Universitat Autònoma de Barcelona

HIGH-RESOLUTION MICRODIALYSIS TO DETECT METABOLIC DYSFUNCTION AFTER TRAUMATIC BRAIN INJURY

Ángela Sánchez Guerrero

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Universitat Autònoma de Barcelona

Surgery and Morphological Sciences Department
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DOCTORAL THESIS

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PhD student: Ángela Sánchez Guerrero

PhD directors: Juan Sahuquillo Barris and Maria Antònia Poca Pastor

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DOCTORAL THESIS

High-resolution microdialysis to detect metabolic dysfunction after traumatic brain injury

Report submitted by Ángela Sánchez Guerrero to qualify for Doctorate degree by the *Universitat Autònoma de Barcelona*.

This thesis has been conducted in the Neurotraumatology and Neurosurgery Research Unit (UNINN) at Vall d'Hebron Research Institute (VHRI).

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“Experience is the worst enemy of innovation”.

Anonymous

“If you want different results, do not do the same things”.

Albert Einstein (1879-1955)

INDEX

ABBREVIATIONS AND ACRONYMS

A	Surface of the microdialysis membrane
ABG	Arterial blood gases
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANLS	Astrocyte-neuron lactate shuttle
ATA	Atmospheres absolute
ATP	Adenosine triphosphate
$AVDO_2$	Arterio-venous difference of oxygen
BTF	Brain trauma foundation
C_0	Matrix concentration of the analyte of interest
CaO_2	Arterial oxygen content
CBF	Cerebral blood flow
CCO	Cytochrome C oxidase
C_{dial}	Analyte concentration in the dialysate
$CjvO_2$	Jugular venous oxygen content
CMRglc	Cerebral metabolic rate of glucose
$CMRO_2$	Cerebral metabolic rate of oxygen
CO_2	Carbon dioxide
CPP	Cerebral perfusion pressure
CSF	Cerebrospinal fluid
CT	Computed tomography
ctHb	Concentration of total hemoglobin
e-	Electron
ECF	Extracellular fluid
EEG	Electroencefalography
ETC	Electron transport chain
F	Infusion flow rate
FAD+	Flavin adenine dinucleotide oxidized form
$FADH_2$	Flavin adenine dinucleotide reduced form
FiO_2	Fraction of inspired oxygen
GCS	Glasgow coma scale
$[Glu]_{brain}$	Brain glucose concentration

GLUT	Glucose transporter
$[Gly]_{brain}$	Brain glycerol concentration
GOSE	Glasgow Outcome Scale Extended
GTP	Guanosine triphosphate
H^+	Proton
Hb	Hemoglobin
HBO	Hyperbaric oxygen therapy
HK	Hexoquinase
HR	Heart rate
ICH	Intracranial hypertension
ICP	Intracranial pressure
KC	Krebs cycle
$[Lac]_{brain}$	Brain lactate concentration
LDH	Lactate dehydrogenase
LGR	Lactate-to-glucose ratio
LPR	Lactate-to-pyruvate ratio
MABP	Mean arterial blood pressure
MAS	Malate aspartate shuttle
MCT	Monocarboxylate transporter
MD	Microdialysis
MMCAI	Malignant middle cerebral artery infarction
MRI	Magnetic resonance imaging
NAD+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
NB	Normal injured brain
NBO	Normobaric oxygen therapy
NIRS	Near-infrared spectroscopy
NMDA	*N*-methyl D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
OEF	Oxygen extraction fraction
OGI	Oxygen-glucose index
$ONOO^-$	Peroxynitrite

OxS	Oxidative stress
$PaCO_2$	Partial pressure of arterial carbon dioxide
PaO_2	Partial pressure of arterial oxygen
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFK	Phosphofructokinase
PFR	PaO_2/FiO_2 ratio
PO_2	Partial oxygen pressure
PPP	Pentose phosphate pathway
$PtiO_2$	Brain tissue oxygen pressure
$[Pyr]_{brain}$	Brain pyruvate concentration
r	Average mass transfer coefficient
REDOX	Reduction-oxidation
RI	Reference interval
ROS	Reactive oxygen species
RR	Relative recovery
SAH	Subarachnoid hemorrhage
SaO_2	Arterial oxygen saturation
$SjvO_2$	Jugular venous oxygen saturation
TBI	Traumatic brain injury
TC	Traumatic core
TIVA	Total intravenous anesthesia
TP	Traumatic penumbra
ZFM	Zero-flow rate method

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of death and disability in the world's population under 45 years of age. Most patients that survive a severe TBI have sequels with major medical, familiar and socioeconomics implications. So far, no neuroprotective treatment has been proven to be effective in controlled clinical trials, and neurological sequelae of TBI survivors have not changed significantly in the last 25 years.[1] The advances in this field have been limited by the lack of knowledge about the biochemical, cellular and molecular changes involved in the pathophysiology of brain injury.

The physiopathological mechanisms of TBI are a combination of primary damage, which are resultant from direct tissue disturbance by biomechanical forces at the moment of the impact, and a secondary damage, characterized by the activation of biochemical cascades in response to primary injury. Among the mechanisms of secondary brain damage are ischemic or non-ischemic cerebral hypoxia, excitotoxicity, energy failure and cell death due to the depression of aerobic metabolism and the inability to maintain ionic homeostasis. The mechanisms of this energy failure are not well established, but it is known that both the substrate reduction and the mitochondrial dysfunction are involved.[2,3]

Continuous monitoring of intracranial pressure (ICP) together with clinical neurological examination and computed tomography (CT) brain scanning have been the traditional methods for monitoring patients with moderate/severe TBI. In recent years, the use of new neuromonitoring systems such as brain tissue oxygen pressure ($PtiO_2$) measurements and cerebral microdialysis (MD) has provided an unprecedented opportunity to explore the pathophysiology of TBI at the cellular and molecular level and has motivated researchers to transfer this knowledge effectively to the bedside. During the last decades, these new monitoring systems ($PtiO_2$ and cerebral MD) have been applied in the clinical practice of TBI patients admitted to the neurointensive care unit at Vall d'Hebron University Hospital (VHUH). With the experience gained, we have developed 2 textbook chapters with a methodological review of both techniques

for its application in neuromonitoring of the neurocritical patient, which have been included in the present doctoral thesis.

Cerebral MD allows to explore the local neurochemistry by the quantification of metabolites involved in energetic metabolism (glucose, lactate and pyruvate), excitotoxicity (glutamate) and cellular damage (glycerol). By neurochemistry monitoring ($PtiO_2$ and MD), it has been determined that the brain undergoes significant alterations in energy metabolism, even with an adequate supply of oxygen (O_2).On the other hand, a reduction in the O_2 supply (hypoxia) is not necessarily accompanied with metabolic changes such as those found in classical cerebral ischemia. In both scenarios, the mitochondrial dysfunction may play an important role.

Despite the great advantage of having these new neuromonitoring tools, there is a lack of knowledge regarding the use of data obtained through advanced brain monitoring systems. One of the crucial steps for any clinical variable is determining its reference interval (RI) in a cohort of disease-free patients. Several published studies have attempted to establish thresholds and patterns that serve as a guide in the treatment of patients with TBI. However, there are still doubts about the thresholds that should be used and how to interpret the values displayed by the different monitors.[4,5] For cerebral MD, this approach is limited by its invasiveness and by ethical issues. Additionally, in MD, technical issues increase the complexity of determining appropriate RIs. This fact remains an important obstacle for adequately evaluating monitoring parameters in neurocritical patients and creates significant uncertainty among clinicians about correct patient management.

This thesis aims to help define the role of multimodal neuromonitoring tools, specifically of cerebral MD, in the definition of the metabolic profiles present in the brain of patients with TBI and to improve its clinical application. In a first phase, we have focused on the two major biomarkers that are most used in the definition of a situation of metabolic energy failure—lactate and lactate-to-pyruvate molar ratio (LPR)—in order to classify the profiles observed in a cohort of patients with moderate or severe TBI. In parallel, we have developed a study to shed light on the ongoing problem of using cerebral MD: the reference intervals. By using the extrapolation to zero-flow rate method (ZFM), we have determined the extracellular fluid (ECF)

concentrations of energy metabolites and glycerol. Based on the results of both studies, we have defined a series of patterns and RIs to be applied in MD data of TBI patients in order to obtain a more robust classification and to integrate cerebral MD into the clinical treatment of patients with TBI.

As part of the work conducted during the present doctoral thesis that has been included in the annexes, we have also studied the metabolic effects of normobaric hyperoxia (NBO) therapy applied in the acute phase of patients with moderate or severe TBI. The aim of this study was to define if this therapeutic maneuver has potential benefits in improving cerebral oxygenation and metabolic disorders. This assessment was accomplished with multimodal monitoring techniques (e.g., MD and $PtiO_2$) and the use of the RIs defined in the present thesis as a guide to interpret the metabolic changes observed with NBO therapy.

1. BRAIN ENERGY METABOLISM

The brain is the organ with higher energy needs. Although it only constitutes 2% of the body, it uses up to 20% of the total generatedenergy.[6,7] Glucose is considered the major fuel for both neurons and astrocytes,[8] leading to a higher glucose consumption in brain compared with other organs of the body. The brain consumes ≈120g of glucose daily from the total of 200g that a normal human body needs per day. Thus, the brain takes up 60% of the circulating glucose of the body.[6,9] Glucose metabolism support different pathways with important functions in energy metabolism, neurotransmission, reduction-oxidation (redox) reactions and biosynthesis of essential brain components with non-oxidative (glycolytic) and oxidative components. Synaptic activity spend the 50% of the energy produced by the brain; 25% is used for ionic gradients restoration, and the rest is inverted in biosynthesis processes. Glucose metabolism is complex due to its multifunctional roles and is tightly regulated by the needs for energy production and synthesis of glucose-derived metabolites.[6,10,11] In the following sections, we briefly review the most relevant aspects of brain energy metabolism in humans.

