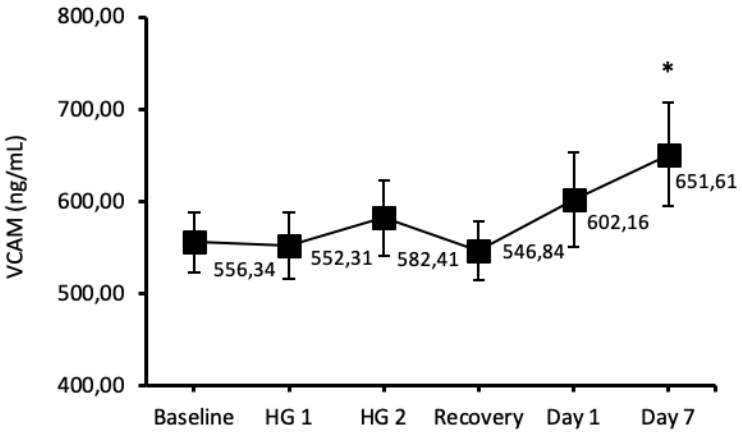

***Figure 8**: Effects of hypoglycemia on inflammatory parameters: (A) IL-6, (B) ICAM, (C) VCAM* p<0.01; ** p<0,001; Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL)*[93]

Fig. 9A

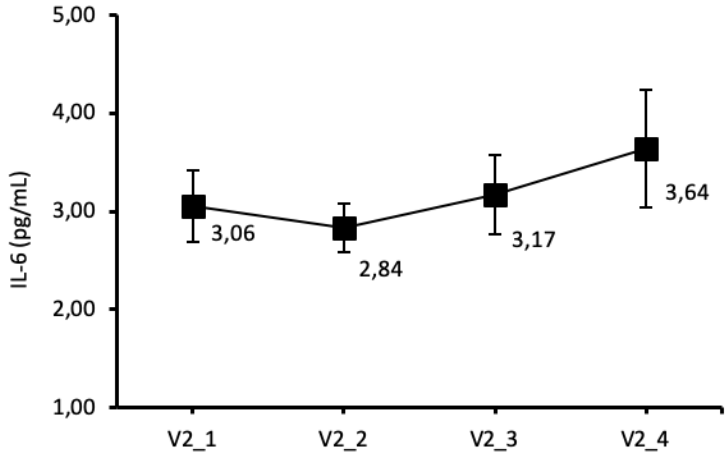

Fig. 9B

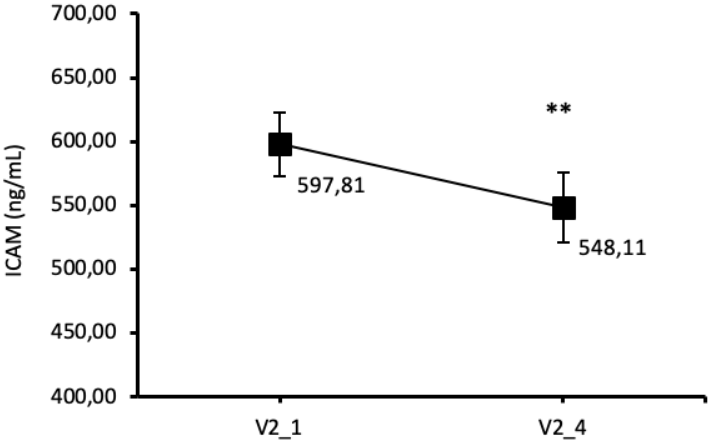

Fig. 9C

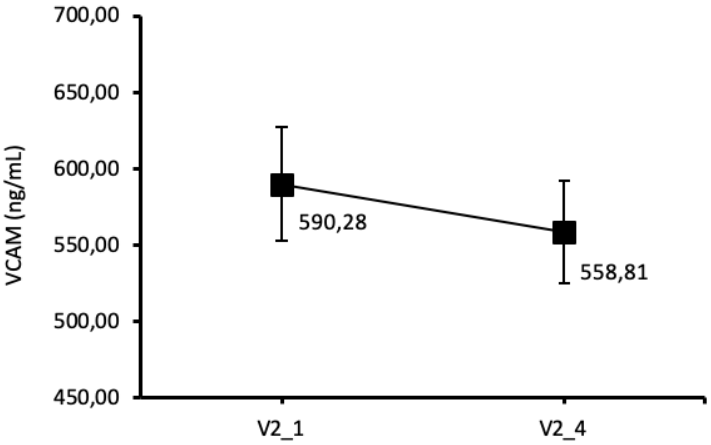

Figure 9: *Effect of the hyperinsulinemic-euglycemic clamp on Inflammatory parameters: (A) IL-6, (B) ICAM and (C) VCAM;* p<0.01; ** p<0,001 ; Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 60 min; V2_3: glucose 100 mg/dL at 90 min; V2_4: glucose 100 mg/dL at 120 min*[93]

