

Effect of lactate administration on cerebral blood flow during hypoglycemia in people with type 1 diabetes

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ABSTRACT

Introduction Impaired awareness of hypoglycemia, clinically reflected by the inability to timely detect hypoglycemia, affects approximately 25% of the people with type 1 diabetes. Both altered brain lactate handling and increased cerebral blood flow (CBF) during hypoglycemia appear to be involved in the pathogenesis of impaired awareness of hypoglycemia. Here we examine the effect of lactate on CBF during hypoglycemia.

Research design and methods Nine people with type 1 diabetes and normal awareness of hypoglycemia underwent two hyperinsulinemic euglycemic-hypoglycemic (3.0 mmol/L) glucose clamps in a 3T MR system, once with sodium lactate infusion and once with sodium chloride infusion. Global and regional changes in CBF were determined using pseudocontinuous arterial spin labeling.

Results Lactate (3.3 ± 0.6 vs 0.9 ± 0.2 mmol/L during lactate infusion vs placebo infusion, respectively) suppressed the counter-regulatory hormone responses to hypoglycemia. Global CBF increased considerably in response to intravenous lactate infusion but did not further increase during hypoglycemia. Lactate also blunted the hypoglycemia-induced regional redistribution of CBF towards the thalamus.

Conclusions Elevated lactate levels enhance global CBF and blunt the thalamic CBF response during hypoglycemia in patients with type 1 diabetes, mimicking observations of impaired awareness of hypoglycemia. These findings suggest that alteration of CBF associated with lactate may play a role in some aspects of the development of impaired awareness of hypoglycemia.

Trial registration number NCT03730909.

INTRODUCTION

Hypoglycemia is the most frequent adverse effect of insulin therapy in people with type 1 diabetes treated with insulin. If glucose levels drop, a hierarchically organized counter-regulatory response is initiated, which includes the release of counter-regulatory hormones and the appearance of hypoglycemic symptoms. The ventromedial hypothalamus is thought to be involved in the

Significance of this study

What is already known about this subject?

► Altered brain lactate handling and increased cerebral blood flow (CBF) during hypoglycemia may be involved in the pathogenesis of impaired awareness of hypoglycemia in type 1 diabetes.

What are the new findings?

► Intravenous lactate infusion increases the global CBF and blunts the hypoglycemia-induced regional redistribution of CBF towards the thalamus.
► Lactate infusion enhances global CBF.
► Lactate infusion blunts the thalamic CBF response during hypoglycemia in patients with type 1 diabetes.
► This mimics observations of impaired awareness of hypoglycemia.

How might these results change the focus of research or clinical practice?

► These results help to unravel the pathophysiology of impaired awareness of hypoglycemia; our findings suggest that local alterations of CBF associated with lactate contributes to the development of impaired awareness of hypoglycemia.

coordination of this sympathetic response to hypoglycemia.¹ Indeed, studies using neuroimaging techniques have shown that hypoglycemia results in a redistribution of cerebral blood flow (CBF) towards the thalamic areas, as a reflection of increased brain activity in patients with type 1 diabetes and normal awareness of hypoglycemia (NAH) and in people without diabetes.^{2–5}

About one in every four patients with type 1 diabetes is diagnosed with impaired awareness of hypoglycemia (IAH), a reduced ability to detect the onset of hypoglycemia.⁶ In patients with IAH, the glucose threshold for the initiation of a counter-regulatory hormone and symptom response is shifted to lower

glucose levels, creating a greater risk for severe, potentially hazardous, hypoglycemia that requires help from another person for recovery.⁷ Results of brain imaging techniques suggest that the hypoglycemia-induced relative increase in thalamic CBF is blunted in patients with IAH, indicating that hypoglycemia fails to activate brain areas that coordinate counter-regulatory responses.^{4,8,9}

Altered CBF response and altered brain lactate handling have both been implicated in the pathogenesis of IAH, and both are probably aimed at protecting the brain from harm induced by hypoglycemia. First, in patients with IAH, moderate hypoglycemia increases global CBF,³ a response that usually requires deeper hypoglycemia.^{10,11} Second, altered lactate handling of the brain may be a key process in the development of IAH.^{12–14} Lactate can be oxidized by the brain during hypoglycemia as an alternative fuel source when glucose supply is low. Our previous studies have indicated that both the capacity to transport lactate across the blood-brain barrier¹⁵ and that of lactate oxidation are upregulated in patients with IAH.¹⁴ The role of lactate in the development of IAH is further supported by the observation that administration of lactate diminishes symptomatic and hormonal responses to hypoglycemia,^{16,17} similarly to the situation seen in IAH.

It is usually assumed that the excess of lactate in the brain is only used for oxidation.^{18,19} However, brain lactate may also regulate cerebral perfusion to maintain brain glucose metabolism.¹³ As such, lactate may be involved in the upregulation of global CBF to enhance the supply of nutrients (ie, glucose) to the brain. To test this hypothesis, we examined the effect of lactate infusion during hypoglycemia on both regional CBF and global CBF, measured by magnetic resonance () techniques, in people with type 1 diabetes and NAH.