1.1. Glycolysis

Glucose and lactate with hydrogen protons are transported from blood and the ECF into the cell by glucose transporters (GLUTs) and monocarboxylate transporters (MCTs), respectively. On the other hand,O_2can diffuse freely into the cells. Cellular ATP production is mainly performed through the glycolytic and oxidative pathway. Once in the cytoplasm, a series of 10 enzymatic reactions, that involve the activity of multiple enzymes, oxidize glucose and generate two ATP, two NADH and two pyruvate molecules per glucose molecule in the absence of O_2 (glycolysis).[7] Glycolysis is divided in 2 phases: the energy investment phase where 2 ATP molecules are used, and the energy harvesting phase in which 4 ATP and 2 NADH molecules are produced. For each glucose molecule, the net production of 2 ATP is achieved by substrate-level phosphorylation (**Figure 1**). During hypoxia, the glycolysis pathway can increase the rate of ATP production through this pathway in order to meet the

metabolic demands, although its velocity is limited by glucose supply and the speed of reaction of the enzymes involved. The availability of ADP and NAD+ molecules is also important in glycolysis. This pathway is regulated by the enzyme phosphofructokinase-1 (PFK-1), which is activated when ATP demand is increased.[6]

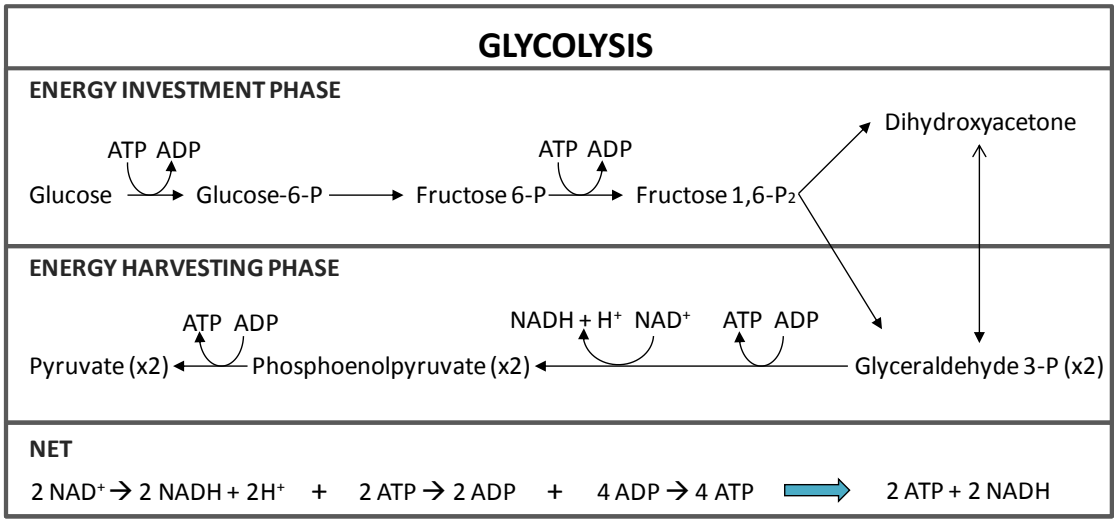

Figure 1. Schematic diagram of glycolysis pathway. Glycolysis is divided in 2 phases: the energy investment phase where 2 ATP molecules are used, and the energy harvesting phase in which 4 ATP and 2 NADH molecules are produced. For each glucose molecule, the net production of 2 ATP is achieved by substrate-level phosphorylation.

1.2. Krebs cycle

The resultant pyruvate molecules from glycolysis can then diverge into different fates as a function of O_2 availability. Under aerobic conditions (aerobic metabolism), pyruvate enters the mitochondria through MCTs, undergoes oxidation and is converted into acetyl-CoA by the enzyme pyruvate dehydrogenase (PDH). In this step, electrons are transferred to NAD+ producing NADH, and carbon dioxide (CO_2) is formed by a carbon loss. The next stage takes place in the Krebs cycle (KC) (also named citric acid cycle or tricarboxylic acid [TCA] cycle), where acetyl-CoA binds oxaloacetate, and through a series of enzymatic redox reactions, it is broken with the by-product generation of CO_2 and water (H_2O). For every molecule of glucose, the KC is completed twice (2 pyruvates), and a net of 8 NADH, 2 $FADH_2$, 2 ATP and 6 CO_2

molecules are produced. The electrons (e-) released in the redox reactions are transferred to the electron transport molecules NADH and $FADH_2$.[11] (**Figure 2**)

1.3. Oxidative phosphorylation

The major part of the energy production by aerobic respiration takes place in the electron transport chain (ETC) (also named respiratory chain) through the oxidative phosphorylation. The ETC is composed of a series of membrane-bound carriers in the mitochondria: ubiquinones (coenzyme Q), cytochromes and Fe-S proteins. All these proteins are organized in separated complexes. Complex I and II catalyze the transfer of the e- of a molecule of NADH (complex I) and succinate (complex II) to the ubiquinone. Complex III transports the electrons that the reduced ubiquinone has received into cytochrome c. Finally, the IV complex completes the sequence by transferring electrons from cytochrome c to O_2. During this process, the electrons provided by NADH and $FADH_2$ are transferred between the membrane proteins, which pump hydrogen ions across the membrane. When hydrogen ions flow back across the membrane through an ATP synthase complex, ATP is synthesized by the enzyme ATP synthase. O_2 acts as the final electron acceptor, and it is reduced to form H_2O. During this phase, NADH is converted to NAD+ assuring the cyclic continuity of glycolysis. This step is known as cellular respiration because O_2 is consumed, and CO_2 and H_2O are released. In hypoxic conditions, the IV complex of ETC could not yield the e- to O_2, leading to a decrease or stop of the e- flow. In summary, for each glucose molecule, a net of 32 to 38 ATP molecules are produced. Four stages convert glucose into ATP: glycolysis, pyruvate oxidation, KC and ETC (**Figure 2**).[11]

Malate-aspartate redox shuttle (MAS) is involved in the generation of both the NAD+ needed during glycolysis and the pyruvate for oxidation by the KC. Likewise, this shuttle transfers reducing equivalents from the cytosol to the mitochondria. The glycerol phosphate shuttle is also involved in the regeneration of NAD+ from NADH and in transporting reducing equivalents as well as lactate dehydrogenase (LDH)that regenerates NAD+ removing pyruvate from the oxidative pathway (**Figure 2**).

