PROMETEUS

preterm brain-oxygenation and metabolic eu-sensing

D3.2 – Results of rats study

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V2	17/07/2026	Clothilde Courivaud	Addition of material and methods and results	Draft
V3	27/07/2026	Clothilde Courivaud	Insertion of last results obtained and description of them	Draft
V4	28/07/2026	Clothilde Courivaud	Minor changes regarding some details and miswriting	Draft

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1. Context

The aim of the animal study is to provide a framework for personalized brain nutrition in premature babies, based on real-time monitoring of cerebral blood flow and oxygenation on the one hand, and blood metabolites on the other. These data will be used to create a neonatal mathematical model linking brain hemodynamic with metabolism. To achieve this goal, we studied the relationship between different key brain energy fuels, cerebral blood flow and cerebral oxygenation based on data obtained in rat pups under different glycemic conditions (euglycemia, hyperglycemia, hypoglycemia). In this deliverable, results of experiments done are reported. Experiments had received the approval of the ethical committee (Deliverable 3.1).

2. MRI experiments

The aim is to study the cerebral blood flow (CBF) and cerebral oxygenation (SO2) under different glycemic conditions on rat pups.

2.1 Animal groups and experiment timeline

The experiment was conducted on young rats after an early weaning at post-natal day 13. All the animals were assigned to three groups, hypoglycemic (n=14), hyperglycemic (n=14) and normoglycemic (n=11). Blood glucose, lactate and BHB concentration were monitored with handhold devices before, during, and after MRI acquisitions (Fig. 1).
The MRI protocol was performed using a Bruker 9.4 T scanner. High resolution anatomical images were acquired with a spatial resolution of 156x156x500 μm^3 and maps with a spatial resolution of 208x208x1000 μm^3 (Table 1). The protocol was performed in three steps. The first two steps correspond to the time required to reach and stabilize the desired glucose level, and the last step correspond to the acquisition of the physiological maps:

- Step1: Between 0 and 30 minutes, the MRI was setup and two anatomical images were obtained using a T2-weighted TurboRARE sequence, one with higher resolution for atlas alignment. The apparent diffusion coefficient (ADC) of water was assessed via Diffusion Tensor Imaging using Echo-Planar Imaging (DTI-EPI). T1 map and inversion efficacy of arterial labelling were measured.
- Step 2: Between 30 and 90 minutes, CBF was measured with continuous arterial spin labelling (CASL) every 10 minutes.
- Step 3: Between 90 and 130 minutes, once the glucose concentration was stabilized, a T2 map was obtained using a Multi-Slice Multi-Echo (MSME) sequence. Blood volume fraction (BVf), vessel size index (VSI) and SO2 were derived from multi gradient-echo and Multi Gradient Echo Sampling of Free Induction Decay and Spin Echo (MGEFIDSE) sequences, acquired before and 1 minute after the injection of ultrasmall particle of iron oxide (USPIO).

