NL64421.091.17/ *Lactate_CBF* **The effect lactate administration on cerebral blood flow during hypoglycemia**

The effect of lactate administration on cerebral blood flow during hypoglycemia

Are the suppressive effects of lactate on counterregulatory responses to hypoglycemia reflected in an altered CBF response?

The effect of lactate administration on cerebral blood flow during hypoglycemia

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
AR	**Adverse Reaction**
CBF	**Cerebral blood flow**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
HC	**Healthy controls**
IAH	**Impaired awareness of hypoglycemia**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
NAH	**Normal awareness of hypoglycemia**
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

NL64421.091.17/ *Lactate_CBF* **The effect lactate administration on cerebral blood flow during hypoglycemia**

SUMMARY

Rationale: It is thought that altered brain lactate handling is involved in the development of impaired awareness of hypoglycemia (IAH), i.e. the inability to timely detect hypoglycemia in people with type 1 diabetes (T1DM). Infusion of lactate diminishes symptomatic and hormonal responses to hypoglycemia in patients with normal awareness of hypolgycemia (NAH), resembling the situation of patients with IAH. It is unknown whether this attenuating effect is due to brain lactate oxidation or the result of lactate-induced alterations of global and regional cerebral blood flow (CBF).

Normally, hypoglycemia causes a redistribution of CBF towards the thalamus, from where the sympathetic response to hypoglycemia is coordinated, but in IAH this effect is absent and global CBF is increased. We hypothesize that lactate infusion in patients with NAH will result in blunting of thalamic activation and/or enhanced global CBF. If so, these results may help delineating the pathogenesis of IAH which eventually creates new avenues to protect against the morbidity associated with hypoglycemia and IAH.

Objectives: To investigate the effect of intravenous lactate administration, compared to placebo, on thalamic (regional) and global CBF during euglycemia and hypoglycemia in patients with T1DM and NAH.

Study design: Single-blind placebo controlled, randomized cross-over intervention study

Study population: T1DM patients with NAH (n=10)

Intervention: On two separate occasions, patients with T1DM and NAH will undergo a hyperinsulinemic euglycemic-hypoglycemic glucose clamp with or without the infusion of exogenous lactate. ASL-MRI will be applied to measure global and regional changes in CBF.

Main study parameters/endpoints: The change in regional thalamic CBF in response to intravenous lactate infusion compared to placebo, during hypoglycemia

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The hypoglycemic condition is likely to induce typical symptoms (e.g. sweating, feeling hungry, palpitations) in T1DM patients with NAH, but is usually well-tolerated and less pronounced when lactate is infused. The risk for more severe hypoglycemia is negligible. The use of venous and arterial catheters may lead to hematomas and/or phlebitis, yet this is self-limiting and has in our hands never led to permanent damage. ASL-MRI is a non-invasive method to determine CBF, involving high magnetic fields, which are not associated with adverse events other than possible claustrophobia due to lying in the small MR-bore.

1. INTRODUCTION AND RATIONALE

Hypoglycemia is the most frequent side-effect of insulin therapy in type 1 diabetes (T1DM). If glucose levels drop, a hierarchically organized counterregulatory response is initiated, including the appearance of hypoglycemic symptoms and counterregulatory hormone responses. Hypoglycemia has also an effect on cerebral blood flow (CBF). Several studies, including our own, show that hypoglycemia results in a redistribution of CBF towards the thalamus in patients with T1DM and normal awareness of hypoglycemia (NAH) and in non-diabetic subjects (1-3). As such, the thalamus is thought to be involved in the coordination of the sympathetic response to hypoglycemia.

About one in every four patients with T1DM, the clinical syndrome of impaired awareness of hypoglycemia (IAH) can be diagnosed (4, 5). In patients with IAH, the glucose threshold for the initiation of a counterregulatory response is shifted to a lower level, which creates a greater risk for severe, potentially hazardous, hypoglycemia that requires help from another person for recovery. Brain imaging techniques have shown that hypoglycemia causes an increase in global CBF in these patients, whereas the abovementioned hypoglycemia-induced increase in thalamic CBF is blunted (3, 6).

The exact pathogenesis of IAH is thus far not known, but brain lactate is likely involved in the development of IAH (7-9). Various studies have shown upregulated capacity to transport lactate across the blood-brain barrier in patients with IAH, so that this lactate can be oxidized during hypoglycemia as an alternative fuel source when glucose supply is low (9). The role of lactate in the development of IAH is further supported by observations, including our own, that the administration of lactate diminishes symptomatic and hormonal responses to hypoglycemia (10, 11), similarly to the situation seen in IAH. It is unknown whether lactate also blunts the thalamic response to and increases global CBF during hypoglycemia. If so, this implies that, apart from its use for oxidation, lactate functions as a ‘master’ regulator of various processes in the brain to maintain energy metabolism and protect the brain from harmful effects of glucose deprivation (8). It is, however, possible that the increase in global CBF is a ‘late’ effect of IAH, thus remaining unaltered after single administration of lactate.