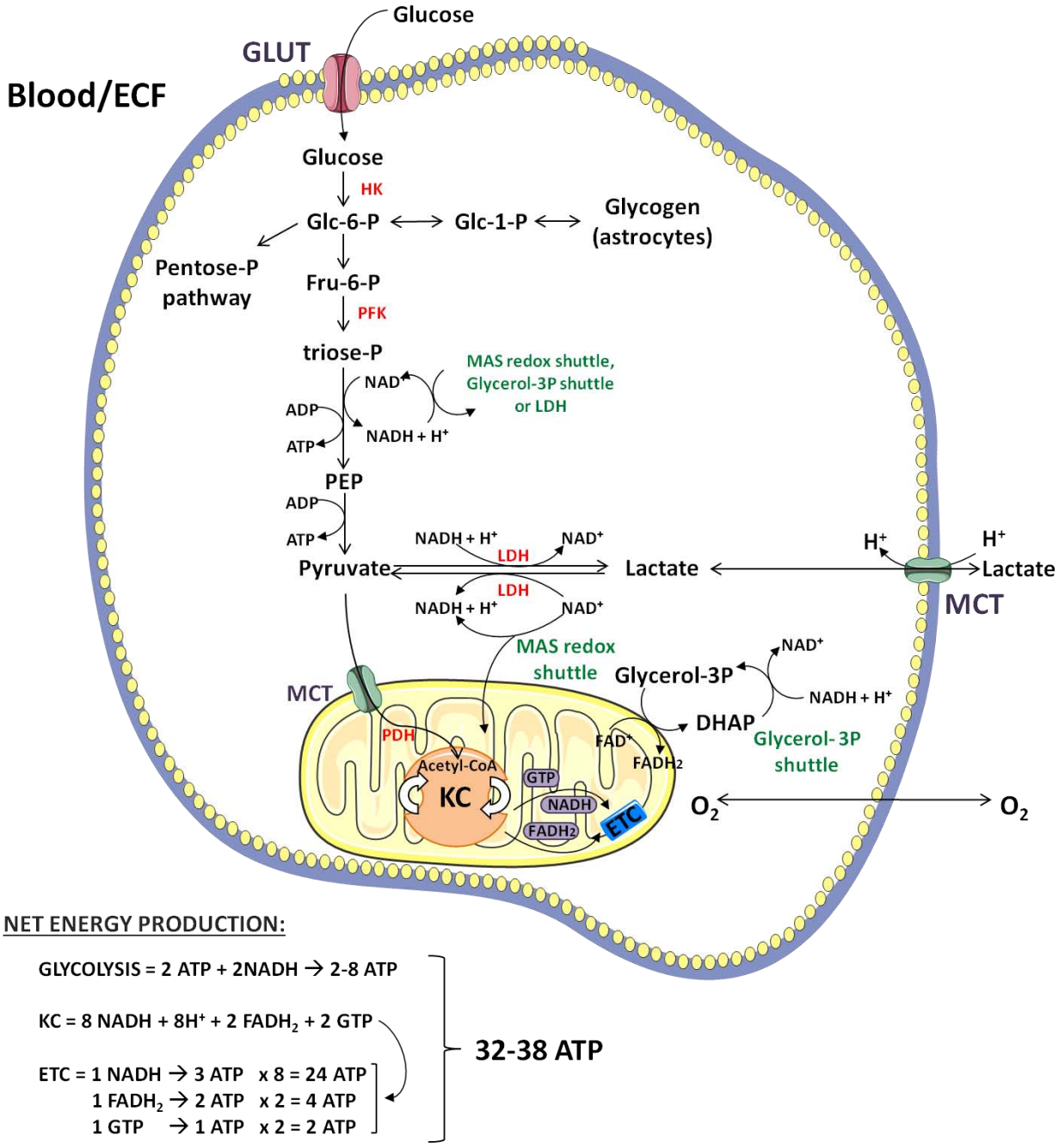

Figure 2. Representation of glucose metabolism pathways. Glucose and lactate with hydrogen protons are transported from blood and the ECF into the cell by GLUTs and MCTs, respectively. On the other hand, O_2 can diffuse freely into the cells. The glycolytic pathway is regulated by HK, PFK, and other enzymes (red).The green color coding indicates the processes of regeneration of NAD+ needed during glycolysis. Four stages convert glucose into ATP: glycolysis, pyruvate oxidation, KC and ETC. For each glucose molecule, a net of 32 to 38 ATP molecules are produced.ECF: extracellular fluid; GLUT: glucose transporter; MCT: monocarboxylate transporter; O_2: oxygen; HK: hexokinase; PFK: phosphofructokinase; LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; MAS: malate-aspartate shuttle; KC: krebs cycle; ETC: electron transport chain.

1.4. Alternative pathways of glucose metabolism

During the glycolysis, intermediate metabolites can also be diverted to other pathways away from the energy production. In the first reaction of glycolysis, glucose is phosphorylated by hexokinase, and most of the resultant substrate (glucose-6-P) follow the glycolytic pathway, but glucose-6-P can also enter into the pentose phosphate pathway (PPP) to produce NADPH and pentoses (5-carbon sugars) as well as ribose 5-phosphate (nucleotide precursor).[11]Furthermore, Glucose-6-P can be converted to glucose-1-P and derived to the synthesis of glycogen through glycogen synthase (**Figure 2**).

Neurotransmitters, neuromodulators and amino acids are other molecules synthesized from glycolytic and oxidative pathways. Pyruvate carboxylase (PC), only found in astrocytes, is involved in the synthesis of aspartate, glutamate, gutamine and GABA, from α-ketoglutarate synthesized in the Krebs cycle.[10,12]

1.5. Control of energy production

Connett et al.[13] defined that the energy production by the mitochondria has three control stages: 1) the state of phosphorylation, 2) the redox state, and 3) the concentration of dissolved O_2. The state of phosphorylation and the concentration of ATP, ADP, AMP, PCr and inorganic phosphate are believed to be the main regulating variables for energy production in most cases.

In brain metabolism, >90% of the glucose is oxidatively degraded via KC and ETC, as can be concluded from the oxygen-to-glucose index (OGI), which is a ratio between rates of utilization of glucose (CMRglc) and of O_2 ($CMRO_2$).[6,11,14] The theoretical ratio for each glucose (6 carbon atoms) would be of 6, because, during glucose oxidation, 6 O_2 atoms would be consumed in the catabolic route. In the real setup, the brain OGI at steady state is equal to 5.5. So, there is a part of pyruvate originating from glycolysis that deviates to lactate synthesis or to biosynthetic routes through the PC enzyme. Depending on the needs for energy production and for synthesis of glucose-derived

metabolites, pyruvate fate will be regulated by the different enzymes that use it as a substrate (PDH, LDH and PC).

Pyruvate and hydrogen ions, the end-products of glycolysis, can also be directed to the formation of lactate, thus allowing high rates of glycolysis. In normal conditions, this pathway represents ≈5-10% of the glucose consumed. Under low O_2 conditions (anaerobic metabolism), pyruvate cannot enter the KC and is converted to lactate by the LDH enzyme. In this reaction, NADH is also converted to NAD+ (**Figure 2**).[6]

2. TRAUMATIC BRAIN INJURY

In TBI, after primary lesions produced by the direct disruption of the brain parenchyma, a secondary brain damage may occur at the tissue and cellular level, which is characterized by the activation of biochemical, molecular and cellular cascades; this damage plays a very relevant role in the pathophysiology of TBI. Beyond the mechanisms involved in this secondary injury are ischemia, excitotoxicity, energy failure, cerebral edema, axonal injury, inflammation and regeneration, extra-cerebral insults (hypotension, hypoxemia), among others.[15,16]

2.1. Brain hypoxia

In the past two decades, cerebral ischemia has been, for most clinicians and researchers, the main cause of the secondary lesions occurring in TBI.[17] During the early stage following TBI, a significant reduction in cerebral blood flow (CBF) is described, which correlates with an early posttraumatic hypoperfusion, ischemia and a poor neurological outcome. With this evidence, all therapies have been classically directed to the management of CBF.[18,19]

Hypoxia is defined as the reduction of tissue oxygenation to insufficient levels for the maintenance of cellular metabolism and function. Most studies consider the term "cerebral hypoxia" to be synonymous with "ischemic cerebral hypoxia" or ischemia, a phenomenon that occurs following a global or regional reduction in partial oxygen pressure (PO_2) and/or CBF. However, the non-ischemic causes of cerebral hypoxia are in fact more frequent than ischemia induced by hemodynamic changes. Although ischemic hypoxia has been extensively studied, many experimental and clinical reports have shown that non-ischemic hypoxia can frequently exist.[20–22]

The real availability of O_2 in the tissue depends on PO_2 but also on other variables such as the hemoglobin concentration, the affinity between hemoglobin and O_2, the CBF, the number of functioning capillaries, the O_2 diffusion through the cell membrane and the characteristics of the extracellular space.[23] Apart from all these factors, O_2 metabolism depends on O_2 reduction in the mitochondria via the ETC. A

failure in one of these factors can show similar clinical manifestations, making it difficult to perform a differential diagnosis between them without the use of multimodal neuromonitoring techniques. In **Table 1**, the types of tissue hypoxia and the neuromonitoring profiles for each of them are summarized.

Table 1. Types of tissue hypoxia

Hypoxia type	Cause	Monitoring profile				
		$SjvO_2$ (%)	**$PtiO_2$ (mmHg)**	**P_{50}(mm Hg)**	**P_X(mmHg)**	**LPR**
Ischemic	↓Cerebral blood flow	↓	≤15	24-29	N	↑ (↑L - ↓P)
Low extractability	- Hypoxemia	↓	≤15	24-29	↓	↑ (↑L - ↓P)
	- Anemia	↓	≤15	24-29	↓	
	- High affinity between Hb and O_2	↑	≤15	<24	↓	
Shunt hypoxia	Arterio-venous shunts	N/↑	≤15	24-29	N	↑ (↑L - ↓P)
Dysperfusion	Difficulties in O_2 diffusion from Hb to the mitochondrion	N/↑	≤15	24-29	N	↑ (↑L - ↓P)
Histotoxicity	Inhibition of the mitochondrial cytochromes	N/↑	N	24-29	N	↑ (↑L - ↓P)
Uncoupling hypoxia	Uncoupling between O_2 reduction and ATP syntesis	N/↑	N	24-29	N	↑ (↑L - ↔P)
Hypermetabolic	Increase in metabolic requirements	↓	≤15	24-29	N	↑ (↑L - ↓P)

Table modified from Sahuquillo et al.[23] Hb: hemoglobin; N: range of normality;$SjvO_2$: jugular venous oxygen saturation; $PtiO_2$: brain tissue oxygen pressure;P_{50}: O_2 pressure at which Hb is 50% saturated; Px: O_2 extraction pressure; LPR: lactate-to-pyruvate ratio; L: lactate; P: pyruvate.

With the oxygen extraction pressure (Px), we can define and detect 3 possible causes of low extractability hypoxia: hypoxemia, anemia and high affinity between Hb and O_2. It has been shown that a moderately low alkalosis combined with hypothermia and moderate anemia– that is, frequent characteristics in patients with TBI –may contribute significantly to a reduced release of O_2 to the brain.[20,24,25] Advanced neuromonitoring methods such as cerebral MD, $PtiO_2$ and global and regional CBF

state allow the implementation of algorithms to identify step-by-step altered brain metabolism and the etiology of tissue hypoxia.[23,26]

2.2. Brain energy metabolism after traumatic brain injury

Brain energy metabolism needs the continuous supply of O_2 and glucose as well as a normal mitochondrial function in order to produce ATP. An insufficient ATP synthesis leads to a deterioration of homeostatic mechanisms, an increase of intracellular calcium concentration and cell death.[6] After TBI, the energy metabolism can be decreased due to ischemia caused by increased intracranial pressure and diminished cerebral perfusion pressure (CPP)or due to the inability of mitochondria to use the available O_2.[6]

On the other hand, due to the excitotoxicity phenomena that frequently occurs in the acute phase of TBI, there is an increase in metabolic demand related to glutamate uptake.[27–29] High amounts of glutamate release after head injury have been demonstrated in animal experimental studies.[29,30] Excitotoxicity is defined as the effect of glutamate and other excitatory amino acids when they are released up to toxic levels. Glutamate stimulates *N*-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic receptors with a subsequent increase in intracellular calcium concentration that leads to the activation of proteases, lipases and endonucleases, the stimulation of nitric oxide synthase (NOS) and the production of reactive oxygen species (ROS). All these factors are the triggers of DNA damage, metabolic dysfunction and cell death.[16] NMDA receptor activation also leads to a massive flux of potassium towards the extracellular compartment. Ionic fluxes after TBI result in an increase in glycolysis to supply energy to membrane ionic pumps and restore ionic gradients and in the accumulation of lactate due to functional mitochondrial alterations.[31] This hyperglycolysis is also triggered in conditions of hypoxia, when the anaerobic metabolism is reinforced to supply the ATP that cannot be generated in the ETC. The pyruvate cannot enter the KC and is diverted to the production of lactate to regenerate NAD^+ molecules, resulting in a high production of lactate (**Figure 3**). This hyperglycolysis is short-lived and can lead to a decrease in the

ECF glucose levels associated with poor outcome.[22,32,33] Bergsneider et al. first detected posttraumatic hyperglycolysis in humans by positron emission tomography (PET) around contusions and hematomas.[28] This hypothesis is supported by an increased lactate production described after TBI in animal models and humans, where maintained high levels have been correlated with a bad outcome.[33–35]

High levels of lactate in the ECF can produce cerebral acidosis, increase the calcium-mediated intracellular damage and can cause a decrease in brain pH, thus being harmful to the injured brain.[6] However, in the last decades, the classical concept that glucose represents the only brain energetic substrate has been questioned. There is a great controversy about the meaning of elevated ECF lactate levels.[36–38] Some studies have considered it as a waste end-product of metabolism, while recent hypotheses such as the astrocyte-neuron lactate shuttle (ANLS) suggested by Pellerin and Magistretti[39] have attributed new roles to lactate.