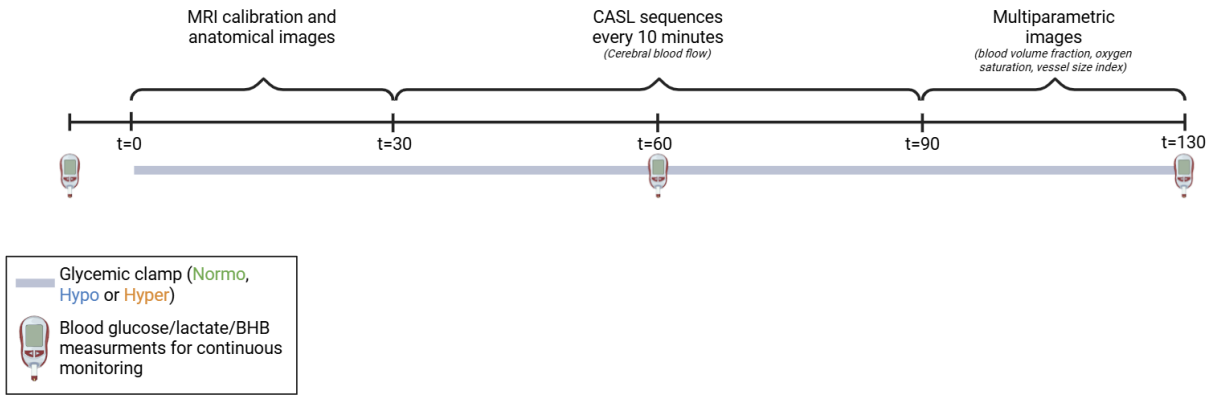


Fig. 1 MRI experiment timeline

Sequence	TR/TE (ms)	Other parameters	Field of view (mm²)	Voxel Size (μm³)	Acquisition Time
T2 TurboRARE	2500/33	RARE-factor=8 N. Avr=2	20x20	208x208x1000	1 min
T2 TurboRARE High_Res	2500/30	RARE-factor=8 N. Avr=8	20x20	156x156x500	5 min 20 s
DTI-EPI	3000/26.5	6 directions b-values=0.8 s.mm²	20x20	208x208x1000	1 min 06 s
T1-MTI	12000/15	12 TI= (14;5000 ms)	20x20	208x208x1000	4 min 26 s
Inv. Eff.	250/3.9	N. Avr=4	17x17	89x89x800	4 min 24s
CASL	4000/8.5	30 repetitions, LD=3000 ms, PLD=300 ms	20x20	208x208x1000	8 min
MSME	2000/TE_1=6	ΔTE=6 ms, 30 echoes	20x20	208x208x1000	3 min 12 s
MGE3D	200/TE_1=13	ΔTE=4.03 ms, 24 echoes	20x20	104x104x500	6 min 54 s
MGEFIDSE	4000/TE_1=2.3	ΔTE=2.3 ms, 32 echoes, spin echo time=50 ms N. Avr=2	20x20	208x208x1000	12 min 48 s

Table 1. MRI sequences parameters. TR: repetition time; TE: echo time; Inv. Eff.: Inversion efficacy; N. Avr.: number of averages; TI: inversion time; LD: label duration; PLD:post labeling delay

2.2 Metabolic monitoring

The three glycemic conditions (i.e. normoglycemia, hypoglycemia and hyperglycemia) were achieved experimentally through the clamp technique. Briefly, intravenous infusions of insulin and glucose were performed to maintain plasma glucose almost constant and in the range of 80-120 mg/dL for normoglycemia, 200-300 mg/dL for hyperglycemia and 30-70 for hypoglycemia.

- Normoglycemia was induced by insulin infusion (5 mU/kg/min bolus for 5 min, followed by a continuous infusion of 1 mU/kg/min),
- Hypoglycemia was induced by insulin infusion (10 mU/kg/min bolus, followed by 5 mU/kg/min continuously)
- Hyperglycemia was induced by glucose infusion (30 mg/kg/min bolus, followed by 5 mg/kg/min continuously).

The rather frequent monitoring of blood glucose allowed us to ensure that target glycemia is reached (Fig. 2). Glycemia was measured using a handhold glucose meter based on a blood drop obtained by cutting the tip of the paw.

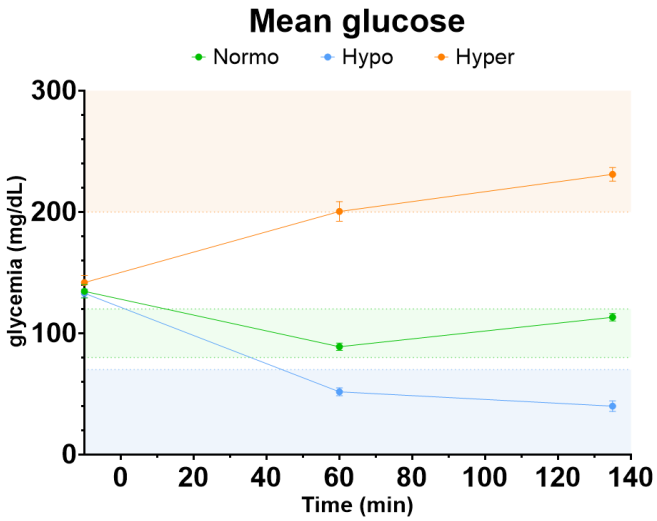


Fig. 2 Blood glucose concentration is maintained as the target value for each group. Results are shown as mean ± SEM.

2.3 MRI data

MRI data were analyzed and different parameter maps were calculated using MP3 software (Brossard et al. 2020). Representative images obtained for ADC, BVf, SO2, VSI and CBF are displayed in figure 3.