MATERIALS AND METHODS

This was a randomized, single-blinded, placebo-controlled crossover study that was performed at the Radboud University Medical Center in Nijmegen. The study was approved by the Institutional Review Board of the Radboud University Medical Center (online supplemental file 1) and performed according to the principles of the Declaration of Helsinki. All participants gave written informed consent before participation.

Study population

Patients with type 1 diabetes and NAH were recruited from the outpatient clinic of the Radboud University Medical Center and via advertisements on online diabetes platforms. The presence of NAH was assessed by a score of 0 or 1 on the Dutch-modified translation of the Clarke Questionnaire.²⁰ Patients with type 1 diabetes were potentially eligible for participation if they were younger than 50 years, had a body mass index below 30 kg/m² and HbA1c levels not exceeding 9.0% (75 mmol/mol). Exclusion criteria were the use of medication other

than insulin, except for oral contraceptives and stable thyroxine supplementation therapy, the presence of any other medical condition, microvascular or macrovascular complications of diabetes and MRI contraindications, such as claustrophobia or the presence of metal parts in the body. All subjects were invited for a medical screening prior to the investigational days to determine eligibility.

Study procedure

Participants underwent two hyperinsulinemic euglycemic-hypoglycemic glucose clamps, once with concomitant sodium lactate infusion (600 mmol/L) and once with saline infusion (500 mL, 0.9%) as placebo. The experimental days were separated by at least 2 weeks. In female subjects, both experiments were performed during equal phases of the menstrual cycle.

On each experimental day, subjects came to the MR research facility in the morning in fasting condition, having abstained from alcohol, smoking and caffeine containing substances for 24 hours and from strenuous exercise for 48 hours. In addition, participants received specific instructions to avoid hypoglycemic incidents the day and night before and the morning of the experimental day. Experiments were rescheduled in case of hypoglycemia (ie, glucose levels <3.0 mmol/L) in the 24 hours before the clamp.

On arrival at the MR research facility, the brachial or radial artery of the non-dominant arm was cannulated under local anesthesia (Xylocaine 2%) for frequent blood sampling. We measured arterial plasma glucose and plasma lactate levels (Biosen C-line, EKF Diagnostics) at 5-min intervals. An intravenous catheter was inserted in the antecubital vein of the contralateral arm for infusion of insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark), glucose 20% (Baxter, Deerfield, Illinois, USA), and sodium lactate (600 mmol/L; Spruyt Hillen, IJsselstein, the Netherlands, and prepared by the Department of Pharmacy, Radboud University Medical Center, Nijmegen, the Netherlands) or sodium chloride (500 mL, 0.9%) as placebo.

Subsequently, the participants were placed, in supine position and headfirst, in the MR scanner and a hyperinsulinemic euglycemic-hypoglycemic glucose clamp was applied. Insulin was infused at a rate of 60 mU/m²/min, and glucose 20% was infused at a variable rate, aiming for stable plasma glucose levels of ~5.0 mmol/L during the euglycemic phase and ~2.8 mmol/L during the hypoglycemic phase. After the acquisition of anatomical images, baseline CBF data and baseline blood samples (~20 min after start of the euglycemic clamp), the infusion of sodium lactate, or an equivalent volume of sodium chloride, was started. Sodium lactate infusion was started in a dose of 40 µmol/kg/min for 15 min, and then continued in a dose of 25 µmol/kg/min for the remainder of the experiment. We aimed for plasma lactate levels of 3.5 mmol/L. Subsequently, after ~25 min, plasma glucose levels were allowed to fall to 2.8 mmol/L over approximately 30 min and were maintained at that level for another 45 min.

Prior to entering the MR and at the end of the hypoglycemic phase, symptoms of hypoglycemia were assessed on a