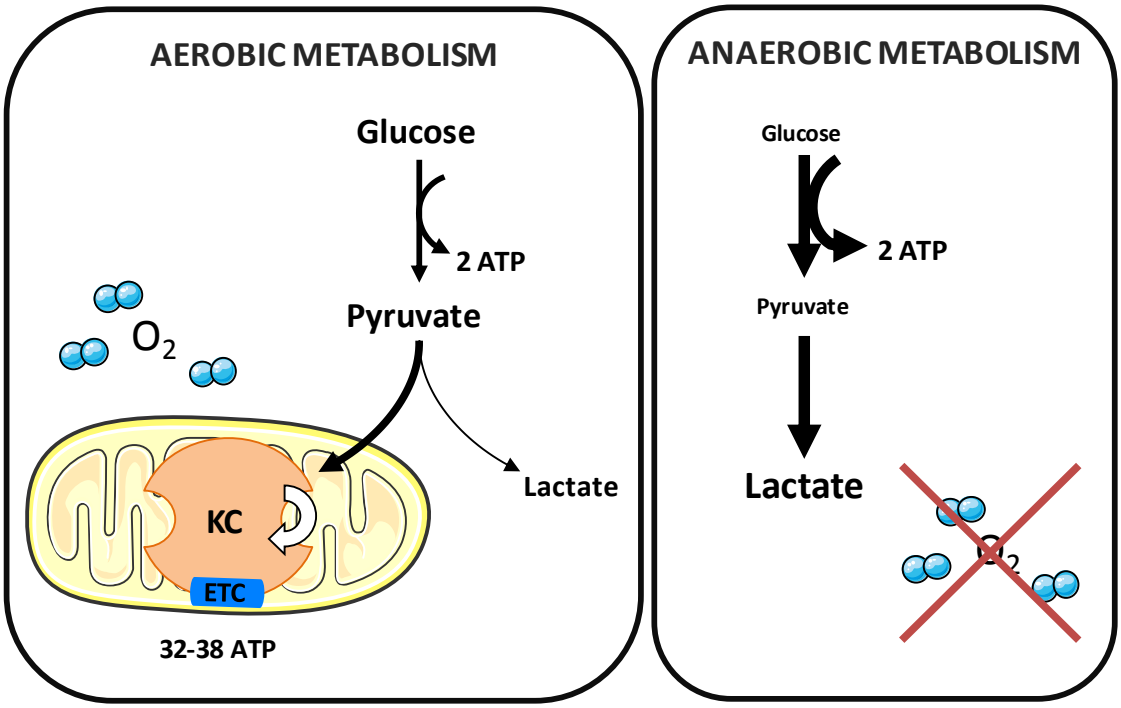

Figure 3. Aerobic and anaerobic metabolism. Under hypoxic conditions, anaerobic metabolism is reinforced to supply the ATP that cannot be generated in the ETC. The rate of glycolysis is also accelerated, resulting in an increased of lactate production and leading to a decrease in glucose and pyruvate levels. O_2: oxygen; KC: krebs cycle; ETC: electron transport chain.

2.2.1. Mitochondrial dysfunction

Mitochondria may play a key role in some of the alterations found in TBI. Studies in animals have shown that mitochondria also suffer structural and functional damage and that brain survival is closely linked to mitochondrial homeostasis. Several lines of evidence support the notion that cellular energetics are deranged in TBI, not because of an inadequate brain O_2 supply, but rather due to an impaired mitochondrial function. There are several factors that can support this theory: 1) $CMRO_2$ is consistently low following severe TBI, possibly due to mitochondrial inhibition.[40] Obrist et al. showed that oxidative metabolism, and therefore $CMRO_2$, is reduced to half the normal levels in severe TBI in proportion to the depth of coma;[41] 2) studies conducted in recent years show that brain energy metabolism is altered in TBI, even when O_2 is available but cells cannot use it; cytophatic hypoxia[42] or the equivalent term histotoxic hypoxia[20] define this situation; 3) metabolic alterations and increased extracellular lactate and LPR levels have been observed in macroscopically normal brain tissue with the presence of normal levels of $PtiO_2$ and CBF. In the absence of ischemia, the brain may experience a "metabolic crisis" possibly related to a mitochondrial dysfunction.[22,32] Nelson et al.[43] applied data mining techniques to microdialysis data and found a weak relationship between ICP, CPP and MD data, which did not explain the observed metabolic alterations, suggesting that factors other than pressure or flow variables were the cause of metabolic dysfunction; 4) mitochondrial swelling is present in early stages of TBI; 5) isolated mitochondria from experimental models and TBI patients present a significant decrease in respiratory rate and ATP production. There is experimental evidence that confirms that mitochondria play a key role in metabolic dysfunction after TBI and contribute to posttraumatic neuronal death.[21] Nevertheless, the causes and consequences of the above mentioned abnormalities remain poorly understood and further studies are needed.[44]

The main causes of mitochondrial failure are the blockage of the ETC and the reduction of pyruvate delivery to the KC. The causes of the ETC inhibition are yet to be identified, but many authors believe that nitric oxide (NO) is the cause. TBI is associated with an increase in NOS and, as a consequence, in NO production. Apart from its role in cell signaling pathways, this molecule is involved in cytotoxic

processes because of its ability to interfere with cellular energy metabolism by reversibly inhibiting the enzymatic activity of the terminal complex of the mitochondrial ECT -Complex IV or cytochrome a,a3-.[42] This inhibition is based on the competition between both O_2 and NO for the same binding site on Complex IV. The formation of peroxynitrite ($ONOO^-$), resulting from the reaction of NO with superoxide radicals (O_2^-), causes an irreversible inhibition of mitochondrial respiration by blocking Complexes I, III and V.[42] ONOO- also blocks aconitase, the enzyme that converts citrate into isocitrate in the KC.[45] The reduction of pyruvate delivery to the KC can be due to the inhibition of the PDH enzyme. Inactivation of PDH, even with sufficient O_2, limits the pyruvate conversion to acetyl-CoA and thus its entry into the KC is blocked, leading to an accumulation of pyruvate and an increased production of lactate.

Activation of the enzyme poly-ADP ribose polymerase-1 (PARP-1) could be another cause of mitochondrial failure. It is a nuclear enzyme that participates in the repair of breaks in nuclear DNA induced by ROS. During its activity, it depletes the NAD+/NADH reserves and impairs the O_2 utilization to support the synthesis of ATP causing a type of histotoxic hypoxia. It has been shown that this enzyme activation may be mediated by the presence of pro-inflammatory cytokines (TNF-α, IL-1β and IFNγ).[46]

2.2.2. Lactate and lactate-to-pyruvate ratio as brain ischemia biomarkers

For decades, lactate has been considered an excellent biomarker for O_2 limitation and therefore of organ ischemia; as a result, lactate is often regarded as an essential biomarker for managing circulatory failure. Most clinical research in hypoperfused organs, including the brain, is based on the “anaerobic threshold” concept introduced by Wasserman and McIlroy in 1964.[47] According to the traditional paradigm, increase in lactate in any organ or in blood, even during muscular contraction or exercise, is a direct consequence of tissue hypoxia. As mentioned above, under conditions of O_2 limitation, the inhibition of the ETC produces an insufficient ATP generation via oxidative phosphorylation and increases the mitochondrial and cytosolic pools of NADH. The increased mitochondrial [NADH]/[NAD] ratio inhibits the KC producing

an accumulation of cytosolic levels of pyruvate and as a consequence an increase in lactate production (**Figure 3**).[48]

In accordance with conventional theory, in patients with acute brain injuries, an increase in brain extracellular lactate levels above a somewhat arbitrary and variable threshold (i.e., 2.0-4.0 mmol/L) has been considered an indicator of increased anaerobic glycolysis and brain hypoxia.[49–52] However, the LPR is considered a more robust indicator of anaerobic metabolism and the redox status of the tissue, and has been found to be an independent predictor of mortality and unfavorable outcome in a multivariate analysis of the largest cohort of TBI patients monitored with MD.[51] In conditions of CBF reductions or ischemia/hypoxia, lactate is produced to support the brain energetic demands, as it has been described in MD studies in TBI with elevated levels of lactate and LPR and a concomitant fall of pyruvate.[51,53] However, in TBI clinical research, both biomarkers have been used as equivalent indicators of ischemic and non-ischemic brain hypoxia. Metabolic alterations with increased lactate and LPR levels are frequently present in the absence of cerebral ischemia, which could be a more or less common phenomena in this type of patients.[22,33,43] This has confounded the discussion regarding brain metabolism impairment and the potential benefit of some therapies. Normobaric hyperoxia, using ≈100% of inspired fraction of oxygen (FiO_2) at 1 ATA at the bedside, could be used as a potential treatment to improve brain oxygenation and consequently the metabolic disorders resulting from TBI. However, contradictory results have raised significant controversy regarding this treatment; in part due to the diverse metabolic criteria used to evaluate the brain's response to the hyperoxic challenge, mostly based in brain lactate levels without taking into consideration changes in pyruvate and in the LPR.[52,54–57]