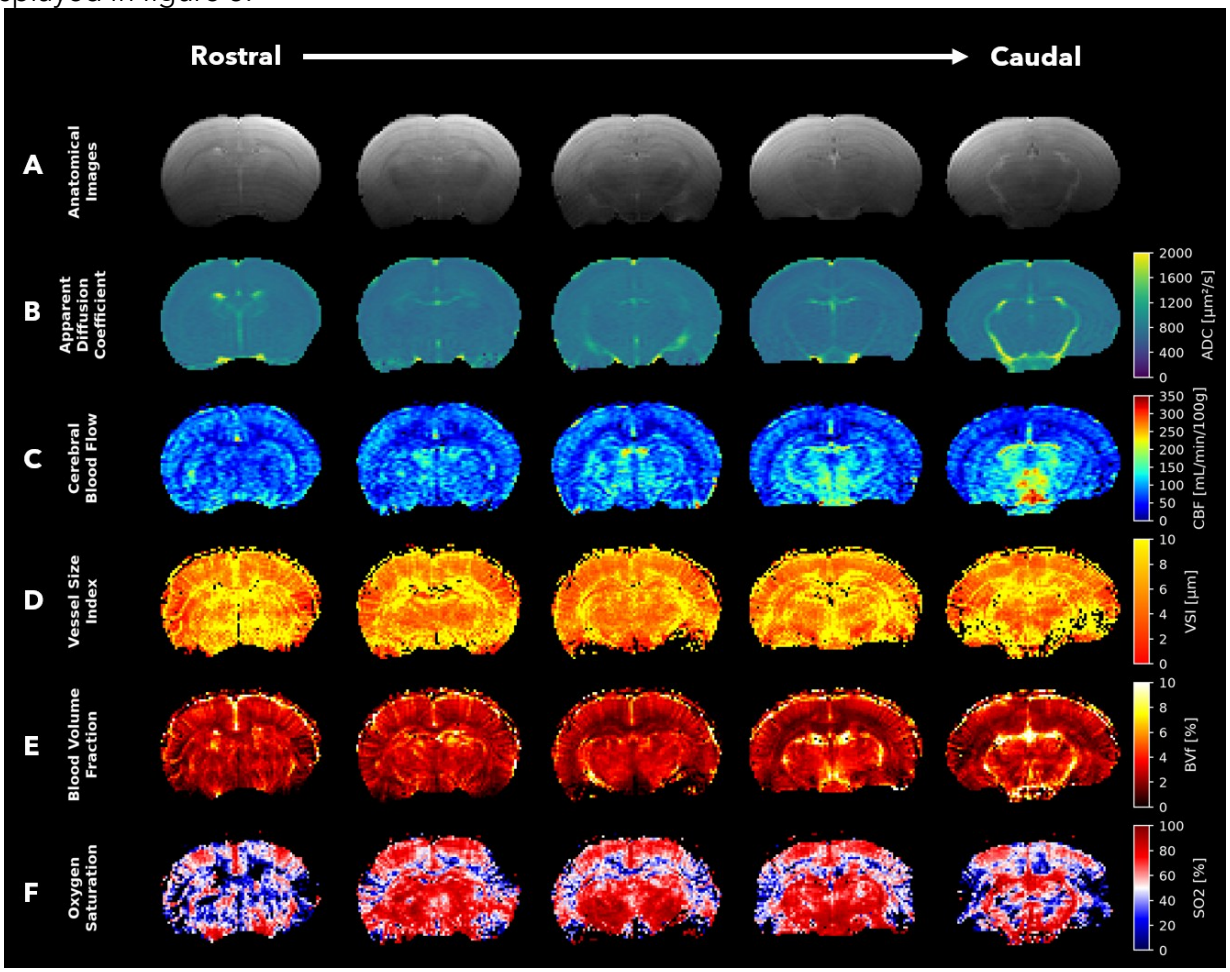


Fig. 3 Representative maps of the different parameters measured. All images are from a normoglycemic subject. Anatomical images (A), ADC (B), CBF (C), VSI (D), BVf (E), SO2 (F). (A) and (B) were acquired before 30 minutes. (C) was acquired between 80 and 90 minutes. (D), (E) and (F) were acquired between 90 and 130 minutes.