Fig.10A

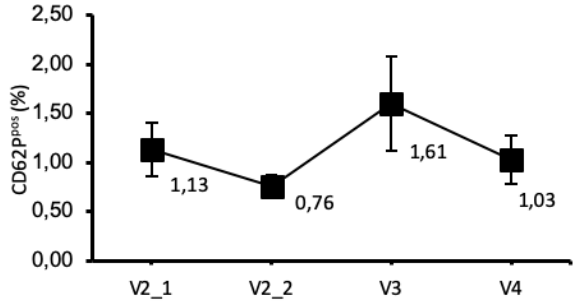

Fig.10B

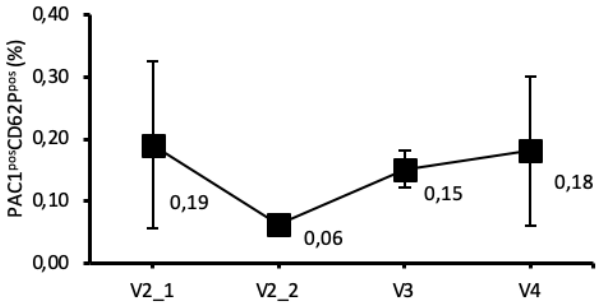

Fig.10C

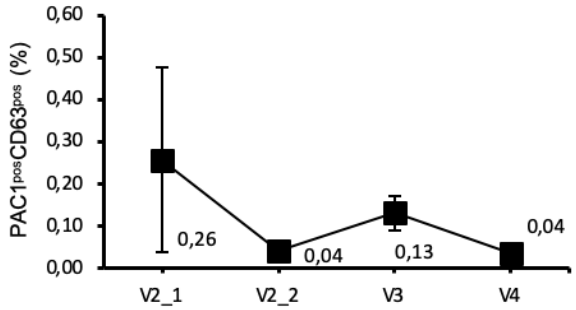

Fig.10D

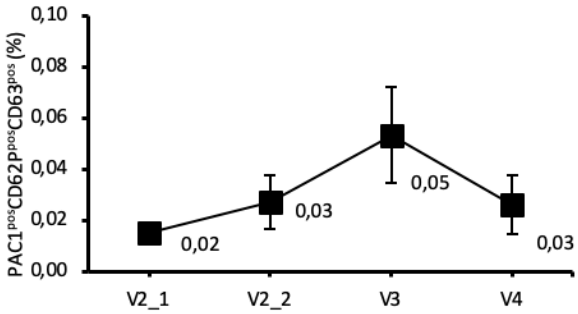

Fig.10E

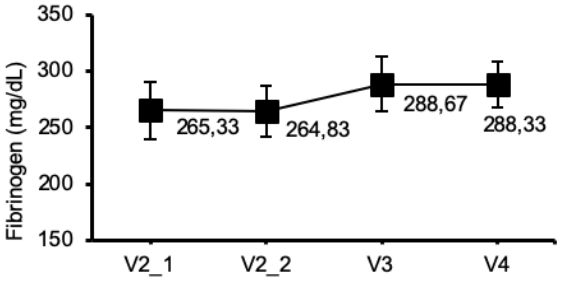

Fig.10F

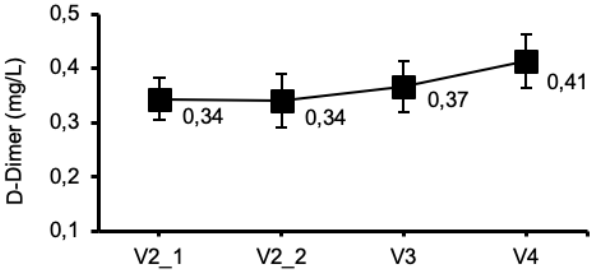

Figure 10: *Follow-up investigation of the hyperinsulinemic-euglycemic clamp (1): (A) CD62pos, (B) PAC1posCD62, (C) PAC1posCD63pos, (D) PAC1posCD62PposCD63pos, (E) Fibrinogen, (F) D-Dimer*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 120 min; V3: day 1 after clamp experiment; V4: day 7 after clamp experiment [93]