To our knowledge, there are no studies that investigated the effect of lactate administration during hypoglycemia on CBF responses. We hypothesize that lactate infusion will result in blunting of the increase in thalamic CBF and enhances global CBF, as seen in IAH. If so,

these results may help delineating the pathogenesis of IAH which eventually creates new avenues to protect against the morbidity associated with hypoglycemia and IAH.

Explorative substudy

Hypoglycemia affects certain metabolite levels, such as glutamate or glucose (12, 13). It is unknown whether lactate infusion might affect these changes. If these metabolite levels change upon infusion of lactate, this may give further insight into the exact role(s) of lactate in the development of IAH. As there is a pause between two CBF measurements, we will use this delay to perform ^{1}H-MRS to explore cerebral metabolite levels.

Substudy

Recent studies indicate that hypoglycemia plays a role in the development of cardiovascular complications . This may be explained by an acute hypoglycemia-induced increase in circulating pro-inflammatory cytokines and pro-atherothrombotic factors (14-16). Our research group found that acute hypoglycemia not only leads to an increase in white blood cell count and demargination of specific immune cells, but also enhances the *ex vivo* inflammatory response of peripheral blood mononuclear cells (17). Moreover, we found that among other cell types, alternative monocytes are recruited upon hypoglycaemia (18). There is substantial evidence supporting the importance of non-classical monocytes in the development of cardiovascular disease (19). These data support the concept that hypoglycemia contributes to the pathogenesis of systemic inflammation and cardiovascular complications in diabetes. However, additional studies are warranted to elucidate the exact underlying mechanisms of these effects of hypoglycemia on immune cells and specifically on monocytes. We therefore aim to use this study to draw additional blood (40 ml) at two time points during the hyperinsulinemic hypoglycemic clamps (only on the placebo day) at moments that blood sampling is scheduled for other measurements.

From 10mL of blood (EDTA tubes) we will obtain around 1x10^{6} monocytes (Ficoll gradient, followed by specific isolation of monocytes with MACS). The blood and the monocytes will be used for the following measurements:

	blood (mL)	**x10^{6} monocytes**	**measurements**
	0,2		leucodiff
	0,2		flow cytometry (monocyte subsets and other immune cell subsets)
	20	2	metabolomics
	2	0,2	flow cytometry (monocyte subsets)
	10	1	transcriptomics
total	32,4		

In addition, we will store plasma for determining metabolites present in the circulation.

In addition to the effects of hypoglycemia on immune cells, we want to study the effects of lactate infusion on immune cells. It has been demonstrated that lactate has broad anti-inflammatory effects on immune cells *in vitro* (20, 21). Moreover, lactate infusion ameliorated inflammation in mouse models *in vivo* (20, 22), highlighting lactate as a potential anti-inflammatory agent. Effects of lactate administration on inflammation in humans are rather unexplored, but insights into the effects of lactate on human immune cells would be highly clinically relevant. Since we will routinely draw blood during the lactate infusion in order to monitor plasma glucose and lactate levels, measurement of immune cell phenotypes before and after lactate infusion require two additional samples (2 x 40 ml, only on the day where patients receive the lactate infusion), during an already scheduled blood draw.
The blood and the monocytes will be used for the following measurements:

	blood (mL)	**x10^6 monocytes**	**measurements**
	plasma		metabolomics
	0,2		leucodiff
	20	2	metabolomics
	2	0,2	flow cytometry (monocyte subsets)
	15	1,5	in vitro stimulations
total	37,2		

In addition, we will store plasma for determining metabolites present in the circulation.

2. OBJECTIVES

Primary objective

To investigate the effect of intravenous lactate administration, compared to placebo, on thalamic (regional) CBF during euglycemia and hypoglycemia in patients with T1DM and NAH.

Secondary Objectives:

To investigate the effect of intravenous lactate administration, compared to placebo, on global CBF during euglycemia and hypoglycemia in patients with T1DM and NAH.

To assess changes in counterregulatory hormone and symptom responses to hypoglycemia in response to lactate infusion, compared to placebo, in patients with NAH.

Objectives substudy:

To determine the effect of acute hypoglycemia on the inflammatory function of monocytes and investigate underlying mechanisms that could explain the pathogenesis of systemic inflammation and cardiovascular complications.

To determine the effect of lactate administration on immune cell function and metabolism.