2.2.3. Non-hypoxic/ischemic causes of lactate increase

Intracranial hypertension (ICH) and brain ischemia have traditionally been considered the main secondary intracranial insults in TBI, and have thus been the subject of most research in these patients. However, evidence accumulated in the last decade indicates that ischemia is the least frequent etiologic factor in TBI-related energetic disturbances.[22,35]

The anaerobic threshold concept has been questioned by different studies during the last 4 decades that offer an alternative explanations for lactate production increases that do not involve O_2 limitation.[58,59] Connett et al. study was focused on describing that an increase in lactate may not be related with tissue anoxia in working muscles. In fact, they proposed that lactate accumulation acts as a buffer of cytosolic redox state and as a substrate for support of the aerobic ATP production in mitochondria during exercise.[58]

The conventional interpretation of the meaning of high brain lactate levels was first challenged by the studies of Vespa et al., which showed that in TBI patients increases in lactate may indicate hyperglycolysis or metabolic crisis and not necessarily ischemia.[22] In this study, the metabolic crisis were defined by the elevation of the LPR>40, and ischemia was defined by the PET criteria of OEF>0.75 or by a LPR>40 combined with glucose levels <0.2 mmol/L. The data recorded in this study showed a 25% incidence rate of metabolic crisis and just a 2.4% incidence rate of ischemia.[22] Sala et al.[35] observed elevated lactate levels predominantly associated with glycolysis, normal values of $PtiO_2$ and with normal or hyperemic brain perfusion in severe TBI patients. A recent study has shown lactate levels increased during glucose depletion episodes associated with normal $PtiO_2$ values.[60]

These studies in acute brain injuries aligns with subsequent evidence indicating that lactate is a non-specific biomarker of an increased glycolytic flux, but that the increase in glycolysis can have multiple etiologic factors other than tissue hypoxia.[61]

2.2.4. Lactate as a fuel and signaling molecule

Knowledge of the role of lactate in bioenergetics has increased in the last years, and the classical view of a waste product has changed toward more important roles as a fuel and signaling molecule. The main findings that lend support to this idea are that lactate accumulation does not correlate with the amount of metabolized glucose and that lactate infusion increases its utilization by the brain and has a glucose-sparing action.[10]

The resting brain releases a small amount of lactate that increases by 3- to 4-fold during brain activation. There is growing evidence that lactate may be a fuel used by neurons under aerobic conditions in agreement with the ANLS hypothesis.[39] Pellerin and Magistretti hypothesized that activation of astrocytes by glutamate can convert glucose into lactate, which is utilized by neurons via the KC. *In vitro* experiments confirmed this theory, suggesting that astrocytes act as a lactate 'source', while neurons may be a lactate 'sink'.[62,63] Schurr et al. studies in rat hippocampal slices highlighted that neuronal energy demands can be supplied by lactate production by increased glial glycolytic flux.[63] Neuronal culture studies have defined the role of lactate as a preferential oxidative substrate over glucose.[64,65] Gallagher et al. were the first to demonstrate that lactate can be used as a fuel via oxidative metabolism in TBI patients, introducing ^{13}C-labelledcerebral MD as a potentially useful tool for studying human brain chemistry.[66]

Cerdán et al.[67] proposed an alternative mechanism for astrocyte-neuron lactate trafficking in which redox shuttles regulate the glycolytic and oxidative metabolic coupling between neurons and astrocytes through the reducing equivalents transference between both cell types. In this model, pyruvate and NADH are generated from lactate oxidation in neurons, and the first is released and metabolized by astrocytes to regenerate NAD+. This results in a coupling of cytosolic NAD+/NADH redox states of both neurons and glial cells. By this mechanism, the glycolytic metabolism in astrocytes can be supported by this redox shuttle cycle between both cell types instead of using the MAS. However, more *in vivo* studies are needed to define the functional role of lactate shuttling coupled to its oxidation or brain release.

2.2.5. Additional potential benefits of lactate

In recent years, the use of lactate in humans as therapy has attracted the attention of clinicians. Studies in healthy subjects have demonstrated that intravenous lactate is metabolized by the brain, and at supra-physiological plasma lactate concentrations, it contributes up to 60% to cerebral metabolism, thus promoting glucose sparing.[68–70] There is experimental evidence that lactate exogenous administration can be neuroprotective after brain injury by glutamate exposure.[71] The role of this molecule in

the improvement of cognitive recovery has also been described in animal models of TBI[72] and cerebral ischemia.[73]

Hypertonic sodium lactate therapy in TBI patients resulted in an improvement of cerebral energy metabolism.[74–76] However, a good mitochondrial function is needed to benefit from this therapy and a previous evaluation of the oxidative capability of each patient should be considered.[77]

Furthermore, lactate has been implicated as a cell-signaling molecule ("lactormone") in the regulation of gene and protein expression involved in glycolysis.[78] The downregulation of glycolysis in TBI patients leads to redirection of glucose to PPP for cell repair and oxidative stress protection, as described by Dusick et al.[79] A recently described hydroxycarboxylic receptor 1 (HCA1) in the central nervous system could be involved in this new lactate role.[80,81]

Lactate has also been involved in memory formation[82] and axonal regeneration.[83] Oligodendrocytes, through glial glycolysis products (lactate or pyruvate) and not glucose, support axonal metabolism.[83] In human studies, lactate supplementation has been implicated in the improvement of cognitive performance and cerebral performance during intense exercise.[84]

3. MULTIMODAL MONITORING

Therapeutic management in TBI patients is focused on the maintenance of an adequate CBF and energy metabolism guided by cerebral multimodal monitoring tools for the early detection of secondary damage. Although these tools do not directly influence a patient's prognosis, they allow us to continuously know the patient's brain state and to control different parameters that help clinicians to assess brain metabolic needs and the effect of therapies to control ICP and to treat intracranial lesions. However, the high heterogeneity of brain lesions and the different clinical evolution of TBI patients increase the complexity of the analysis of the information provided by these tools, which should be analyzed, in each case, in an individualized and dynamic way.

There are different advanced cerebral techniques involved in the neuromonitoring of neurocritical patients that conform to the recent new concept of multimodal neuromonitoring. The main objective of this strategy is to try to prevent and/or detect the occurrence of secondary lesions early and to guide clinicians in the most appropriate therapeutic interventions for each patient. Among the many standard-of-care procedures available, we focus on ICP, CPP and $PtiO_2$ monitoring, cerebrovascular reactivity (autoregulation) and brain energy metabolism study by cerebral MD.[77]

3.1. Intracranial Pressure

ICP is the force that brain tissue, blood and cerebrospinal fluid (CSF) exert inside the cranial vault. Monro-Kellie hypothesis states that the sum of volumes of these components is constant.[85] ICP can be influenced by an increase in brain volume (brain swelling) and cerebral blood volume and an elevated CSF volume due to an increase in its production or a decrease in its clearance. Mass lesions can also raise ICP.[86] If the intracranial volume increases, there are compensatory mechanisms that allow to maintain ICP within the normal range.[4] As the pressure-volume curve defines, if a critical point of ICP is reached, a small volume increment in any of the mentioned components will generate large increases in ICP levels when it exceeds 20mmHg.[87]

Continuous ICP monitoring is the mainstay of the therapeutic guide for neurocritical patients. Between 50% and 75% of severe TBI patients present ICH at some point in their clinical course, although incidence varies according to the type of brain injury and is dramatically reduced with an aggressive surgical approach to treat focal lesions.[88]

ICP monitoring allows to: 1) determine absolute values of ICP, 2) assess the presence of pathological waves, 3) have information about brain compliance through the wave amplitude parameter, 4) calculate and manage the CPP, 5) diagnose the ICH and serve as a guide for its treatment, 6) help getting information of the patient's prognosis, and 7) recognize the worsening of intracranial lesions.

In neurocritical patients, ICP values less than or equal to 15-20 mmHg are considered normal. Values above 20 mmHg (22 mmHg according to the last edition of the Brain Trauma Foundation [BTF] guidelines for severe TBI) are associated with increased death and disability in these patients.[4,5] The BTF guidelines recommend, with a level of evidence IIB, that the management of severe TBI patients according to the continuous ICP monitoring information reduce in-hospital and 2-week post-injury mortality.[4]

3.2. Brain oxygenation

The determination of cerebral oxygenation can be performed using invasive or non-invasive methods. Within the invasive methods, the determination of the $PtiO_2$, a regional method, or the determination of the jugular bulb oxygen saturation ($SjvO_2$), a global method, are the most frequently used techniques.

3.2.1. Partial brain tissue oxygen tension

Partial brain tissue oxygen tension monitoring is a regional and invasive method that measures brain oxygenation continuously. This method consists of the introduction of an O_2-sensitive catheter in the cerebral parenchyma, which through different

physicochemical phenomena gives us information that is considered as an average O_2 concentration of the area monitored, comprised by three compartments (vascular, intracellular and extracellular) of the cerebral white matter.[89]

$PtiO_2$ monitoring can help the following: 1) early detection of cerebral ischemia phenomena, 2) optimization of O_2 supply to ischemic penumbra areas, 3) guidance in the overall treatment of the patient and 4) understanding of the mechanisms causing secondary events.