Fig.10G

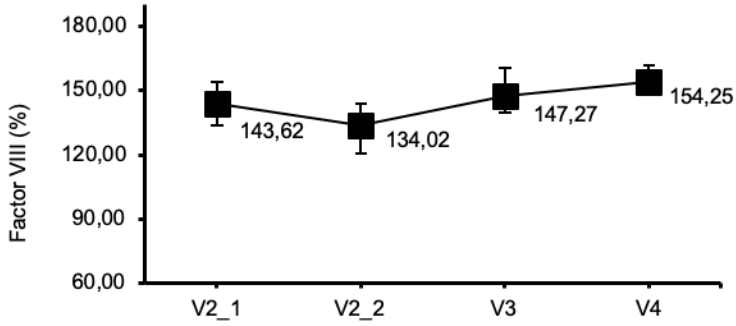

Fig.10H

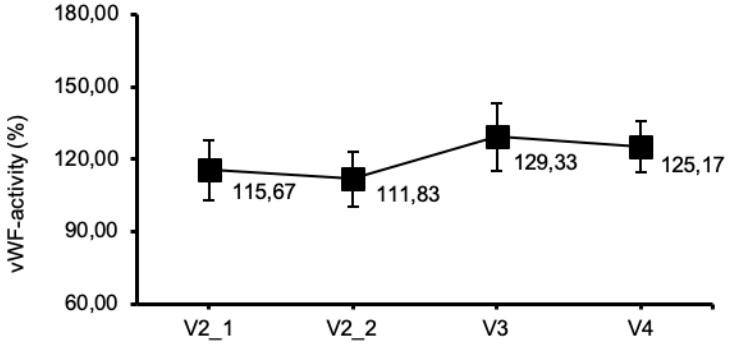

Fig.10I

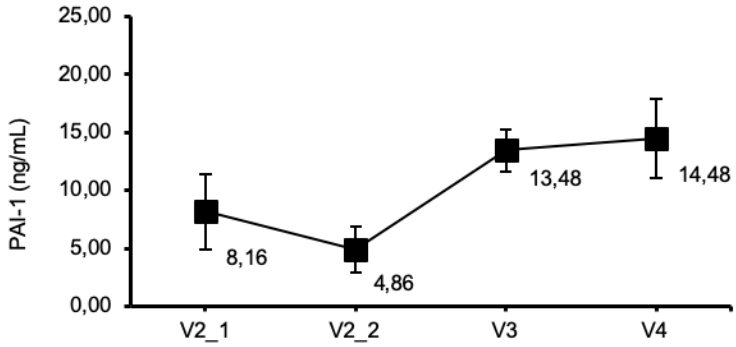

***Figure 10**: Follow-up investigation of the hyperinsulinemic-euglycemic clamp (2): (G) Factor VIII, (H) vWF, (I) PAI-1*

** p<0.01; ** p<0,001*

Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 120 min; V3: day 1 after clamp experiment; V4: day 7 after clamp experiment[93]

3.5 Counter regulatory hormones

Adrenaline, cortisol and glucagon levels showed to be increased significantly during the hypoglycemic clamp experiment, without a sustained elevation the days after the investigation.

During the hyperinsulinemic, euglycemic investigation, adrenaline and cortisol did not display any relevant alteration whilst glucagon levels decreased significantly during hyperinsulinemia.