3. STUDY DESIGN

The study is designed as a randomized single-blind, placebo-controlled, cross-over intervention study. The study will be conducted at the Radboud university medical center in Nijmegen and includes patients with T1DM with NAH. The duration of the study depends on the inclusion of the required number of subjects, with an expected duration of 10 months.

All subjects will be invited for a medical screening prior to the investigational days to determine eligibility. A validated questionnaire will be used to assess hypoglycemic awareness. On the experimental days, participants will be invited to visit the research facility at 08.00h in fasting condition. A euglycemic-hypoglycemic glucose clamp will be initiated with or without the infusion of exogenous lactate, during which CBF measurements are performed. Each subject will be tested under both conditions, the order of which will be randomized.

4. STUDY POPULATION

4.1 Population

For this study, T1DM patients with NAH will be recruited (n=10). Awareness status will be assessed by the Dutch modified version of the Clarke questionnaire, where a score of 0-1 classifies patients as having NAH (23, 24). Patients with T1DM will be recruited from the outpatient diabetes clinic of the Radboud university medical center, by approaching them if they had previously participated in experiments and had consented in being approached for future research, or by using social media related to the Radboud university medical center (e.g. www.umcn.nl/ meedoenaanonderzoek) or to funding agencies (e.g. www.diabetesfonds.nl).

The diabetes clinic of the Radboud university medical center is visited annually by ~800 patients with T1DM and ~70% of these patients are thought to be classified as NAH. Thus, we expect no problems with the recruitment of the planned number of participants.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Diabetes duration ≥ 1 year
- Age: 18-50 years
- Body-Mass Index: 18-30 kg/m^2
- HbA_{1c}: <75 mmol/mol (<9%)
- Outcome Clarke questionnaire: 0-1
- Blood pressure: <160/90 mmHg

4.3 Exclusion criteria

A potentially eligible subject who meets any of the following criteria will be excluded from participation in this study:

- Inability to provide informed consent
- Use medication other than insulin, except for oral contraceptives or stable thyroxin supplementation therapy
- Presence of any other medical condition that might interfere with the study protocol, such as brain injuries, epilepsy, a major cardiovascular disease event or cardiac failure, known liver disease, anxiety disorders or a history of panic attacks.
- Microvascular complications of T1DM:
    - Proliferative retinopathy
    - Symptomatic diabetic neuropathy (including autonomic neuropathy)
    - Nephropathy; clinical/overt albuminuria or an estimated glomerular filtration rate <60ml/min/1.73m^2
- MR(I) contraindications (pregnancy, severe claustrophobia, metal parts in body)

4.4 Sample size calculation

In a previous study, we found that CBF in the thalamus increased in response to hypoglycemia by 14 ± 4% in patients with type 1 diabetes and NAH (3). The difference in thalamic CBF between the placebo arm and lactate infusion arm we aim to detect is 6%. With a power of 80% ($Z_\beta = 0.84$) at a significance level of 0.05 ($Z_\alpha = 1.96$), the number of subjects thus required is 7:

$$N = 2\frac{(Z_\alpha + Z_\beta)^2 * SD^2}{\delta^2} = 2\frac{(1.96 + 0.84)^2 * 0.04^2}{0.06^2} = 7$$

To correct for the small number of participants, we will include three extra subjects (n=10).

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

For this study, stepped hyperinsulinemic euglycemic-hypoglycemic glucose clamps will be conducted, as described previously (9), with and without intravenous administration of lactate. Briefly, subjects will receive an intravenous insulin infusion, at a continuous rate of 60 $mU \cdot m^{-2} \cdot min^{-1}$, as well as glucose 20% w/w (Baxter B.V., Deerfield, IL) intravenously at a variable rate, adjusted by arterial plasma glucose levels, measured at 5 minute intervals, to clamp plasma glucose values at predetermined levels. On one occasion, sodium lactate 600 mmol/L (prepared by the Department of Pharmacy, Radboud university medical center, Nijmegen) will be administrated intravenously at a variable rate (aiming at plasma lactate levels of 3.5 mmol/L) and on the other occasion 500 mL of sodium chloride, 0.9% will be administered (placebo).

5.2 Use of co-intervention

All experiments will be started after an overnight fast. Subjects will be required to abstain from smoking, alcohol and caffeine containing substances (i.e. caffeinated coffee, cola, tea or chocolate) for 24 hours prior to the experiment, and from strenuous exercise for at least two days before the experiment.

In addition, patients will be instructed to avoid hypoglycemia the evening before the experiment and if necessary, to reduce the basal insulin dose the evening before the experiment, and to check their blood glucose level at ~2 AM and in the morning prior to the experiments. They will also be asked to omit the morning dose of rapid-acting insulin and, if applicable, to disconnect the insulin pump one hour prior to the experiment.