Currently, there are two measurement systems: 1) Licox (Integra Neurosciences) and 2) Neurovent-PTO (Raumedic). The Licox system is the most used in neurocritical care. This system, using a polarographic method, determines the partial pressure of O_2 through redox reactions produced at the end of the catheter ("revoxode"). These reactions generate an electric current that is subsequently transformed to a numerical value. On the other hand, the Neurovent-PTO system determines the partial pressure of O_2 by a phenomenon known as O_2 quenching, where fluorescent labeled molecules absorb and emit light of a certain wavelength, depending on the presence or absence of O_2.Since $PtiO_2$ varies according to the temperature at the measurement site, the Neurovent-PTO system allows the determination of the temperature in the same catheter as well as the ICP. In the case of Licox, this parameter must be provided to the monitoring device either automatically, in those devices that have a temperature sensor in addition to oxygen sensor, or manually, through the determination of the core body temperature and incorporating that value into the monitor.[89]

The normal range is considered to be between 15 and30 mmHg. Values below 15 mmHg indicate cerebral tissue hypoxia of any type (ischemic hypoxia, shunt hypoxia, low-extractivity hypoxia, etc.) which can be moderate (10-15 mmHg) or severe (<10 mmHg).[90] Low $PtiO_2$ values have been related to poor functional outcome.[91]

3.2.2. Jugular bulb oxygen saturation

$SjvO_2$ is an invasive monitoring method that allows to globally estimate the brain metabolism and oxygenation and provides information on the relationship between CBF and metabolic O_2 consumption.

The clinical application of $SjvO_2$ determination is mainly in severe TBI patients or other neurocritical patients, including those with subarachnoid hemorrhage (SAH) or malignant middle cerebral artery infarction (MMCAI). It is also used during neurosurgical and cardiovascular procedures. Through the Seldinger technique, a catheter is inserted at the level of the internal jugular vein bulb. The catheter position must be verified by a lateral cervical spine X-ray. The end of the catheter should be located at the level of the mastoid process.

At present, the BTF guidelines for severe TBI recommend maintaining values of this variable above 50% in order to reduce mortality and improve outcomes (level III recommendation).[4] Values below 50% are consistent with brain ischemia due to: CPP decrease, ICP increase, systemic pressure decrease or hypocapnia. Values above 75% are related to cerebral hyperemia, in which the O_2 supply exceeds the metabolic demands of brain due to an increase in CBF or a decrease in O_2 consumption.[92]

CBF may also be estimated through the measurement of the arterio-venous differences of oxygen ($AVDO_2$) or through other hemometabolic variables derived from oxyhemoglobin values obtained from samples taken at the internal jugular vein bulb.[93,94] $AVDO_2$ may be considered as a source of information for clinicians in order to reduce mortality and improve outcomes at 3 and 6 months post injury (level III recommendation by BTF).[4]

3.3. Cerebral microdialysis

A better understanding of the metabolic events that occur in the acute and subacute phases of TBI is essential for detecting these abnormalities in clinical practice and to be able to reverse or modulate such changes.

Microdialysis is based on the principle of solute exchange through a semi-permeable membrane that is located at the distal end of a catheter. It functions like a blood capillary where the solutes are exchanged between a solution of known composition (perfusate) and the fluid surrounding the MD catheter (e.g. ECF) due to an osmotic gradient. This technique applied to the brain allows to monitor different tissue metabolites in the extracellular space and to obtain information about the biochemistry of brain cells and its changes due to hypoxia, mitochondrial dysfunction, ischemia, hyperemia, etc. Therapeutic interventions can also be reflected in metabolite levels.[95]

3.3.1. Methodological considerations

MD catheters are flexible, with a small diameter (< 1 mm) and contain a double lumen with a semi-permeable membrane at the distal end (**Figure 4**). The internal lumen is perfused with a metabolite-free solution (perfusion fluid) contained in a syringe placed in an infusion micropump which perfuses the catheter with a constant perfusion flow. At the semi-permeable membrane, solutes with a particular molecular weight (< 20 KDa in standard catheters and < 100 KDa in high-resolution catheters) are exchanged. Then the microdialysate is recovered in a small container (microvial) that is placed at the end of the outlet tube and that is changed hourly by the nurse in charge of the patient. Once the microvial is recovered, it is inserted in the microdialysis analyzer (e.g. ISCUS Flex analyzer, M Dialysis AB, Stockholm, Sweden) (**Figure 4**) where metabolites are analyzed through enzyme kinetic methods and colorimetric measurements.

There are two types of cerebral MD catheters, CMA-70 (20-kDa cut-off membrane; MDialysis AB) and CMA-71 (100-kDa cut-off membrane). In both models, the 60-mm shaft is made of polyurethane and has an outer diameter of 0.9 mm. The polyarylethersulphone dialysis membrane is 10-mm long with an outer diameter of 0.6 mm. The catheter is perfused with a sterile isotonic CNS fluid containing 147 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 2.7 mmol/L KCl and 0.85 mmol/L $MgCl_2$ (M Dialysis AB) at a fixed flow rate of 0.3 μL/min using a CMA-106 pump (M Dialysis AB). A subcutaneous catheter (CMA61/63, M Dialysis AB) is also implanted in the adipose

tissue to monitor the systemic levels of the metabolites of interest. Glucose, lactate, pyruvate, glycerol and glutamate are routinely monitored at the bedside. After these hourly measurements are completed, the microvials are placed in a rack (M Dialysis AB) to seal them and to prevent evaporation. All racks are stored at -86ºC until further analysis. Metabolic abnormalities detected by MD catheter may help to screen the cause of potential secondary intra- or extra-cranial insults.

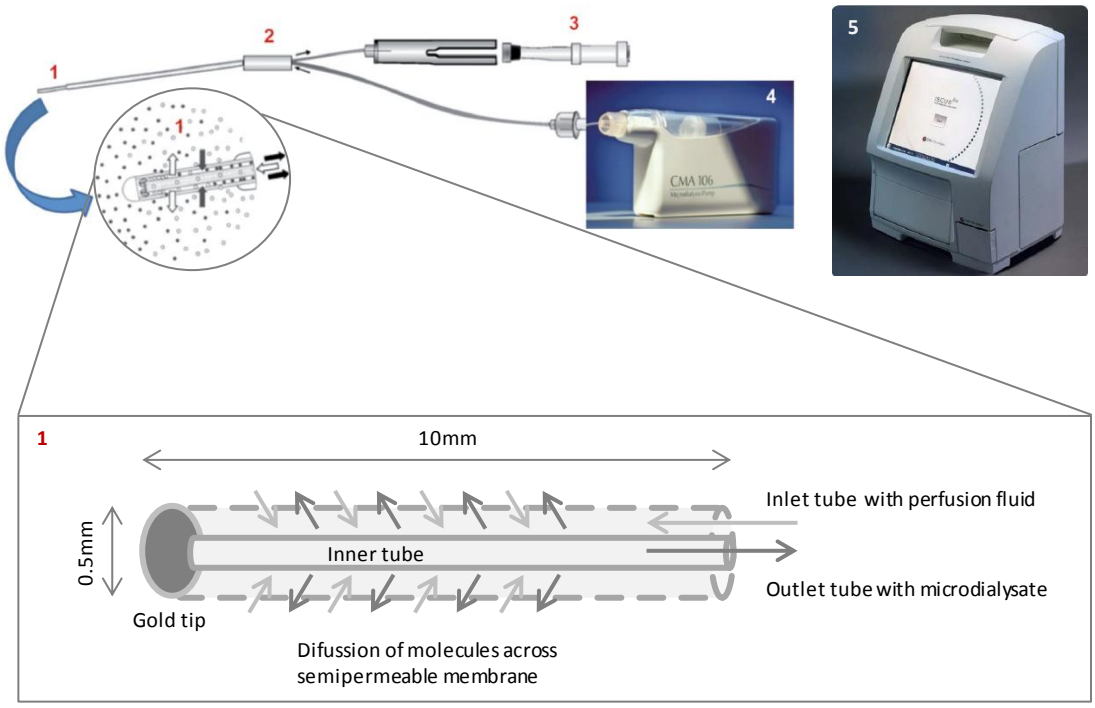

Figure 4. Cerebral microdialysis technique elements: 1) dialysis membrane, 2) double lumen catheter, 3) microvial, 4) infusion micropump and 5) microdialysis ISCUS Flex analyzer.