Fig.11A

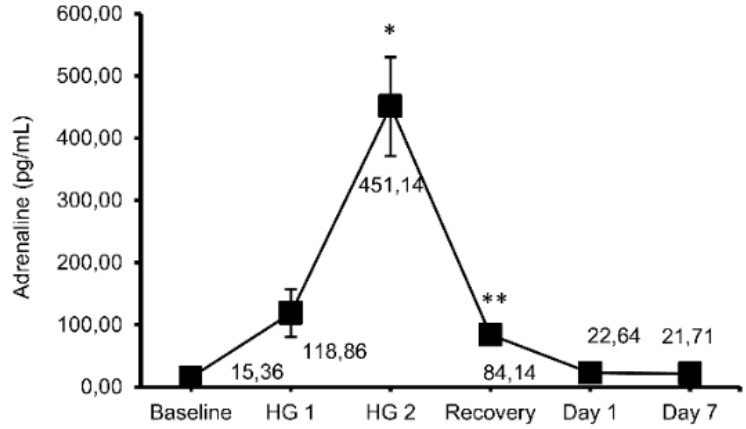

Fig.11B

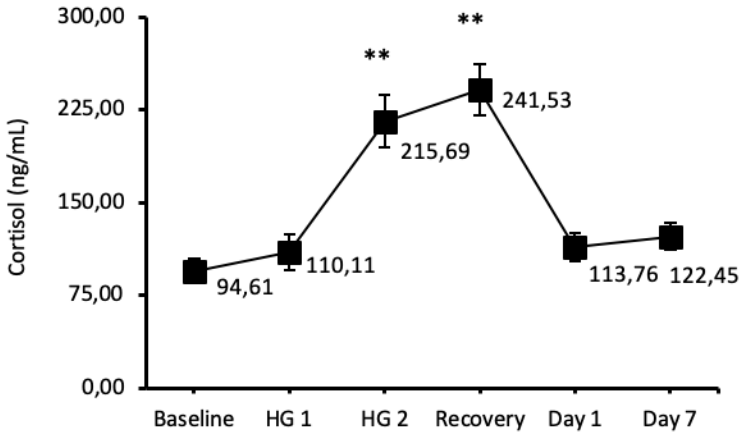

Fig.11C

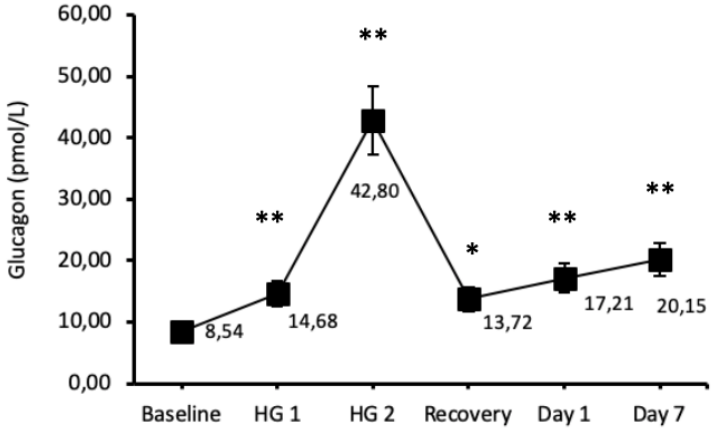

Figure 11: *Effects of hypoglycemia on counter regulatory hormones: (A) Adrenaline, (B) Cortisol, (C) Glucagon * p<0.01; ** p<0,001; Data is displayed as mean +/- standard error (SE). Data is displayed as mean +/- standard error (SE); HG 1, hypoglycemic plateau 1 (63 mg/dL); HG 2, hypoglycemic plateau 2 (45 mg/dL)*