5.3 Escape medication

Additional glucose 20% will be infused as required to avoid that glucose levels will fall too deep or to restore plasma glucose concentrations to euglycemic values at the end of the clamp or upon request of the participant or should his/her (medical) condition require such action to be taken. All participants will be given a meal high in carbohydrates upon termination of the experiments.

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

Sodium lactate solution (50%), 600 mmol/l; prepared for intravenous infusion and, as a placebo, sodium chloride solution (0.9%); prepared for intravenous infusion. For a detailed description of the investigational products we refer to section 2.1 (Chemische Farmaceutische gegevens) of the IMPD.

6.2 Summary of findings from clinical studies

Endogenous lactate is abundantly produced and metabolized by the body. Exercise, for example, raises plasma lactate levels easily above 10 mmol/L.

In a previous study, approved by the CMO Arnhem-Nijmegen (2016-2731/NL58348.091.16), we used a similar concentration and duration of sodium lactate, without adverse effects. Furthermore, several studies have been performed with intravenous sodium lactate infusion, in both healthy subjects and T1DM patients (7, 10, 11, 25-30). These studies do not report side effects related to sodium lactate infusion. We enquired about side effects with one of the authors, who confirmed this (10).

Potential side effects are mainly related to irritation at the infusion site.

6.3 Summary of known and potential risks and benefits

It is expected that subjects will not benefit from the sodium lactate infusion. Potential risks include hematomas and/or phlebitis following infusion, yet this is self-limiting. To reduce the risk of phlebitis, we will flush the catheter with 100 ml NaCl 0.9% after the lactate infusion. Some early studies have described a panicogenic effect when a high dose of sodium lactate was rapidly infused in patients with (a history of) panic disorders (31), but substantial methodological problems (i.e. lack of specificity and sensitivity, disregard of baseline cofounders) render the evidence highly questionable (32). Such adverse effects have not been reported in later studies involving healthy volunteers and people with diabetes (10), including our own study (manuscript submitted; 2016-2731/NL58348.091.16). Nevertheless, we will exclude subjects with panic disorders or a history of panic attacks. In theory, sodium lactate infusion could lead to hypervolemia, but this risk is negligible when infusing for a short duration (i.e. ~120 min) in otherwise healthy people with T1DM (without a history of heart failure).

6.4 Description and justification of route of administration and dosage

Sodium lactate will be infused into the antecubital vein, using a standard intravenous pump system. Although the solution is hyperosmolar, previous studies have shown that sodium lactate, in approximately equal concentration and dosage, can be administered through a peripheral vein without adverse effects. Furthermore, although sodium lactate is hyperosmolar, it is not very hypertonic. When sodium lactate is infused intravenously, lactate will enter the intracellular compartment, and only the osmolarity of sodium has to be taken into account (i.e. will stay intravascular).

The expected rate of infusion is 25 µmol/kg/min after a priming/bolus dose of 40 µmol/kg/min for 15 min.

6.5 Dosages, dosage modifications and method of administration

Sodium lactate will be administrated intravenously and we will use a primed (40 µmol/kg/min for 15 min) continuous (25 µmol/kg/min for the remainder of the experiment) infusion scheme, aiming at plasma lactate levels of 3.5 mmol/L. Dosage modifications will be performed if plasma lactate levels fall below 3.0 mmol/L or rise above 4.0 mmol/L. In the unlikely event of a very small antecubital vein, glucose 20% and sodium lactate will be separately infused (see section 8.3).

6.6 Preparation and labeling of Investigational Medicinal Product

The sodium lactate solution will be prepared in 500 ml infusion bags at the pharmacy department of the Radboud university medical center. For information about the preparation see chapter 2.1.P.2 (Farmaceutische ontwikkeling) and 2.1.P.3 (Bereiding) of the IMPD.
Labeling will be done according to the relevant GMP guidelines (annex 13) by the pharmacy department of the Radboud university medical center.

6.7 Drug accountability

The sodium lactate solution will be stored according to GMP guidelines, at the clinical research center, Radboud university medical center. The coordinating investigators will document receipt of lactate or placebo, including the date and dosage. Remaining sodium lactate solution will be destructed at the end of the study.

7. NON-INVESTIGATIONAL PRODUCT

7.1 Name and description of non-investigational product(s)

Insulin aspart (Novorapid®): rapid-acting analogue of human insulin; glucose-lowering agent used for the treatment of type 1 and (insulin-requiring) type 2 diabetes.