A preliminary analysis was performed on the right cortex which is easily identifiable and can be isolated to draw manually a region of interest, especially since it is an area with a strong MRI signal. The mean value obtained in this region for each animal is plotted figure 4.

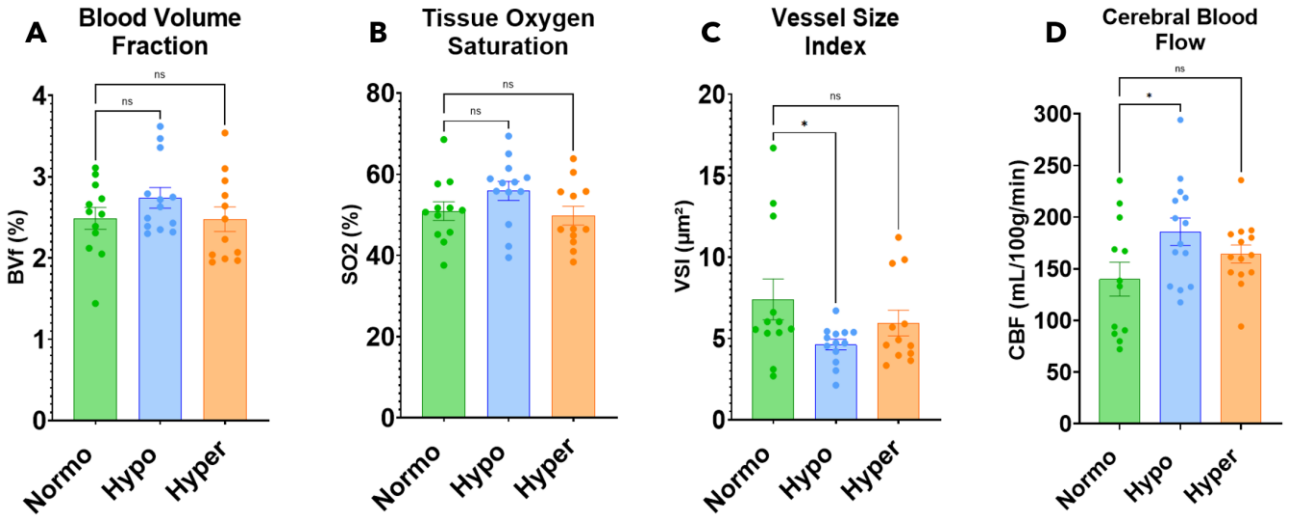


Fig.4 Mean values measured in the right cortex for BVf (A), SO2 (B), VSI (C) and CBF (D). Results are shown as mean ± SEM. One-way ANOVA post-hoc Dunnett's multiple comparison test; *p<0.05, ns: not significant.

The CBF values correspond to that of the last acquired CASL, i.e. between 80 and 90 minutes and just before the acquisition of the multiparametric images. We observed a significant increase of CBF in the hypoglycemic group compared to the normoglycemic group, mean value (185.8 ± 50.0 vs 140.0 ± 56.6 mL/100g/min, p=0.0325). No change was observed for the hyperglycemic group. Regarding BVf, SO2 and VSI, we observed a reduction in the microvessel diameter under hypoglycemia (5.94 ± 2.71 vs 4.61 ± 1.19 µm, p=0.049). No significant differences were observed between groups for BVf and SO2.

Cerebral blood flow (CBF) was measured every 10 minutes from 30 to 90 minutes after the induction of the glycemic clamp. The temporal evolution of CBF is presented in figure 5. Throughout the 60-minute measurement period, CBF remained consistently higher under hypoglycemic conditions compared with euglycemic conditions. Statistical analysis revealed significant differences between the two conditions at 50–60 minutes (p = 0.0443) and 70–80 minutes (p = 0.0265). No statistically significant differences were observed at the other time points. Altogether, these data suggest that both hypo- and hyperglycemia induce an increase in CBF, the one related to hypoglycemia being larger than that related to hyperglycemia.