3.3.2. Catheter implantation and tissue classification

Cerebral microdialysis catheters can be surgically implanted through a burr hole or through the cortex after a lesion evacuation. They can also be implanted at the bedside through a cranial screw or a percutaneous technique (**Figure 5**).[96] MD allows to monitor a small area surrounding the catheter membrane, so it is considered a focal technique. For this reason, the microdialysis catheter must be implanted in the brain region that provides the most useful information for patient management. Catheter implantation in healthy tissue provides the possibility of monitoring the tissue with

the highest probability of recovery, while implantation in areas of "penumbra" (areas surrounding focal lesions) allows monitoring of brain regions at greatest risk of ischemia.[97,98]

In TBI, one or more brain catheters should be applied according to the type of lesion. In diffuse lesions, the implantation of a single brain catheter in the right frontal region is recommended. In focal lesions, two catheters are recommended, one in the macroscopically non-lesioned region and the other in the penumbra. The position of the catheter is confirmed by a control computed tomography (CT) scan in which a gold-tip in the distal end of the catheter is visible (**Figure 5**).[97,98]

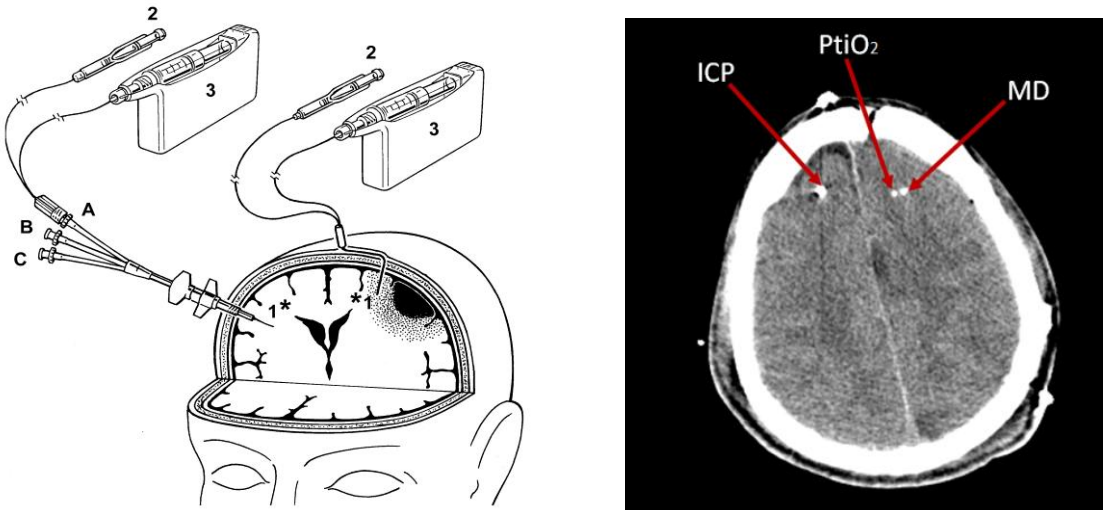

Figure 5. Left. Cerebral microdialysis catheters (1*) implanted in macroscopically normal injured brain (right hemisphere) and in traumatic penumbra (left hemisphere). The catheter implanted in the right hemisphere is introduced through a cranial screw (A), with two additional lumens (B and C) to implant ICP, $PtiO_2$ or temperature probes. The catheter placed in traumatic penumbra is introduced through a percutaneous technique. The figure shows a scheme of the microvial collector (2) and the infusion micropump (3). **Right.** Radiological artifacts produced by an ICP sensor, a $PtiO_2$probe and a MD catheter. ICP: intracranial pressure; $PtiO_2$: tissue oxygen partial pressure; MD: microdialysis.

The region of the brain sampled by the probes can be classified in one of the following categories: apparently normal injured brain (NB), traumatic penumbra (TP) and traumatic core (TC).[99] NB is defined when the probes tips are inserted in a region of the brain without any macroscopically visible abnormality (blood or hypodense lesion). To consider the brain to be "normal", the closest hemorrhagic/hypodense

lesion must be located at least 30 mm from the tip of the cerebral MD catheter. TP arbitrarily defines the position of the probes in the brain parenchyma without any changes in attenuation and that is at least 20 mm away from any intraparenchymal lesion (contusions, hematomas, etc.). TP is also considered when the probes are located in the brain immediately below any significant extra-cerebral hematoma. TC is defined when the probes are placed in areas of brain tissue that has macroscopically obvious lesions such as contusions and hemorrhages or when the probes are located in a small hemorrhagic lesion.

3.3.3. Relative recovery

One inherent problem to MD is the fact that the dialysate concentration of any substance is never a perfect mirror of the extracellular levels but instead represents a variable fraction of the actual values.[7,100–102] Relative recovery (RR) is defined as the ratio of the concentration in the dialysate to the true *in vitro* concentration of a given substance.[103] The RR depends primarily on the physical properties of the membrane, the temperature of the medium, the perfusion flow rate, the characteristics of the sample matrix and the diffusion coefficient of the substance of interest.[7,100,101,103]

3.3.4. *In vitro* calibration

The most frequently used approach to evaluate cerebral MD efficiency for any substance is to use the *in vitro* RR. The determination of *in vitro* RR involves immersing a MD catheter in a matrix with a known concentration of the metabolite of study under controlled conditions of agitation and temperature. The metabolite levels are analyzed in the dialysate and matrix samples and the RR is calculated using the following equation:

$$RR = (C_{md}/C_{matrix}) \times 100 \qquad \textbf{(Equation 1)}$$

where C_{md} and C_{matrix} are the analyte concentrations in the microdialysate and in the matrix, respectively.

In vitro RR results are difficult to extrapolate to the brain, due to the effect of the tissue properties on the diffusion of analytes. The ECF space in any organ is an inhomogeneous and tortuous medium; therefore the *in vitro* RR can only provide a rough estimate of the efficiency of cerebral MD probes *in vivo*.[101,104]

3.3.5. *In vivo* calibration

Since its introduction, a recurrent problem with MD has been the lack of a reliable *in vivo* calibration method, which prevents accurate *in vivo* concentrations from being established.[105] In clinical practice, the true substance concentration in the ECF is calculated by measuring the concentration in the dialysate and dividing that concentration by the *in vitro* RR expressed as a fraction of 1.[101] The validity of this approach rests on the principle that the *in vitro* and *in vivo* conditions are similar; however, this assumption is far from true.[101]

To overcome the limitations of *in vitro* studies, three different methods have been proposed to estimate the true ECF concentration of any analyte of interest: 1) the extrapolation to zero-flow rate method,[106] 2) the no-net flux method,[107] and 3) the slow perfusion rate.[105] All three methods are considered reliable and yield similar results, as shown in the experimental studies conducted by Menacherry et al.[105]

3.3.6. Extrapolation to zero-flow rate method

Through the extrapolation to zero-flow rate method, the extracellular concentration of metabolites can be estimated by determining the relationship between the flow rate variation and the recovery over the microdialysis membrane.[106] As it is not necessary to change the perfusion fluid composition, the ZFM can be easily applicable to clinics.

This method, described by Jacobson et al.[106], determines the analyte concentration through the determination of the relation between the perfusion flow rate, the probe's

membrane area and the transference coefficient. This relation is defined by the following equation:

$$C_{dial} = C_0 - C_0 [\exp(-rA/F)] \quad (\textbf{Equation 2})$$

where C_{dial} is the analyte concentration in the dialysate, C_0 the matrix (e.g., ECF) concentration of the analyte of interest, r the average mass transfer coefficient, A the surface of the microdialysis membrane, and F the infusion flow rate. The matrix concentration (C_0) can be estimated by fitting a nonlinear regression model to the dialysate concentrations obtained at different perfusion rates.[105,106] According to this method, at a theoretical flow rate of 0 μL/min (y-intercept), the metabolite concentration of the dialysate is considered to be equal to the true concentration in the matrix.[106,108]

Hutchinson et al.[109] applied this method on patients with head injury or poor-grade SAH. Two adjacent microdialysis catheters were inserted into the frontal cortex: one reference catheter perfused at a constant rate of 0.3 μL/min and a second catheter perfused at different flow rates (0.1-1.5 μL/min) during 16h. A metabolite recovery of approximately 70% of the real content of the cerebral extracellular space was obtained when using catheters with a dialysis membrane of 10 mm and a perfusion flow velocity of 0.3 μL/min.[109] Abi-Saab et al.[110] also used the ZFM to monitor conscious human subjects with complex partial seizure disorder. A MD catheter was attached to a depth electrode and perfused at different flow rates between 0.25 and 2.5 μL/min, and basal levels were determined using regression analysis.[110]

3.3.7. Normal ranges for brain metabolites

The main study reporting the RIs for analytes involved in the monitoring of the brain energy metabolism in neurocritical patients was conducted by Reinstrup et al. in patients who underwent neurosurgical procedures under general anesthesia.[49] Other authors have reported brain metabolite levels in the normal brain of patients with central nervous system tumors,[111] in awake epileptic patients[110,112] and in patients with

spontaneous SAH (**Table 2**).[113,114] However, in most of these studies, the true ECF concentrations were unknown, and the reference limits were estimated from the concentrations in the dialysate and from the *in vitro* RR for the specific metabolite. Consequently, the normal ranges for brain lactate, the LPR, and what has been considered the 'anaerobic threshold' are still quite arbitrary. This threshold is usually extrapolated from clinical studies, animal studies, and studies of other organs, or they are based on reference levels obtained from CSF samples or from cerebral MD conducted in patients who underwent neurosurgical operations for posterior fossa lesions or epilepsy.[49,110]

Table 2. Extracellular concentrations for energy metabolites and glycerol defined in the literature

Study	Condition	Glucose (mmol/L)	Lactate (mmol/L)	Pyruvate (µmol/L)	LPR	Glycerol	Pathology
1	Anesthetized (1 µL/min)	1.2 ± 0.6	1.2 ± 0.6	70 ± 24	22 ± 6	28 ± 16	Posterior fossa tumor
	Awake (1 µL/min)	0.9 ± 0.6	1.4 ± 0.9	103 ± 50	21 ± 6	42 ± 29	
	Awake (0.3 µL/min)	1.7 ± 0.9	2.9 ± 0.9	106 ± 47	23 ± 4	82 ± 44	
2	Anesthetized (0.3 µL/min)	2.1 ± 0.2	3.1 ± 0.3	151 ± 12	19 ± 2	82 ± 12	SAH (nonischemic area)
3	Awake (2.5 µL/min)	0.8 ± 0.3	1.4 ± 1.2	NA	NA	NA	Epilepsy (nonepileptogenic area)
	Awake (ZFM))	1.6 ± 0.8	5.1 ± 1.4	NA	NA	NA	
4	Awake (ZFM)	1.6 ± 0.3	4.7 ± 0.5	NA	NA	NA	Epilepsy (nonepileptogenic area)

Values are expressed as mean ± SD.**1**:Reinstrup et al., 2000; **2**: Schulz et al., 2000; **3**: Abi-Saab et al., 2002; **4**: Cavus et al., 2005; ZFM: zero-flow rate method; SAH: subarachnoid hemorraghe; NA: not applicable; LPR: lactate-to-pyruvate ratio.