7.2 Summary of findings from non-clinical studies

For the findings from non-clinical studies, we refer to sections 5.3 (Gegevens uit het preklinisch veiligheidsonderzoek), 6.1 (Lijst van hulpstoffen), 6.2 (Gevallen van onverenigbaarheid), 6.3 (Houdbaarheid) and 6.4 (Speciale voorzorgsmaatregelen bij bewaren) on pages 20-21 of the attached SPC document on Novorapid® insulin (D2).

7.3 Summary of findings from clinical studies

For the findings from clinical studies, we refer to sections 5.1 and 5.2 on pages 17-20 of the attached SPC document on Novorapid insulin, describing the pharmacodynamic (5.1) and pharmacokinetic (5.2) characteristics of Novorapid® insulin.

7.4 Summary of known and potential risks and benefits

Novorapid® insulin is indicated (hence beneficial) for the treatment of diabetes mellitus (any type) in adults, adolescents and children above the age of 2 years (section 4.1 [Therapeutische indicaties], page 12 of the SPC). For a summary of known and potential risks, we refer to sections 4.4 (Bijzondere waarschuwingen en voorzorgen voor gebruik), 4.5 (Interacties met andere geneesmiddelen en andere vormen van interactie), 4.8 (Bijwerkingen) and 4.9 (Overdoseringen) on pages 14-17, which specifically address the risk of hypoglycaemia and other potential, but rare, adverse effects. However, it should be acknowledged that the risk of severe hypoglycemia during the experiment is negligible, since glucose is measured at 5-minute intervals and additional glucose will be administered should glucose levels tend to drop below the predetermined levels of ~5.0 or 2.7 mmol/l.

7.5 Description and justification of route of administration and dosage

Hyperinsulinemic glucose clamp studies require that insulin is administered at a steady continuous rate to achieve stable levels of hyperinsulinemia. To this end, insulin needs to be infused intravenously using a standard intravenous pump system. The insulin dose will be adjusted according to the body surface area, aiming for insulin levels of ~120 mU/l, which is within the physiological range. Thus, for a subject with a body weight of 70 kg, body length of

180 cm and – consequently – a body surface area of 1.936 m^2, the required insulin infusion can be calculated as: 1.936 x 60 x 60 ÷ 1000 = 7.0 units per hour.

7.6 Dosages, dosage modifications and method of administration

As stated above, the insulin dose will be individually adjusted according to the body surface area, aiming for insulin levels of ~120 mU/l. Thus, for a subject with a body weight of 70 kg, body length of 180 cm and – consequently – a body surface area of 1.936 m^2, the required insulin infusion can be calculated as: 1.936 x 60 x 60 ÷ 1000 = 7.0 units per hour. The insulin dose will not be adjusted during the clamp. However, when this dose appears insufficient to achieve hypoglycemia, a repeat test may be scheduled on a case-by-case basis using a dose of 120 mU·m^2·min.

7.7 Preparation and labeling of Non Investigational Medicinal Product

Aspart insulin (Novorapid®) will be ordered in prefilled ampoules of 3 ml (containing 100 units/ml) from the pharmacy department of the Radboud university medical center. No specific alterations for preparation or labeling of the product are required. Preparation of the insulin pumps will be done by the investigator and checked by a research nurse or vice versa.

7.8 Drug accountability

Not applicable (product ordered from pharmacy department by usual in-hospital prescription form).

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

The main study parameter of the study is the change in regional thalamic CBF in response to intravenous lactate infusion compared to placebo, during hypoglycemia.

8.1.2 Secondary study parameters/endpoints

- Change in global CBF in response to intravenous lactate infusion compared to placebo, during hypoglycemia.
- Change in plasma levels of counterregulatory hormones in response to hypoglycemia and euglycemia with and without lactate infusion (adrenaline, noradrenaline, growth hormone and cortisol) (pmol/L, nmol/L, mU/L, µmol/L)

- Change in glucose infusion rate (GIR): the amount of glucose 20% necessary to maintain plasma glucose at steady state euglycemic or hypoglycemic values (mg/kg/min) with and without lactate infusion
- Change in hypoglycemic symptoms scores in response to hypoglycemia with and without lactate infusion

8.1.3 Other study parameters

- Plasma glucose concentration (mmol/L)
- Plasma lactate concentration (mmol/L)
- Plasma insulin concentration (mU/L)
- Plasma pH values

8.1.4 Explorative study parameters

- Change in brain metabolite levels (eg. glutamate, glutamine, aspartate, NAA, lactate) in response to intravenous lactate infusion compared to placebo during euglycemia
- Change in brain metabolite levels (eg. glutamate, glutamine, aspartate, NAA, lactate) in response to intravenous lactate infusion compared to placebo during hypoglycemia

8.1.5 Substudy parameters

- Cytokine production capacity of stimulated monocytes in response to lactate infusion or hypoglycemia
- Measurements of metabolites in cell lysates or supernatants of the cultured immune cells
- Analysis of intracellular metabolic pathways on protein (Western Blot, e.g. HIF1α, AMPK, mTOR) and gene expression level (qRT-PCR, e.g. glycolytic enzymes, fatty acid oxidation enzymes) of the immune cells

8.2 Randomization, blinding and treatment allocation

This study has a randomized placebo-controlled, singe-blind cross-over design. Randomization will be done by a computer program, to ensure that equal numbers of subjects will start with intravenous lactate or saline infusion (placebo).