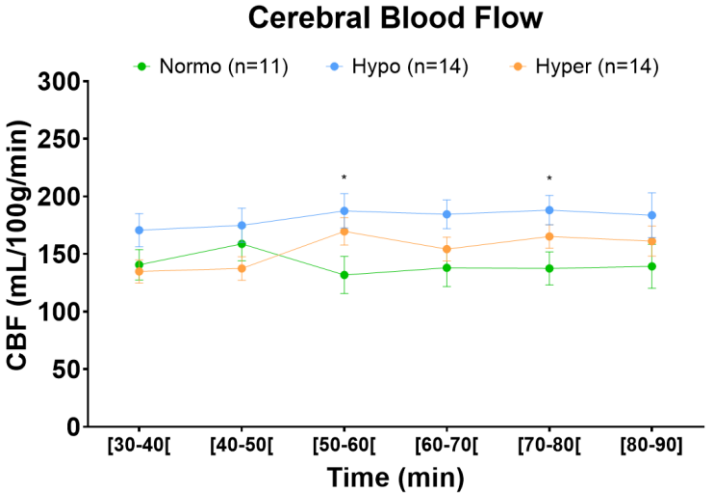


Fig. 5 Evolution of CBF measured during a 10-minute interval for 60 minutes. Measurements started 30 minutes after the induction of glycemic clamp. Results are shown as mean ± SEM. Two-way ANOVA post-hoc Dunnett's multiple comparisons test; *p<0.05, ns: not significant.

Note that these results are restricted to the right cerebral cortex. Future work will extend these measurements to other brain regions through the application of a dedicated brain mask and atlas. This step has required additional methodological development, as the immature rat pup brain could not be analyzed using the standard processing pipelines developed for adult rats or mouse brains.

Raw data were gathered on an Excel file named "MRI_Raw_Data.xlsx". This file is composed of two sheets, one named "Glossary" with all acronyms used in the second sheet. The second sheet, named "Raw_Data", is composed of 37 columns representing a parameter and 40 rows, one per animal. Parameters are listed below, with meaning in brackets:

- Animal (name of the animal)
- Group (normo-, hypo or hyperglycemia)
- Sex (male or female)
- Age (age in days when MRI was performed)
- Weight (weight in grams when MRI was performed)
- Clamp_time (when the glycemic clamp starts, set as t=0)
- ADC-value (value of the ADC in the right cortex)
- T1_value (value of T1 in the right cortex)
- CASL1_time (start of the first CASL acquisition)
- CASL1_value (value of T1 in the right cortex)
    - Those two columns are repeated until CASL6
- T2_time (start of multiparametric images acquisitions)
- T2_value (value of T2 in the right cortex)
- BVf_value (value of BVf in the right cortex)
- SO2_value (value of SO2 in the right cortex)
- VSI_value (value of VSI in the right cortex)
- Glu1_time (time when first metabolites measurements were done)
- Glu1_value (value of the first glucose measurement, before MRI)
- Lac1_value (value of the first lactate measurement, before MRI)
- BHB1_value (value of the first BHB measurement, before MRI)
    - These four parameters are repeated for the second measurement done during MRI and the third one done after MRI.

3. Tracers experiment

The aim is to study glucose, lactate and BHB turnover under different glycemic conditions.

3.1 Set up of the experiment

For this experiment, different optimizations steps had to be performed, for the reasons exposed here after. First, it is necessary to insert in a reproducible way 3 catheters on rat pups: one in caudal vein to infuse insulin and glucose to induce glycemic clamp, a second one in femoral vein to infuse tracers and the last one in femoral artery to sample blood. This surgery is not at all straightforward due to the very small size of rat pups, as well as to the necessity that all 3 catheters are successfully placed, otherwise the rat is lost and must be sacrificed. During this experiment, a mix of three different tracers was infused, D7-glucose, 3^{13}C-lactate and $^{13}C_4$-BHB. The infusion rates that were evaluated are not described here and the infusion rates used for the final experiment are described below (section 3.3).
Once mastered, we started feasibility tests with mass spectrometry (MS) platform GExiM in

Grenoble (France). Metabolites detection in plasma samples was difficult, and for glucose, the detection mode used was not appropriate. At the end, after 3 campaigns of feasibility experiments, i.e. 8 months later, the GExiM platform gave us a "NO-GO" decision. Therefore, another platform was approached.