Fig.12A

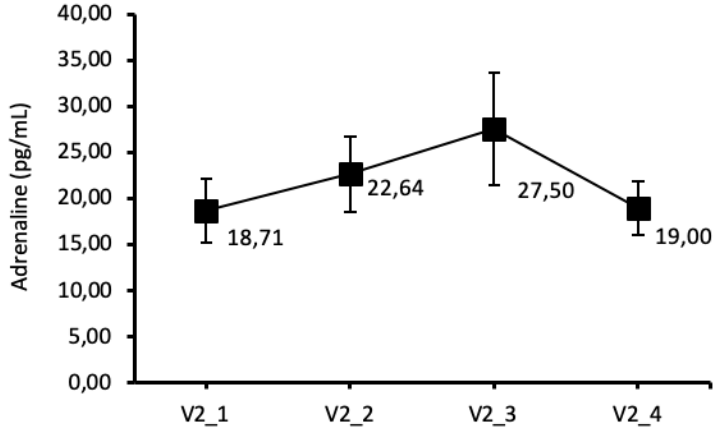

Fig.12B

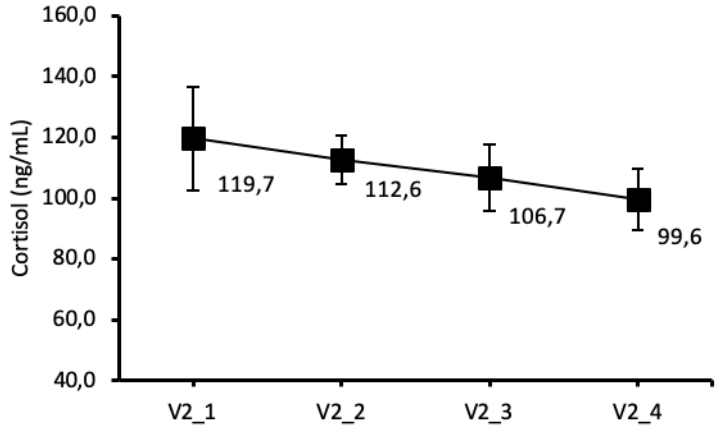

Fig.12C

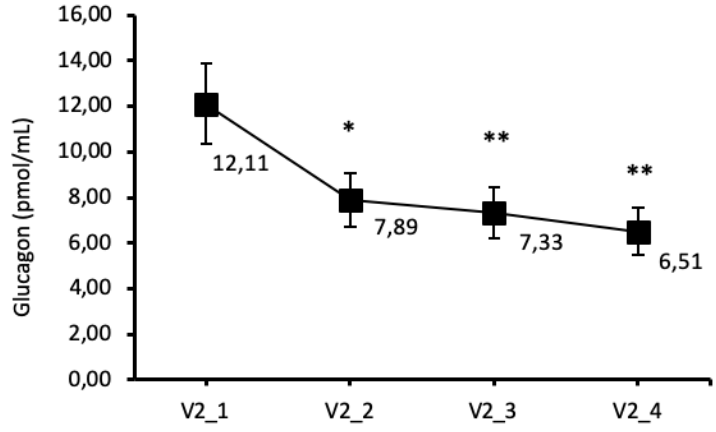

Figure 12: *Effects of the hyperinsulinemic-euglycemic clamp on counter regulatory hormones: (A) Adrenaline, (B) Cortisol, (C) Glucagon*

** p<0.01; ** p<0,001; Data is displayed as mean +/- standard error (SE). Data is displayed as mean +/- standard error (SE); V2_1: glucose 100 mg/dL at 0 min; V2_2: glucose 100 mg/dL at 120 min; V3: day 1 after clamp experiment; V4: day 7 after clamp experiment*

4 DISCUSSION

The purpose of this experimental study was to investigate acute and persisting effects of a single stepwise induced hypoglycemic event on platelet activation, coagulation, endothelial function and inflammation in T2DM.
The results highlight a heterogenous picture of platelet function activation with LTA by showing a numerical increase in platelet activation the day after the hypoglycemic clamp without reaching statistical significance.
Although LTA is accepted as a valuable method for assessing platelet activation, one single method does not provide an extensive portrait of platelet function and for that reason a better overview only can be achieved through taking various procedures into consideration leading to a different approach to determine platelet function.[95] Therefore tests based on flow cytometry, on platelet aggregation measured by light transmission (LTA) as well as based on platelet adhesion under shear stress (PFA-200) have been performed.
However, in this trial an increase in platelet activation by the expression of P-selectin, CD63 and PAC-1 after the hypoglycemic event including a sustained effect up to 7 days after the clamp has been demonstrated. Platelet activation assessed by PFA-200 displayed a decreased closure times one day as well as one week after the hypoglycemic clamp. These findings are supported and enhanced by a similar clamp experiment carried out by Chow et al. also displaying increased platelet activity during acute hypoglycemia.[47] DIAPLATE included a stepwise hypoglycemic clamp with specific glucose plateaus (30 minutes of 63 mg/dL and 30 minutes of 45 mg/dL) to investigate whether there is a certain glycemic threshold to activate platelets or coagulation. As there were no significant results present during the hypoglycemic episodes, it cannot be assumed whether there is a difference in platelet activity within these two hypoglycemic plateaus. In contrast, Chow et al. carried out two subsequent hypoglycemic clamps (60 minutes in the morning and 60 minutes in the afternoon) with a 45 mg/dL glucose target in each individual. Compared to Chow et al. examining subjects on various antidiabetic agents including insulin and 2 individuals taking aspirin, the DIAPLATE participants had a short duration of diabetes and were using metformin only and no subject was taking

platelet aggregation inhibitors.

Our finding of platelet activation 24 hours after the hypoglycemic stimulus is supported by the observation of a diminished platelet sensitivity to prostacyclin also 24 hours after a hypoglycemic clamp.[55] Physiologically, prostacyclin is a potent inhibitor of platelet aggregation and thrombus formation.[96]

Moreover, parameters of coagulation display an intensified activity response following hypoglycemia.

Changes in vWF represent endothelial dysfunction, as it is capable of luring platelets and leukocytes to the site of vascular damage. In this trial vWF-antigen levels rose to a prior hypoglycemic episode which enhances the findings of Wright et al. showing similar hypoglycemic vWF properties in T1DM.[50]

Factor VIII, a major key player in the intrinsic pathway of the coagulation cascade, is recently suggested to be of predictive value for coronary heart disease and stroke, especially in individuals with diabetes mellitus.[68] In our study, significantly increased F VIII levels have been found 24 hours after hypoglycemia and remained on an elevated level for one week.