Participants will be unaware of the intravenous infusions used (sodium lactate or saline) and the label on the infusion bag of sodium lactate or placebo will be covered. Researchers cannot be blinded, since plasma lactate levels are measured simultaneously with plasma glucose levels, necessary to clamp plasma glucose values at predetermined levels.

In case of severe adverse events or other clinical conditions for which unblinding is thought to be inevitable by the care-providing physician, the participant will be unblinded. Data obtained will be excluded from the analysis, except for the safety analysis.

8.3 Study procedures

Participants will be asked to come to the research facility for a medical screening, including medical history and standard physical examination. If not determined in our hospital in the last 6 months, we will determine kidney function (MDRD, serum creatinine) and HbA_{1c}.

Subjects will be examined on two occasions, separated by at least two weeks. In female subjects, both experiments will be performed during equal phases of the menstrual cycle.

On each experimental day, subjects will come to the MR research facility in the morning in fasting condition, having abstained from alcohol and strenuous exercise for 48 hours and caffeine containing substances for 24 hours before the start of the experiments. In addition, participants will receive specific instructions to avoid hypoglycemic incidents the day (and night) before and the morning of the experimental day. Patients will be asked to omit their usual morning insulin dose. Experiments will be rescheduled in cases of hypoglycemia in the 24 hours before the clamp.

Upon arrival at the MR research facility, the brachial or radial artery of the non-dominant arm will be cannulated under local anesthesia (Xylocaine 2%) for frequent blood sampling. An intravenous catheter will be inserted in the antecubital vein of the contralateral arm for infusion of glucose, lactate (or placebo) and insulin. In the unlikely event of very small veins, a second intravenous catheter will be inserted. Subsequently, the subject will be placed, in supine position and headfirst, in the MR scanner and baseline CBF data and blood samples will be acquired. Then, sodium lactate or saline will be infused and a hyperinsulinemic euglycemic-hypoglycemic glucose clamp will be applied as previously described in detail (9, 33). In short, insulin will be infused at a rate of 60 $mU/m^2/min$ and glucose 20% will be infused at a variable rate, aiming for stable plasma glucose levels of ~5.0 mmol/l during the euglycemic phase (40 minutes) and ~2.8 mmol/l during the hypoglycemic phase (45 minutes). The infusion rate of glucose will be adjusted by arterial plasma glucose levels, measured at 5-minute intervals. Plasma lactate levels will also be measured at a 5-minute interval. Prior to entering the MR and immediately after the hypoglycemic phase, symptoms will be assessed on a linear analogue scale with a validated questionnaire. Additional blood will be sampled for measurement of plasma insulin and counterregulatory hormones, and for

cytokine measurements (sub-study), at the end of the eu- and hypoglycemic phase. The clamps will be terminated after 45 minutes of hypoglycemia, and sufficient glucose 20% will be administrated to quickly restore euglycemia. After decannulation, a meal will be offered to the subjects to prevent late hypoglycemia.

All MR experiments will be conducted on a 3T MR system (MAGNETOM Prisma, Siemens, Erlangen, Germany), equipped with a ^{1}H volume head coil. A transversal T_1-weighted anatomical image will be acquired as reference. Before each perfusion measurement, low-resolution 3D time of flight angiogram will be acquired to detect the brain feeding arteries. Baseline CBF data will be obtained before the start of the lactate infusion. During lactate (or saline) infusion, CBF data will be obtained at several time-points during euglycemia and hypoglycemia (see fig. 1).

In between the CBF measurements we will perform ^{1}H-MR spectroscopy (MRS) to determine the effects of lactate infusion on cerebral metabolite levels. This will be an explorative substudy, using the same volume head coil.

Subjects will be contacted one week after the experiments, to ask about adverse effects or any other discomfort potentially related to the experimental procedure.