3.2 Mass spectrometry feasibility

To calibrate and optimize the tracer experiment, we performed feasibility tests with the MS platform MetaToul, located in Toulouse (France). They could confirm, after 3 tests, that tracers were detectable and reached equilibrium. This allows us to calculate metabolic turnovers (Fig. 6). From these results, the final experiment time was increased by 1 hour and the infusion rate of glucose was also optimized.

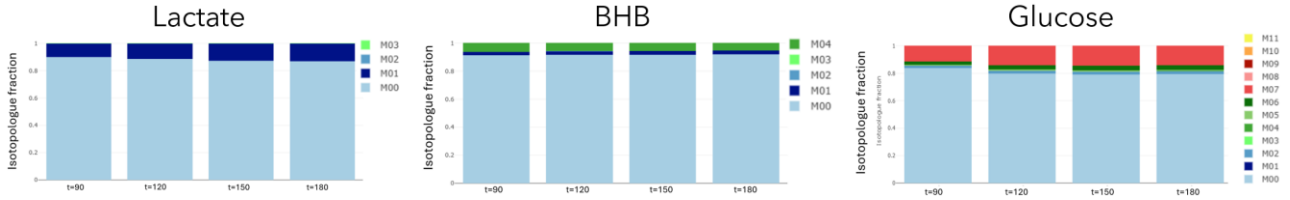


Fig. 6 Isotopologue fraction of lactate, BHB and Glucose in arterial blood sample at 90, 120, 150 and 180 minutes after the beginning of tracer perfusion.

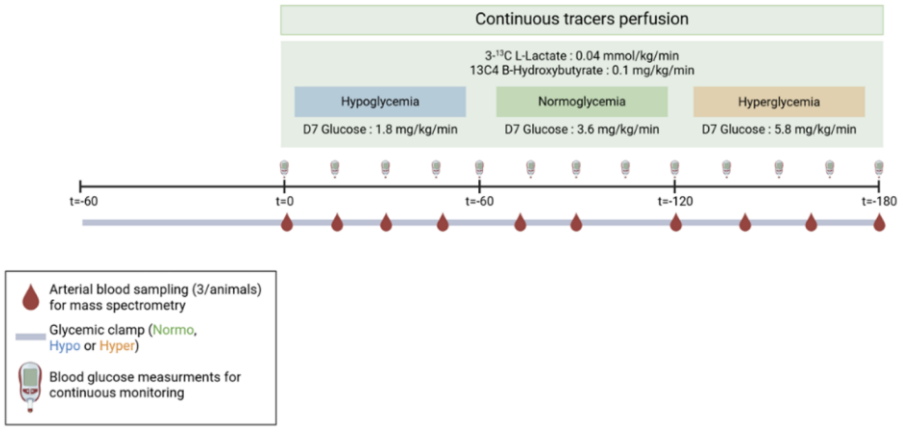
3.3 Animal groups and experiment timeline

The experiment was conducted on young rats after an early weaning at post-natal day 13. All the animals were assigned to three groups; hypoglycemic (n=12, 6 males), hyperglycemic (n=12, 6 males) and normoglycemic (n=12, 6 males). Animals underwent a glycemic clamp 1 hour prior to the perfusion of labelled glucose, lactate and BHB. Tracers were infused at different rates:

- Normoglycemia: 3^{13}C-lactate 0.04 mmol/kg/min; ^{13}C4-BHB 0.1 mg/kg/min; D7-glucose 3.6 mg/kg/min
- Hypoglycemia: 3^{13}C-lactate 0.04 mmol/kg/min; ^{13}C4-BHB 0.1 mg/kg/min; D7-glucose 1.8 mg/kg/min
- Hyperglycemia: 3^{13}C-lactate 0.04 mmol/kg/min; ^{13}C4-BHB 0.1 mg/kg/min; D7-glucose 5.4 mg/kg/min

Blood glucose concentration was monitored every 15 minutes (Fig. 7A). To track the incorporation of the tracers over time—and since a maximum of three samples can be collected per animal (10% of total blood volume)—we choose a sampling schedule to distribute the data over the three-hour tracer infusion period. At least two samples were collected per timepoint, and we made sure that the gender distribution was balanced for each group and each timepoint (Fig. 7B).