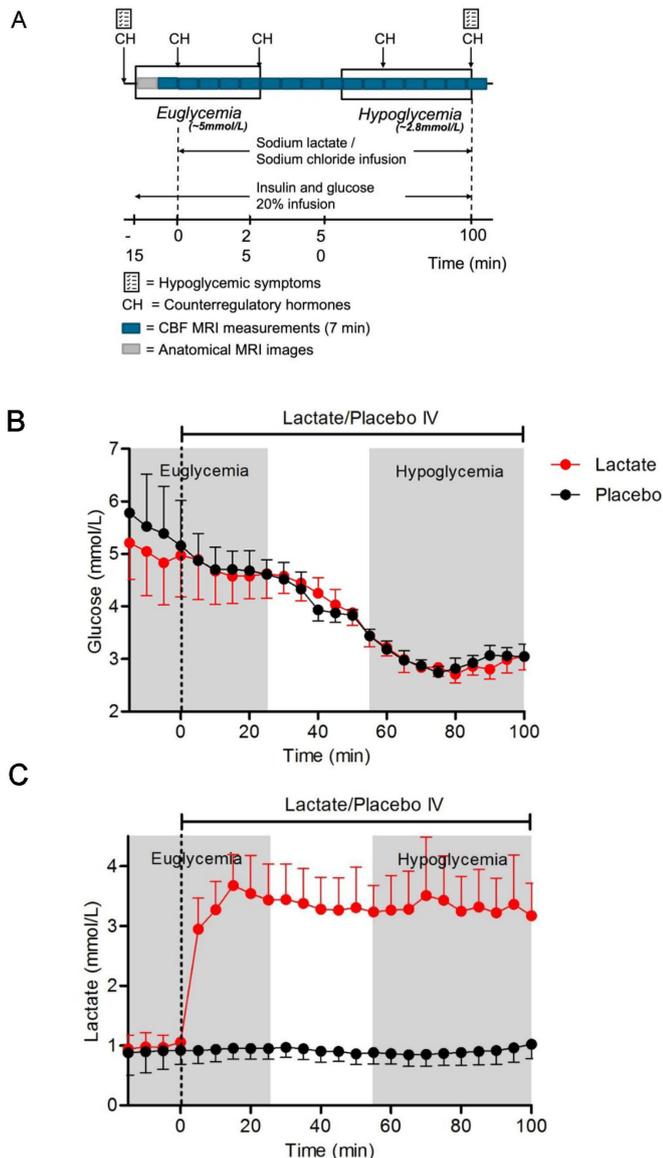


Figure 1 (A) Schematic overview of the study protocol, (B) Arterial glucose levels during lactate infusion (red circles) and placebo infusion (black circles) and (C) arterial lactate levels during lactate infusion (red circles) and placebo infusion (black circles). CBF, cerebral blood flow.

linear analog scale with a validated questionnaire.²⁰ Patients were asked to score 18 symptoms (autonomic, neuroglycopenic, general and dummy) from 0 to 6 (none to most severe). Additional blood was sampled for the measurement of plasma insulin, pH, glucagon, catecholamines, cortisol and growth hormone at several timepoints during the euglycemic and hypoglycemic phase, as indicated in figure 1A.

MRI protocol

MR measurements were performed at 3T (MAGNETOM Prisma-fit; Siemens, Erlangen, Germany) using a body coil for excitation and a 12-channel head coil for signal reception. After initial acquisition of localizer images, a T1-weighted magnetization-prepared rapid acquisition with gradient-echo MRI (3D MPRAGE) was acquired at 1

mm isotropic resolution. A time-of-flight angiogram was acquired to detect the brain-feeding internal carotid and vertebral arteries for accurate positioning of the 17-mm thick ASL labeling slab. The labeling slab was positioned perpendicularly to the feeding vessels, about 7–10 cm below the anterior commissure-posterior commissure line (AC-PC line).

The perfusion images were obtained with a pseudo-continuous arterial spin labeling (pCASL) MRI research sequence (Fraunhofer Institute for Digital Medicine MEVIS, Bremen, Germany). The postlabeling delay was set to 1800 ms, the labeling duration to 1800 ms and background suppression was applied with two hyperbolic secant pulses. A three-dimensional gradient and spin echo readout module was used (Field of View (FOV): 230×173 mm; in-plane resolution: 3.6 mm² and slices of 4.5 mm thickness; partial-Fourier factor of 7/8; turbo-factor 11). Echo time was 29.56 ms and repetition time (TR) was 4800 ms. We acquired 16 pairs of label and control images per timepoint, with a scan duration of 5:22 min. Subsequently, two reference images (proton density, TR: 7000 ms), with opposing in-plane phase-encoding directions were acquired without spin labeling and without background suppression for CBF quantification and distortion correction. The acquisition of pCASL MRI data and reference images was repeated continuously (every ~7 min) while the subjects stayed in the MR system.

At the end of euglycemic phase and at the beginning of hypoglycemia, a localizer and a time-of-flight angiogram was acquired to visually detect possible subject motion. If necessary, the pCASL MRI settings were readjusted accordingly to 7–10 cm below the AC-PC line.

Analytical methods

HbA1c was measured by the TOSOH G8 HPLC-analyzer, distributed by Sysmex. Plasma insulin was assessed by an in-house radioimmunoassay.²¹ Plasma glucagon was measured by radioimmunoassay (Eurodiagnostica, Malmö, Sweden). Plasma epinephrine and norepinephrine were analyzed by high-performance liquid chromatography combined with fluorometric detection.²² Plasma growth hormone and cortisol were determined using a routine analysis method with Electrochemiluminescent Immunoassay on a Modular Analytics E170 (Roche Diagnostics, Mannheim, Germany). pH was measured by routine arterial blood gas analysis on the RapidPoint 500 (Siemens Nederland, Den Haag, the Netherlands).

Processing of perfusion images

Images were processed using the FMRIB Software Library (FSL, V.6.0.0)²² and Advanced Normalization Tools (ANTs (2.2.0)).²³ Reference images, label and control images were motion corrected using linear affine registration (*MCFLIRT*²⁴). The motion-corrected label and control images were subtracted pairwise and averaged per timepoint. A susceptibility-induced off-resonance field was estimated²⁵ from the averaged two

proton density-weighted images (acquired with opposed phase-encoding directions), which was used to correct for susceptibility distortions in the proton density as well as in perfusion-weighted images.²⁶ The CBF maps were registered with the subject's anatomical T1-weighted images, which in turn were registered to the MNI152 atlas (MNI152_T1_0.5mm), using linear and non-linear registration (*antsRegistration*). Resampling the CBF maps to the MNI152 atlas allowed a voxel-wise comparison over all timepoints. CBF was quantified voxel-wise according to the guidelines from Alsop *et al.*²⁷ Global CBF values were obtained by averaging the CBF values in the gray matter.