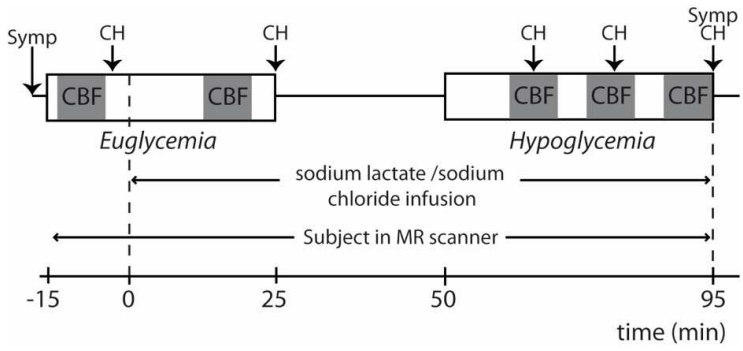

Fig. 1 *Schematic overview of the study protocol. Cerebral blood flow (CBF) will be determined before the infusion of sodium lactate/placebo and as indicated during euglycemia and hypoglycemia.*
CH: determination of counterregulatory hormones; Symp: hypoglycemic symptom questionnaire.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.5 Replacement of individual subjects after withdrawal

If a subject withdraws from the study, an additional study participant will be recruited, to a maximum of two participants.

8.6 Follow-up of subjects withdrawn from treatment

There will be no follow-up of subjects who withdraw from the study, unless the subject's withdrawal was a consequence of urgent medical reasons.

8.7 Premature termination of the study

The study will be terminated prematurely when unexpected serious adverse events are experienced which endanger other subjects. If the trial is prematurely terminated the investigator will promptly inform the trial subjects, the METC and competent authority. A detailed written explanation of the termination will be provided. Appropriate follow up and treatment of trial subjects will be assured.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product / trial procedure/ the experimental intervention. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;

- is life threatening (at the time of the event);
- requires hospitalization or prolongation of existing inpatients' hospitalization;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgment by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 9.2.2);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the medicinal product under investigation, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:

- Summary of Product Characteristics (SPC) for an authorized medicinal product;
- Investigator's Brochure for an unauthorized medicinal product.

The sponsor will report expedited the following SUSARs through the web portal ToetsingOnline to the METC

- SUSARs that have arisen in the clinical trial that was assessed by the METC;

- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.

The expedited reporting of SUSARs through the web portal Eudravigilance or ToetsingOnline is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

9.3 Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, and competent authorities of the concerned Member States.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

10. STATISTICAL ANALYSIS

All parameters will be presented as continuous variables and will be presented quantitatively. Only subjects who complete the entire study will be counted towards the final results (per-protocol analysis). The assumption of negligible carry-over effects will be tested on the within-subject sums of the results obtained during lactate and placebo infusion using unpaired *t* test (34, 35). In the case of missing data, the data will be reconstructed if possible, using interpolation techniques. All statistical analyses will be performed using SPSS 20.0 or higher. A p-value of <0.05 will be considered as statistically significant.

10.1 Primary study parameter(s)

To assess hypoglycemia-induced changes in regional CBF, voxel-wise univariate general linear modeling (GLM) statistical analysis with cluster significance correction will be performed in FMRI Expert Analysis Tool (FEAT, version 6.0) (36).

10.2 Secondary and other study parameters

The difference in global CBF between euglycemia and hypoglycemia within groups will be calculated and expressed relative to euglycemia and compared with a two-sided Student's t-tests. All other study parameters obtained during the experimental and control intervention will be compared with a unpaired t-test or with Mann-Whitney U test if data are not normally distributed.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

The study will be conducted in accordance with the principles of the Declaration of Helsinki (8th amended version, October 2008) and in accordance with the Medical Research Involving Human Subjects Act (WMO). Prior to participation, all potentially eligible study participants will have to provide written informed consent

11.2 Recruitment and consent

Patients with T1DM will be recruited (see chapter 4 for details). Potentially eligible subjects will be fully informed about the study by the project leader or principal investigator. The project leader or principal investigator will ask for their consent for which they will be allowed sufficient time, but at least 24 hours, to consider participation.

11.3 Objection by minors or incapacitated subjects

Not applicable.

11.4 Benefits and risks assessment, group relatedness

In order to investigate the effect of hypoglycemia on CBF, subjects have to undergo a hypoglycemic clamp. As a consequence, subjects may experience hypoglycemic symptoms, such as sweating, shaking, palpitations, hunger and concentration problems. However, this condition is generally well tolerated by subjects. Consequent to the clamp conditions, the risk of developing more severe hypoglycemia leading to loss of consciousness or worse is negligible. As soon as euglycemia is restored, these symptoms are known to quickly disappear, although a feeling of fatigue may persist for some hours. The investigators have ample experience with the use of hyperinsulinemic hypoglycemic clamps. As the plasma glucose level will be monitored by an arterial line, accurate values will be obtained avoiding blood glucose levels which are too low.