The observed hypoglycemia-induced elevation of fibrinogen goes along with well investigated findings, which highlight a strong association with T2DM and raised levels of fibrinogen secretion as well as changes in fibrin structures. In patients with T2DM fibrinogen levels are elevated, which is well investigated in various studies.[47,62,63] As a consequence, an upregulation of fibrinogen activity leads to prothrombotic circumstances and contributes to cardiovascular complications.[14]

The observed elevation of D-Dimer after hypoglycemia represents increased cleavage of clots, which suggests enhanced clotting activity during hypoglycemia. However, to my knowledge, data on hypoglycemia related changes in D-Dimer properties in T2DM is rare and requires further investigation in future research.

PAI-1 represents an essential molecule to maintain hemostatic balance as it acts as an inhibitor of fibrinolysis via suppressing the splitting of plasminogen to plasmin.[77] Our results demonstrate a significant elevation of PAI-1 concentration 24 hours after the hypoglycemic clamp sustained up to day 7. These findings propose reduced fibrinolytic activity following a prior hypoglycemic stimulus and go along with data from the Framingham Heart Study, in which PAI-1 has been identified to be of fierce predictive value for CVD even after adjusting for established risk factors. [78] In comparison to that, Joy et al. described an increase of PAI-1 levels following

hypoglycemia in healthy subjects and in subjects with T2DM. [6,7,52] However, Chow et al. measured a decrease of PAI-1 after hypoglycemia but delayed clot lysis to a prior hypoglycemic episode.[47] Taking these findings into consideration, the picture of PAI-1 responses remains heterogenous but a tendency towards hypoglycemia triggered increases can be drawn. From this point, further trials investigating PAI-1 in T2DM need to be performed to generate a comprehensive overview. A fall in PAI-1 levels has been observed in the DIAPLATE study shortly after the start of insulin injection, which was also noticed in previous investigations.[7,47]

Changes in inflammatory properties such as Interleukin-6, VCAM-1 and ICAM-1 are supported by several investigations.[47,52] These findings display hypoglycemia related aggravation of vascular inflammation and conclusively the promotion of atherogenesis in T2DM, in which chronic low-grade vascular inflammation is already ongoing.

This trial has limitations. First, a group of 14 subject could be seen as a small number but considering serious adverse side effects of clamp induced (severe) hypoglycemia, sample size needed to be kept at minimum. Other studies performed in subjects with T2DM using similar ranges of sample numbers support and enhance our results of platelet and coagulation activation after hypoglycemia.[47,55] Second, light aggregometry is merely one method of measuring platelet activation for which this analysis is based on. To attempt a comprehensive understanding of platelet activation, various assessment methods need to be taken into consideration. Results suggest a higher sensitivity of flow cytometry based on platelet activation assays as scrutinized during hypoglycemia. Third, the hypoglycemic clamp could be criticized to be an artificial set-up, in which hyperinsulinemia alone could trigger platelet activation and coagulation. To rule out this possibility, an additional euglycemic clamp in each patient was performed, in which no changes towards above mentioned activation were observed. Fourth, as only prolonged (Day 1 and 7) effects after hypoglycemia were observed, one could argue that the exogenous insulin in the hypoglycemic clamp is accountable for the delayed measured activation. Reacting to that a supplemental examination including a follow-up assessment at day 1 and day 7 after a euglycemic has been performed in 6 patients willing to participate again with no significant changes found.(Fig. 10) Fifth, still, no statement can be made, at which glycemic threshold an

activation occurs.

The strengths of this trial consist of the study design, for which a stepwise hypoglycemic clamp was performed, and the homogenous study population of all taking only metformin and no antiplatelet agent.

In conclusion, the DIAPLATE trial delivers evidence to the hypothesis of hypoglycemia causing proaggregatory, procoagulant and proinflammatory responses in diabetes mellitus. These findings are characterized not only by acute effects but also suggest lasting cardiovascular consequences up to one week after hypoglycemia. These findings may provide an attempt to understand the consequences of hypoglycemic episodes on cardiovascular outcome, which is heavily affected by intensive glucose control as previously shown in trials like the ACCORD study.[41] Stabilizing glucose ranges and minimizing hypoglycemic episodes are crucial elements in treating patients with diabetes mellitus.

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