Statistical analysis

Differences between study conditions (lactate vs placebo) were analyzed with paired Student's t-tests or Wilcoxon signed rank tests if data were not normally distributed. The difference in global CBF between periods of euglycemia and hypoglycemia within groups was calculated and expressed relative to baseline (ie, before the start of infusion of lactate or placebo started). Mean regional CBF values of caudate, cerebellum, putamen, insula and thalamus, as well as frontal, occipital, parietal and temporal lobes were extracted using region of interests from the MNI-maxprob-thr50-2mm atlas (*fslmeants*). These serial data, as well as average plasma glucose levels, counter-regulatory hormone responses and symptom scores, were compared with two-way repeated measure analysis of variance with a Bonferroni post hoc test using IBM SPSS Statistics 25. All data are expressed as mean±SD. Localized changes in CBF between the two infusion conditions was assessed with a voxel-wise analysis performed together with a parametric general linear model (*fsl_glm*). We generated a design matrix modeling infusion condition (lactate or placebo) and glycemic state (euglycemia or hypoglycemia). In addition, we controlled with covariates for the total gray-matter CBF individual and the subjects' intercepts to account for the repeated measurements. We tested for glycemic effects between infusion conditions. Voxel-wise statistics were corrected at a cluster level (*easythreshold*) and are represented as color-coded thresholded z-statistics overlaid on the MNI template (MNI152_T1_0.5mm). A p value <0.05 was considered statistically significant.

Data and resource availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

A total of 10 patients with type 1 diabetes and NAH were recruited. One patient withdrew after the first MRI because of a lack of time to plan the second MRI, two were withdrawn, one because of inability to perform arterial cannulation and the other because of insufficient quality

Table 1 Baseline characteristics

	n=9
Age, years	23.0±3.6
Male gender, n (%)	4 (44.4)
Weight, kg	75.1±13.7
Body mass index, kg/m ²	23.6±2.8
Score on modified Clarke Questionnaire	0.0 (0.0–0.5)
Duration of diabetes, years	7.0 (3.0–10.5)
Insulin therapy, n (%)	
CSII	6 (66.7)
MDI	3 (33.3)
Insulin dose, IU/day	47.9±19.2
HbA1c, % (mmol/mol)	7.1±1.0 (54.2±11.1)
Creatinine, µmol/L	67.1±12.0

Data are presented as n (%), mean±SD or median (IQR). CSII, continuous subcutaneous insulin infusion; MDI, multiple daily injections.

of CBF maps. These two participants were replaced. As a result, nine participants completed the study and were analyzed. Baseline characteristics of these patients are shown in [table 1](#).

Arterial blood glucose levels averaged at 4.7±0.5 mmol/L vs 4.8±0.4 mmol/L during the euglycemic phase (difference −0.09 mmol/L, p=0.66) and 3.0±0.0 mmol/L vs 3.0±0.1 mmol/L (difference −0.04 mmol/L, p=0.041) during the hypoglycemic phase for lactate and placebo days, respectively ([figure 1B](#)). Baseline plasma lactate levels were similar on both experimental conditions (0.7±0.2 mmol/L vs 0.6±0.2 mmol/L, lactate vs placebo, p=0.54). Plasma lactate levels increased within 15 min after start of lactate infusion to 3.8±0.5 mmol/L (p<0.001), but did not change when sodium chloride was infused (p=0.45 vs baseline levels) ([figure 1C](#)). Insulin levels were lower on the lactate infusion day at baseline (12.2±5.3 vs 18.1±9.3 mIU/L, p=0.049), but increased during the clamp to levels similar to those obtained on the placebo day (lactate vs placebo, p=0.51). Plasma pH increased from baseline to the end of hypoglycemia, but the increase was higher during lactate than during saline infusion (+0.09±0.03 vs +0.02±0.01, p=0.001). Among the three participants for whom such data were available, Pao₂ (arterial oxygen tension) levels fell during lactate infusion when compared with placebo infusion (15±0.6 vs 14.5±0.4 kPa and 14.4±0.4 vs 15.1±0.5 kPa, respectively, p for interaction=0.018), whereas PaCO₂ (arterial carbon dioxide tension) levels did not differ between the 2 infusion days (p=0.55). Glucose infusion rates during hypoglycemia were not significantly different between the 2 clamp days (3.7±2.8 vs 3.5±1.2 mg/kg/min, lactate vs placebo, p=0.75).