A



B

Animal	Timepoint 1	Timepoint 2	Timepoint 3
♂	0	50	180
♀	0	50	180
♂	0	70	180
♀	0	70	180
♂	15	90	180
♀	15	90	180
♂	15	120	180
♀	15	120	180
♂	30	140	180
♀	30	140	180
♂	30	160	180
♀	30	160	180

Fig. 7 (A) Tracer experiment timeline. (B) Arterial blood sample timepoint for each animal.

3.4 Physiological monitoring

The monitoring of blood glucose allows us to ensure that target glycemia is reached and stable over time (Fig. 8). Glycemia was measured using a handhold glucose meter based on a blood drop obtained by cutting the tip of the paw. Briefly:

- normoglycemia was induced by insulin infusion (5 mU/kg/min bolus for 5 min, followed by a continuous infusion of 1 mU/kg/min)
- hypoglycemia was induced by insulin infusion (10 mU/kg/min bolus, followed by 5 mU/kg/min continuously)
- hyperglycemia was induced by glucose infusion (30 mg/kg/min bolus, followed by 5 mg/kg/min continuously).

As each clamp lasted 4 hours, insulin and glucose infusion rates were manually adjusted (increased, decreased, or temporarily stopped) as needed. Additional boluses of insulin or glucose were administered when required to counterbalance the effect of the other infusion and maintain the target glycemic level. We considered that normoglycemia was reached when blood glucose concentration was between 80 and 120 mg/dL, hyperglycemia when glycemia was between 200 and 300 mg/dL, and hypoglycemia when glycemia was between 30 and 70 mg/dL.

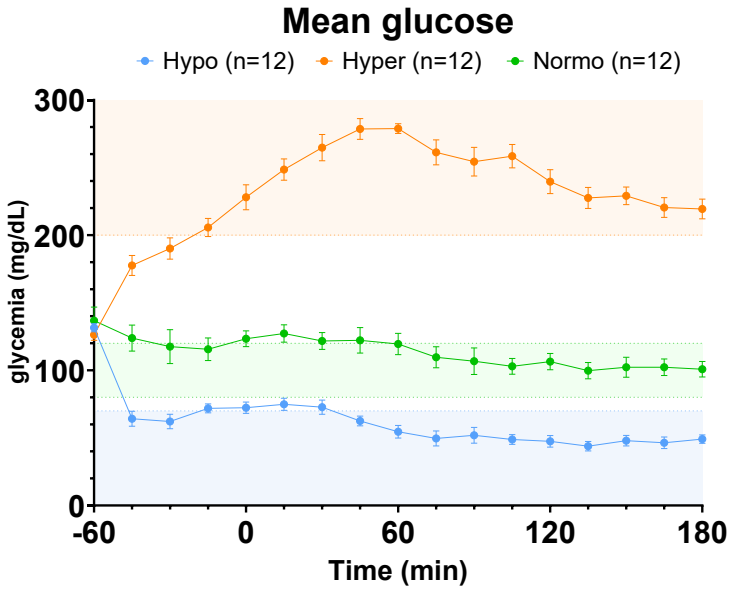


Fig. 8 Blood glucose concentration is maintained as the target value for each group. Results are shown as mean +/- SEM.

3.5 Mass spectrometry analysis

During experiment, 108 samples were collected and stored at -80°C. They were finally all sent to MetaToul. MS analysis and data processing are now completed by the platform. The MS analysis includes a preparation step, i.e. metabolite extraction with organic solvent for all samples, and a purely analytical step, i.e. the liquid-chromatography MS (LC-MS) analysis. Data processing is the necessary step that must be applied to extract the relevant information from raw MS spectra (which contains thousands of peaks). This includes annotation of peaks, identification of isotopologues, isotopologues incorporation kinetics, calculation of tracer-to-tracee ratio and calculation of turnovers. At the end, the platform provided an excel file, the 13th of July, which gathers all these data. We are currently further analyzing the results.
The first results obtained was the turnover of the three metabolites, glucose, lactate and BHB, depicted in figure 9. Turnovers were calculated based on the amount of tracer measured and its natural equivalent, called tracee. Glucose turnover in hypoglycemia is 149.1 ± 9.03 nmol/g/min, which is significantly higher than normoglycemic group (104.7 ± 8.26 nmol/g/min, p=0.0046). Contrary to glucose, lactate turnover decreased during hypoglycemia compared to normoglycemia (229.2 ± 11.87 vs 346.7 ± 35.9 nmol/g/min, p=0.0022). For both glucose and lactate turnover, no significant change was measured for hyperglycemia. The analysis of BHB turnover revealed no change in hypoglycemic condition compared to normoglycemia but revealed a decrease during hyperglycemia (35.88 ± 4.1 vs 22.83 ± 1.68 nmol/g/min, p=0.01).
Further analysis will also allow us to investigate the kinetics of tracer incorporation as well as the tracer-to-tracee ratio.