For the purpose of this study we need to infuse sodium lactate. Some early studies have described a panicogenic effect when a high dose of sodium lactate was rapidly infused in patients with (a history of) panic disorders (31), but substantial methodological problems (i.e. lack of specificity and sensitivity, disregard of baseline cofounders) render the evidence highly questionable (32). Such adverse effects have not been reported in later studies involving healthy volunteers and people with diabetes(10), including our own (2016-2731/ NL58348.091.16). Nevertheless, we will exclude subjects with panic disorders or a history of panic attacks.

Arterial blood sampling is necessary to determine plasma glucose levels accurately. Complications of an arterial line include hemorrhage, thrombosis and embolization, arteriovenous fistulas, infections and injury of the median nerve. However, these complications are very rare (37) and have not occurred in the past >15 years that we apply this technique in our research group.

Although ALS-MRI is a safe technique, MR measurements require in addition to the magnetic field also non-ionizing radio frequent (RF) irradiation. Official FDA limits for the specific absorption rate (SAR) of the RF irradiation are taken into account. These limits cannot be exceeded due to software (and partly also hardware) protections. Other potential risk factors regarding MR are claustrophobia or back problems as a result of the long

measurement time during which the subject has to lay in a supine position. Since there is continuously contact between subjects and investigators, these burdens will be minimized.

Subjects participating in this study will derive no direct benefit from the study. However, the outcome of this study may help delineating the pathogenesis of IAH and create new avenues for its treatment.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.6 Incentives

Participants will be reimbursed with €100 per visit as a compensation for participating in this study. Travel expenses for participants living outside Nijmegen will be covered separately.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

The investigator will preserve confidentiality of all subjects taking part in the study, in accordance with GCP and local regulations. The investigator will ensure that the subject's anonymity is maintained. All data and material from one individual subject will be coded by a unique identification code. The key to the code is maintained by the investigator. A validated data management system will be used for data handling, according to GCP.
Data will be stored for 15 years. Human material will be stored for 15 years at the department of internal medicine and will be used for additional research, related to the aims of the current research, if deemed necessary. If additional research is not directly related to the aims of the current research or if it may result in incidental findings, we will ask the METC for approval. The principal investigators are responsible for the storage, coding and release of the human material.

12.2 Monitoring and Quality Assurance

Source document and database verification will be performed by an independent monitor, according to a predefined monitoring plan. There will be minimal monitoring, once a year, as the study is judged to cause only marginal potential risk to the participants (38).

12.3 Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

12.6 Public disclosure and publication policy

The results of this study will be disclosed unreservedly. Both positive and negative results will be submitted for publication to peer-reviewed scientific journals, trail registers, databases or websites (for instance www.biomedcentral.com).
The trial is registered at a public trial registry (www.clinicaltrials.gov; Identifier NCT: not yet known).

13. STRUCTURED RISK ANALYSIS

13.1 Synthesis

This study is judged to cause negligible additional risk to the participants, provided that participants are adequately instructed and potential risks associated with this study are monitored correctly. Inclusion criteria preclude that only T1DM subjects in otherwise good health and in a vital age range, can participate, minimizing potential problems related to the experiments. The induced hypoglycemia can cause typical symptoms like sweating, hunger and palpitations, but this is not harmful for the participants.

In this study we will infuse sodium lactate, to raise plasma lactate levels or placebo. Sodium lactate may cause local irritation of the vein, but this is self-limiting. Lactate infusion will likely reduce the severity of hypoglycemic symptoms. We refer to section 6.3 for a more detailed description of other potential risks. Subjects will be closely and continuously monitored during the clamp studies by a research nurse.

In addition, we will use Insulin aspart (Novorapid®) during the clamps, which is a rapid-acting analogue of human insulin used for the treatment of type 1 and (insulin-requiring) type 2 diabetes. Insulin administration lowers glucose levels, and is used in this study to perform the hypoglycemic clamps, and thus carries the risk of causing severe hypoglycemia. However, as described in section 11.4, plasma glucose levels will be closely monitored which virtually eliminates the risk of severe hypoglycemia.

Subjects will have to lie in the MRI system for approximately 2 hours, which can be inconvenient but is not associated with any risks, since patients with contra-indications for MRI will be excluded from this study.

The participants will also undergo arterial blood sampling. As described in section 11.4, the complications associated with arterial blood sampling are very rare and have not occurred in the past >15 years that this technique is applied in our research group. We have ample experience with inducing arterial catheters, both inside and outside the MR facilities (n>200).

The outcome of this study may help delineating the pathogenesis of IAH and may create new avenues for its treatment. At the moment, very little is known about this condition. The remaining (small) risks for the subjects, as described in section 11.4, are therefore acceptable.

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