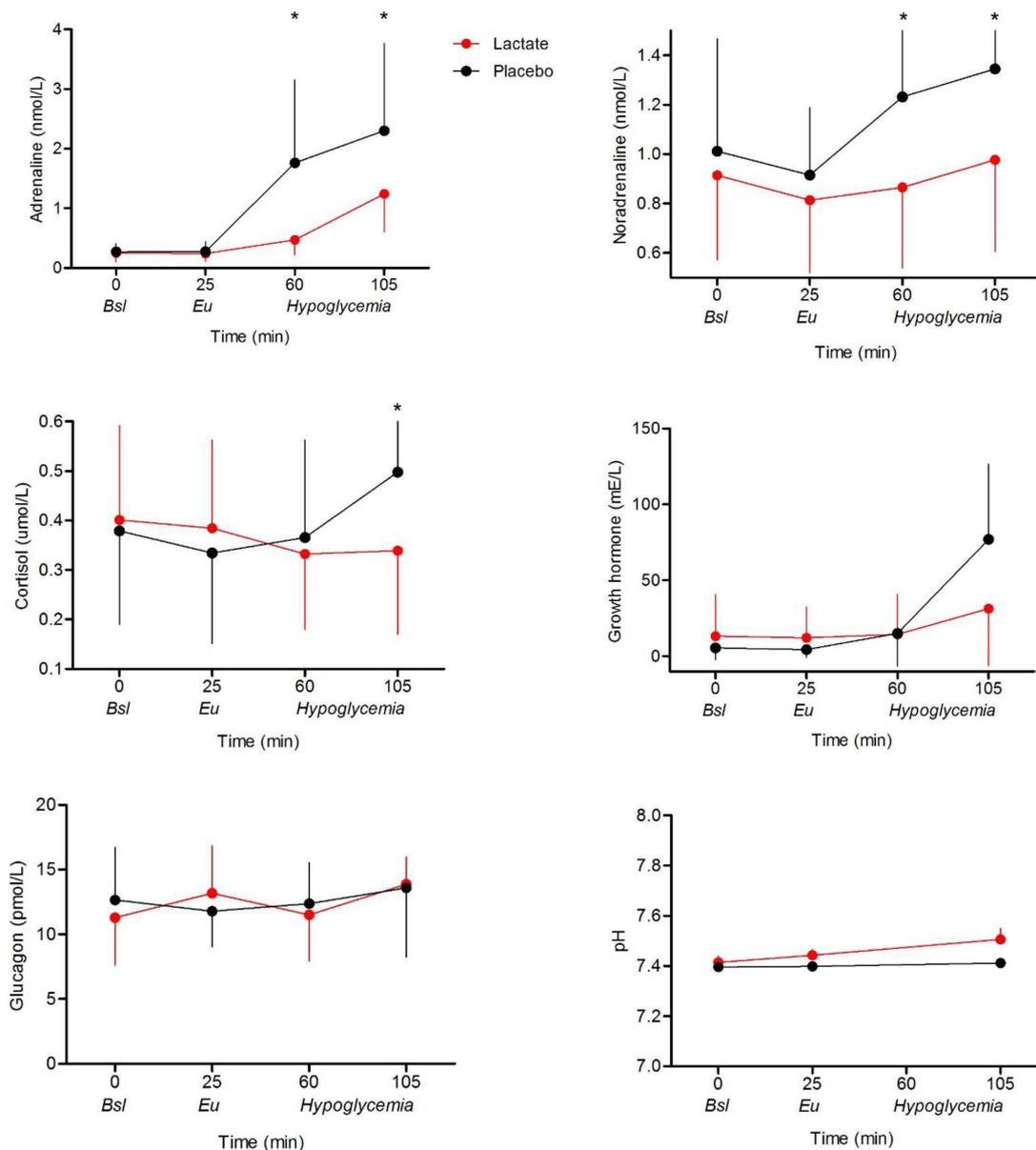


Figure 2 Counter-regulatory hormone levels at baseline (Bsl), during euglycemia (Eu) and during hypoglycemia, with lactate infusion (red) and placebo infusion (black), * $p < 0.05$.

Hypoglycemic symptoms and counter-regulatory hormone responses

Lactate infusion significantly suppressed epinephrine, norepinephrine, cortisol and growth hormone responses to hypoglycemia as compared with placebo (figure 2), while glucagon levels did not differ. The increase in total symptom scores in response to hypoglycemia was similar during infusion with lactate (from 7.5 to 26.7, $p = 0.002$) and saline (from 7.0 to 26.1, $p = 0.001$). This was also the case when symptoms were broken down to autonomic and neuroglycopenic scores (data not shown). Clamp order (lactate first, placebo second or the other way around) did not affect hormonal or symptom responses to hypoglycemia.

Global cerebral blood flow

Global CBF did not differ at baseline between 2 experimental days. Global CBF increased significantly during lactate infusion as compared with saline infusion ($17.4\% \pm 10.3\%$ vs $-2.0 \pm 6.5\%$, $p = 0.001$), but global CBF did not change further in response to hypoglycemia during either lactate ($15.2\% \pm 7.3\%$ vs $17.4\% \pm 10.3\%$, euglycemia vs hypoglycemia, $p = 0.46$) or saline infusion ($2.6\% \pm 9.9\%$ vs $-2.0\% \pm 6.5\%$, euglycemia vs hypoglycemia, $p = 0.16$) (figure 3A,B).