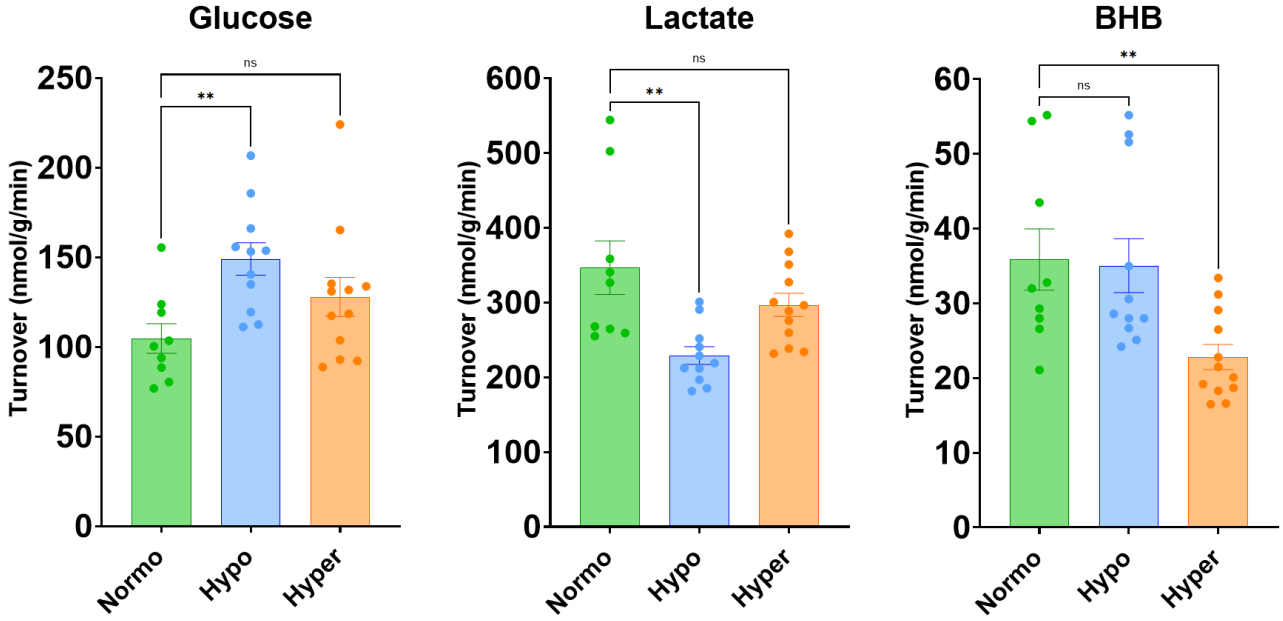


Fig. 9 Turnover of glucose, lactate and BHB. Turnover is calculated when isotopic enrichment reaches equilibrium, at 180 min. Results are shown as mean +/- SEM. Kruskal-Wallis test; **p<0.01, ns: not significant.

4. Conclusion

The rat pup study was performed as planned. MRI and MS results were sent to the UNIPD partner. Preliminary results of quantitative MRI maps on the right cerebral cortex show an effect of hypoglycemia on CBF which increases, and a decrease on vessel radius. BVf and SO2 do not change under glycemic conditions compared to normoglycemia group. Hyperglycemia also shows a trend towards an increase in CBF. Further analysis must be performed to evaluate regionally specific changes under hyperglycemic and hypoglycemic conditions.
First analysis of MS results shows changes in metabolites turnover. During hypoglycemia, glucose turnover increases, meaning that glucose is more produced and consumed, conversely to lactate,

whose turnover decreases during hypoglycemia compared to normoglycemia. For those two metabolites, hyperglycemia does not impact the turnovers, however, elevated blood glucose concentration decreases the turnover of BHB. Future analysis will provide the kinetics of tracer incorporation as well as the tracer-to-tracee ratio.