Regional cerebral blood flow

To assess localized changes in CBF across the whole brain in association with glycemic state and/or infusion type, we leveraged a voxel-wise statistical analysis of the CBF maps. Because lactate infusion leads to overall elevated CBF levels (figure 3A,B) and to determine regional

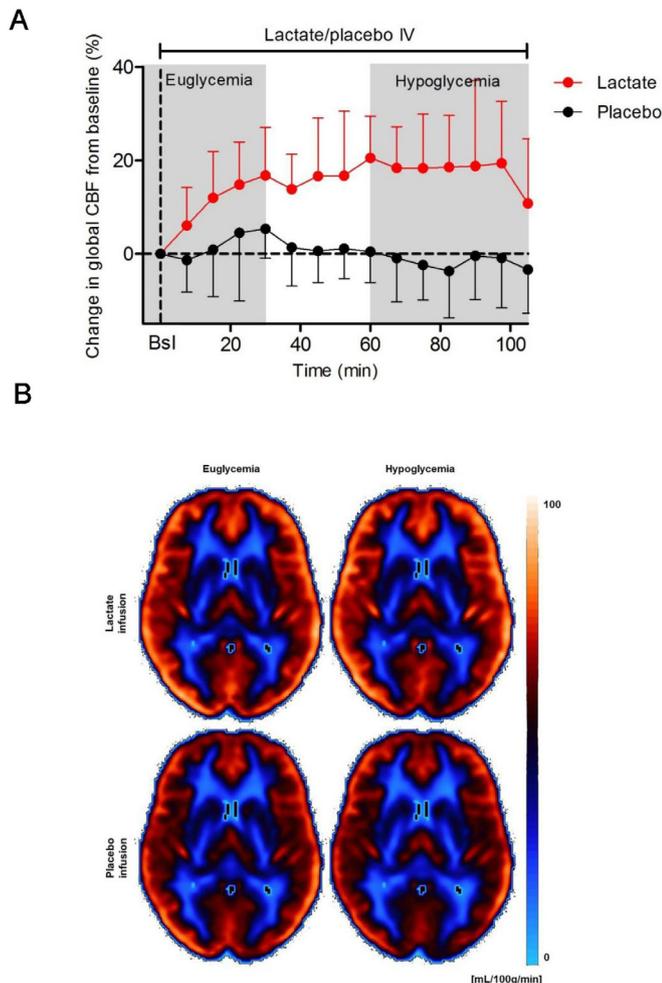


Figure 3 (A) Change in global cerebral blood flow (CBF) from baseline during lactate infusion (red circles) and placebo infusion (black circles). Bsl=baseline measurement; mean CBF value of three euglycemic CBF measurements without lactate/placebo infusion. (B) Quantitative CBF maps averaged over all subjects with type 1 diabetes mellitus and normal awareness of hypoglycemia during euglycemic and hypoglycemic state and during infusion of lactate and placebo. CBF values are color coded with the Flow(Brain colors) (iso) lookup table and range from 1 to 100 mL/100 g tissue/min.

responses, global CBF was accounted as a covariate in our analysis. The increase in CBF in response to lactate infusion (figure 4) was specifically prominent in the posterior cingulate, precuneus and parietal cortex. Significant clusters overlap with the thalamus, denoting a decrease in regional CBF under lactate relative to placebo infusion, during hypoglycemia. These localized effects were only present when gray-matter CBF was present as a covariate, indicating that thalamic and cingulate CBF changes are only relative to the global CBF levels. Thus, in addition to elevating global CBF, lactate infusion leads to localized CBF redistribution specifically within the thalamus and cortical areas and is dependent on the glycemic status (hypoglycemia vs euglycemia).

DISCUSSION

This study shows that lactate infusion significantly increases global CBF, to similar extent under euglycemic and hypoglycemic conditions in people with type 1 diabetes and NAH. The regional CBF in the thalamus region is blunted during hypoglycemia while CBF in the cingulate, precuneus and parietal cortex is enhanced during infusion of lactate. Altogether these findings suggest that local alterations of CBF associated with lactate may play a role in some aspects of the development of IAH.

Previous studies have described an increase in CBF in the thalamic area in response to hypoglycemia in patients with type 1 diabetes and NAH and in healthy controls, as a reflection of stimulated brain activity that drives the autonomic response.^{2 3 28} This increase in thalamic CBF is reduced or absent in patients with type 1 diabetes and IAH,^{4 8 9 29} mirroring the reduced autonomic response to hypoglycemia in these people. Here, we used a lactate infusion to blunt the autonomic response to hypoglycemia in patients with type 1 diabetes, thus mimicking the situation seen in people with IAH. Pathogenetic involvement of thalamic blood flow is further supported by the fact that lactate infusion resulted in a similar blunting of thalamic activation during hypoglycemia. Interestingly, using [¹⁵O]water positron emission tomography, Arbe-laez *et al* showed that antecedent hypoglycemia, a model for IAH, enhanced, rather than reduced, CBF response in the dorsal midline thalamus during subsequent hypoglycemia in healthy young adults.² Whether this points towards different pathophysiological mechanisms underlying counter-regulatory failure or reflect differences in level of detail, neuroimaging technique used and/or study population is uncertain.

We also found an overall increase in global CBF in response to the infusion of lactate, which is in accordance with previous studies.³⁰ For example, Stewart *et al* showed that infusion of a similar dose of lactate as used in the present study resulted in a comparable 20% increase in global CBF under otherwise physiological circumstances.³¹ The lactate-induced increase in global CBF might be caused by a shift in the NADH/NAD⁺ ratio and production of reactive oxygen species, making lactate a regulator of the cerebral microcirculation and thus of CBF.^{32 33} Previously, we found an increase of 8% in global CBF during hypoglycemia, in patients with type 1 diabetes and IAH.⁴ An increase in CBF could be seen as a neuroprotective response, as it enhances the supply of glucose and other energy nutrients to the brain. We speculate that the consequent maintenance of brain energy metabolism may contribute to the suppressed counter-regulatory hormone responses, symptoms of hypoglycemia and impeded hypoglycemic sensing by the brain, as seen in IAH. Similarly, the (twice as large) lactate-induced increase in global CBF may have contributed to the diminished counter-regulatory hormone responses to hypoglycemia, as seen in the current study.

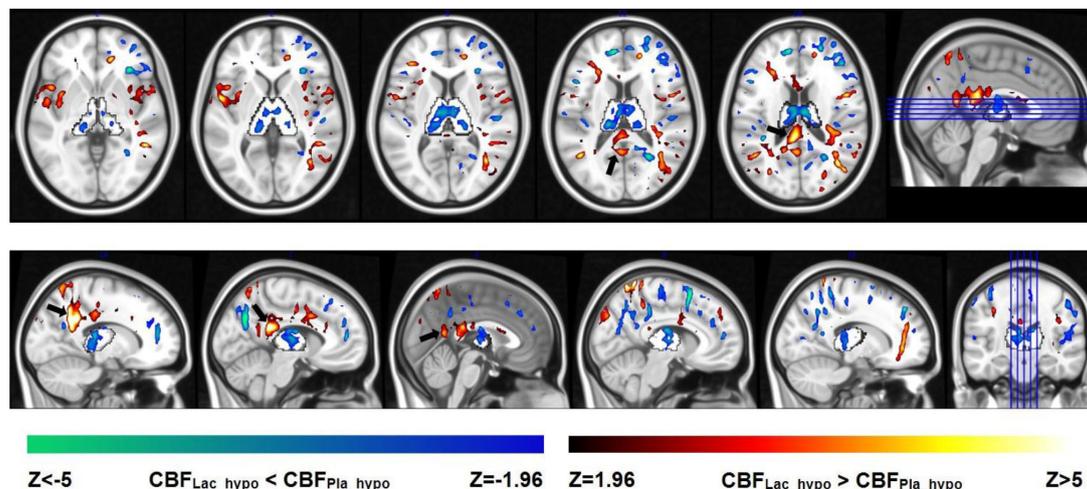


Figure 4 Redistribution of cerebral blood flow (CBF) in response to hypoglycemia by type of infusion in subjects with type 1 diabetes mellitus and normal awareness of hypoglycemia. Z-scores of infusion-induced significant changes ($p < 0.05$) in regional CBF are superimposed on the axial and sagittal views of the MNI-152 atlas and indicate a significant increase (red to yellow, 2 to 5 times SD) or decrease (dark to light blue, -2 to -5 times SD) in regional CBF in lactate relative to placebo infusion. The white underlay indicates the thalamus, and the black arrows point to the cingulate gyrus.

It has been assumed that brain lactate functions primarily as an energy substrate and as a useful alternative for situations of glucose deprivation. Indeed, we previously showed that brain lactate levels fall in response to hypoglycemia in people with type 1 diabetes and IAH,¹⁴ probably as a result of lactate oxidation. Another study also showed increased brain transport and increased metabolism of non-glucose fuels (ie, acetate and lactate) in people with type 1 diabetes and IAH.³⁴ Previous studies suggest that lactate also has a regulatory function with respect to brain perfusion.¹²⁻¹³ This may explain, at least in part, the increase in CBF during lactate infusion in our study and in turn the consequent greater capacity to transport lactate (and other energy substrates) over the blood-brain barrier, as we earlier observed in people with IAH.⁴⁻¹⁵ Whether the increase in pH contributed to the change in CBF cannot be derived from our study. However, the change was small and studies in people with traumatic brain injury show that respiratory alkalosis (through hyperventilation) reduces rather than increases CBF.³⁵ Pao_2 levels were somewhat lower during lactate infusion, which might have contributed to the increase in CBF.³⁶ However, the clinical relevance is uncertain, since the difference with placebo was small, Pao_2 levels remained within the physiological range and we were unable to measure Pao_2 locally in the brain.

Our study has limitations. First, the sample size is rather small although this is typical for this type of study. Furthermore, while the hormonal response to hypoglycemia was clearly blunted on lactate infusion, surprisingly, hypoglycemic symptoms were not. Participants and personnel were blinded to the type of infusion, but participants were informed that hypoglycemia would be induced during both experimental days, although not when this would occur. However, this knowledge and the consequent expectations may have influenced the

subjective reporting of symptoms. The discomfort of lying in the MR scanner may also have contributed to the experience of symptoms. Furthermore, previous studies have shown hormonal and symptom responses to hypoglycemia may not change in parallel in response to an intervention.³⁷ Also, the difference in electrolyte concentration between the lactate and placebo solution needs to be considered, in that the sodium lactate solution is hypertonic and the sodium chloride is isotonic. Although hypertonic solutions may cause fluid shifts over the blood-brain barrier, this is unlikely to have had a major impact and since the glucose solution used for the clamp in both study arms is also hypertonic. Furthermore, saline loading has been shown to enhance rather than suppress hormonal responses (of particularly glucagon) to hypoglycemia.³⁸ Infusion of lactate in our study increased plasma lactate to supraphysiological levels. Therefore, the effect on CBF and counter-regulatory hormones may not be comparable to the situation seen under physiological conditions. However, previous studies also administered pharmacological doses of lactate, making our results comparable to other studies. Finally, although we measured pH, Pao_2 and $PaCO_2$ in arterial blood, we did not measure other parameters that could explain the effect of lactate on CBF, such as cerebral perfusion pressure, cerebral metabolic rate of oxygen, nitric oxide, cardiac output, hematocrit or plasma electrolytes.

In conclusion, we found that lactate infusion increases global CBF and reduces the thalamic CBF response during hypoglycemia in patients with type 1 diabetes and NAH, which mimics previous findings in patients with IAH. These results strengthen the suggestion that alteration of CBF associated with lactate may play a role in some aspects of the development of IAH. Further studies are needed to explain mechanism(s) underlying this effect of lactate.

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Contributors LAVM, ECW and BEdG designed the study, with input from MvdG, CJT, AH, JJAva and JG. LAVM recruited the participants and performed the glucose clamps. LAVM, JJAva and ECW collected the data. ECW, JJAva and JG analyzed the MRI data, with input from MvdG and AH. LAVM analyzed all other data, with input from BEdG and CJT. All authors discussed the results and implications and commented on the manuscript at all stages. BEdG is the guarantor of this work.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was approved by the Institutional Review Board of the Radboud University Medical Center (CMO Arnhem Nijmegen), reference no 2017-3968. All participants gave written informed consent before participation.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

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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?

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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 productinformatie 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)

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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 hypoglycemia (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.

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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,

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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 ¹H-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 1×10^6 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)	x10⁶ 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		

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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. OBJECTIVESPrimary 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.

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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/meedoeaanonderzoek) 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.

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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/m²
- 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:
 - o Proliferative retinopathy
 - o Symptomatic diabetic neuropathy (including autonomic neuropathy)
 - o Nephropathy; clinical/overt albuminuria or an estimated glomerular filtration rate <60ml/min/1.73m²
- 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 $14 \pm 4\%$ 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).

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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 $60 \text{ mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$, 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 administered 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.

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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).

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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 $\mu\text{mol}/\text{kg}/\text{min}$ after a priming/bolus dose of 40 $\mu\text{mol}/\text{kg}/\text{min}$ 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 $\mu\text{mol}/\text{kg}/\text{min}$ for 15 min) continuous (25 $\mu\text{mol}/\text{kg}/\text{min}$ 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.

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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 (Gevalen 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 (Overdoseringsen) 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

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180 cm and – consequently – a body surface area of 1.936 m², the required insulin infusion can be calculated as: $1.936 \times 60 \times 60 \div 1000 = 7.0$ 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 m², the required insulin infusion can be calculated as: $1.936 \times 60 \times 60 \div 1000 = 7.0$ 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·m²·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)

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-
- 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. HIF1 α , 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, single-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.

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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/m²/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

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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 administered 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 ^1H 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 ^1H -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.

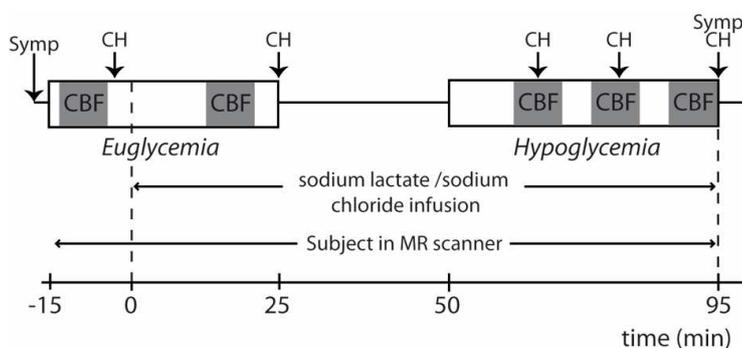


Fig. 1 Schematic overview of the study protocol. Cerebral blood flow (CBF) will be determined before the infusion of sodium lactate/placebo and as indicated during euglycemia and hypoglycemia. CH: determination of counterregulatory hormones; Symp: hypoglycemic symptom questionnaire.

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.

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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;

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-
- 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;

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-
- 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.

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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.

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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

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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.

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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.

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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, trial 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.

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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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14. REFERENCES

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