In human studies, the upper reference limit for lactate in normal brain tissue is unexpectedly variable and ranges from 1.50 to 5.1 mmol/L.[51,52,61,110,111] This variability remains an important obstacle for adequately evaluating metabolic findings in neurocritical patients, as well as for making physiopathological interpretations and

assessing clinical relevance. A recent study for the reference ranges in the CSF established an upper 95% percentile for lactate at 2.6 mmol/L.[115] Reinstrup et al. measured energetic metabolism in 9 awake and anesthetized patients using the same probes (10 mm length and 20 kDa cut-off) and flow rate that were used in the present project (0.3 μL/min), and that are used by most groups in the clinical setting.[49] The metabolite levels obtained in this study are also summarized in **Table 2**.

Perfusion speed is known to influence the RR of any ECF metabolite, whereas the LPR remains stable and is less prone to be affected by methodological issues. In most studies that have provided reference ranges the LPR has a more consistent upper threshold ranging from 15 to 25.[51,116]

3.3.8. Clinical application

Cerebral MD is a technique that allows to explore the regional chemistry of the human brain in almost real time. It was introduced in 1966 by Bito et al. for *in vivo* dialysis of the canine brain[117] and is now used for an extensive array of applications. The first known application of MD in humans was reported in 1990 by Meyerson et al., who implanted microdialysis probes during thalamotomy procedures in patients with Parkinson's disease.[118] In the same year, the first report of changes in energy-related metabolites during frontal lobe resection in five human patients was published.[119] Since that time, cerebral MD has been increasingly used as a neuromonitoring technique in neurocritical patients with TBI, MMCAI and SAH to monitor cerebral energy metabolism during the acute phase after injury or stroke.[7]

Cerebral MD can inform us about brain energy status (glucose, lactate and pyruvate), excitatory amino acid (glutamate) levels and cell membrane integrity (glycerol). Through the determination of glucose, lactate and pyruvate, we can obtain information about the relative contribution of the aerobic or anaerobic metabolism in energy production. Cerebral metabolism can be studied by the availability of glucose that can be metabolized aerobically or anaerobically. High lactate and LPR levels indicate an increase in anaerobic metabolism due to an impairment in the oxidative one. The change to anaerobic metabolism can be due to two circumstances: 1) the lack of O_2

supply resulting from a hypoxic or ischemic process; 2) the failure in mitochondrial oxidative phosphorylation (mitochondrial dysfunction). LPR is considered a good indicator of cerebral energy metabolism deficiency.[97] On the other hand, glutamate can give us information about the excitatory amino acids release to the extracellular space and about energetic dysfunction present in certain pathologies. The membrane state and the level of cellular stress can be evaluated by glycerol levels.

Brain metabolite levels have a high diagnostic value in the detection of metabolic crisis. Identification of metabolite patterns can lead to the distinction of ischemia and mitochondrial dysfunction, and can be helpful in clinical management and therapeutic guidance of TBI patients.[22,33,77,120] The discrimination between this two physiopathological conditions is important in order to avoid the application of therapies that may be detrimental for the patient. Currently, there is no consensual terminology for classifying the pattern with elevated LPR and normal O_2 levels.[121]

Besides its clinical application, cerebral microdialysis is a powerful research tool with several applications whose aim is to increase the knowledge of cerebral biology and pathophysiology. Among its applications are: 1) the understanding of lactate as a brain energetic substrate; 2) the measurement of larger molecules in order to identify new biomarkers or study pathological processes; 3) the use of ^{13}C-labeled substrates to study the fate of specific molecules through metabolic pathways; 4) the study of drugs local effects on neurochemistry and their penetration across the blood-brain barrier (BBB); 5) the monitoring of the ionic profile of the brain tissue in different situations; 6) the development of on-line analysis systems to perform continuous monitoring.[97]

HYPOTHESES AND OBJECTIVES

TBI can lead to cerebral hypoxia, energy failure and cell death due to a depression of aerobic metabolism and an inability to maintain ionic homeostasis. The mechanisms of this energy failure are not well established. Neuromonitoring with $PtiO_2$ and cerebral MD can be helpful to understand the pathophysiology of TBI and to define different patterns of metabolic alteration, where both the reduced contribution of substrates and mitochondrial metabolism may be altered. There is a lack of knowledge regarding the use of data obtained through advanced brain monitoring systems, which creates uncertainty among clinicians about the correct application of these techniques in the patient management and in the guidance of therapeutic interventions for each patient.

The hypotheses, which were intended to be verified or refuted, included the following:

1. Continuous monitoring of $PtiO_2$ and metabolites involved in cerebral energetic metabolism (glucose, lactate, pyruvate) as well as the determination of brain tissue redox status (LPR) by high resolution MD can allow to identify abnormal metabolic patterns of ischemic/non-ischemic etiology (ischemic hypoxia, hypoxia due to mitochondrial dysfunction, cytotoxic hypoxia or hyperglycolysis).

2. The extrapolation to zero-flow rate method can be applied to determine the real concentrations of metabolites in the fluid surrounding a MD catheter. *In vitro* experiments simulating *in vivo* conditions can confirm the applicability of this methodology *in vivo.*

3. NBO is an effective therapeutic maneuver in patients with TBI who present a metabolic pattern of mitochondrial dysfunction, since this maneuver can partially or totally reverse the alterations in cerebral energetic metabolism.

On the basis of the above assumptions, the specific outlined objectives included the following:

1. To define the reference interval of glucose, lactate, pyruvate, glycerol, LPR and lactate-to-glucose ratio (LGR) in the normal interstitial brain through high-resolution MD in patients who will undergo surgical treatment to treat lesions in the posterior fossa and in whom a MD catheter will be implanted. The RIs currently used are based on a single study of only 9 patients.[49] To define the RIs of metabolites involved in brain metabolism, more robust studies are needed.

2. To conduct *in vitro* experiments to study the RR of energy metabolites (glucose, lactate, pyruvate) and glycerol, and to reproduce the ZFM that will be used in the *in vivo* study of RIs.

3. To study the frequency of brain metabolic alterations and to identify the metabolic patterns present in moderate or severe TBI patients admitted to the ICU and monitored with ICP, $PtiO_2$ and cerebral MD.

4. To evaluate the metabolic response within 4 hours of inducing NBO in patients with TBI and within 72 hours after the placement of the MD and $PtiO_2$ catheters, and to evaluate the different cerebral metabolic profiles, determined with cerebral MD and $PtiO_2$, in order to objectify if NBO improves brain metabolism.

STUDIES CONDUCTED

This doctoral thesis is presented as a compendium of publications with 2 textbook chapters and 2 original articles published in international journals:

1. Poca MA, Sahuquillo J, Sánchez-Guerrero A, Vidal-Jorge M. **Brain tissue oxygen pressure ($PtiO_2$) monitoring.** In: Rodríguez-Villar S, Amballur D, editors. *Critical Care*. Madrid: Marbán Libros S.L.; 2017. p. 795–801. [In press]

   Early detection of ischemic or non-ischemic brain hypoxia is one of the goals of TBI patient monitoring. Brain tissue oxygen pressure monitoring can provide information of the availability of O_2 in the brain parenchyma. The aim of this chapter was to review the $PtiO_2$ monitoring technique for its application in the neurocritical patient.

2. Poca MA, Sahuquillo J, Vidal-Jorge M, Sánchez-Guerrero A. **Cerebral microdialysis in neurocritical patients.** In: Rodríguez-Villar S, Amballur D, editors. *Critical Care*. Madrid: Marbán Libros S.L.; 2017. p. 830–837. [In press]

   As mentioned above, one of the main aims of the treatment of TBI patients is to prevent secondary lesions with the early detection of brain metabolic dysfunction. MD allows to monitor brain neurochemistry at the bedside in almost real time. The aim of the present chapter was to conduct a methodological and conceptual review of cerebral MD in its clinical application.

3. Sahuquillo J, Merino MA, Sánchez-Guerrero A, Arikan F, Vidal-Jorge M, Martínez-Valverde T, Rey A, Riveiro M, Poca MA. **Lactate and the lactate-to-pyruvate molar ratio cannot be used as independent biomarkers for monitoring brain energetic metabolism: a microdialysis study in patients with traumatic brain injuries.** *PLoS One*. Jul 2014; 9(7): e102540.

   For decades, lactate has been considered an excellent biomarker for O_2 limitation and therefore of organ ischemia. The aim of the present study was to evaluate the frequency of increased brain lactate levels and the LPR in a cohort of patients with moderate or severe TBI subjected to brain MD monitoring, and to analyze the agreement between these two biomarkers to indicate brain energy metabolism dysfunction. Based on these two biomarkers, we defined a series of metabolic patterns observed in TBI patients in order to guide clinical MD application in neurocritical care patients.

4. Sánchez-Guerrero A, Mur-Bonet G, Vidal-Jorge M, Gándara Sabatini D, Chocrón I, Cordero E, Poca MA, Mullen K, Sahuquillo J. **Reappraisal of the reference levels for energy metabolites in the extracellular fluid of the human brain.** *Journal of Cerebral Blood Flow and Metabolism*. Oct 2016; [Epub ahead of print]

   In this original article, we present the results of a human study in which the reference intervals for analytes involved in energy metabolism (i.e., glucose, lactate and pyruvate) and for glycerol (a biomarker of cell damage) were determined in a cohort of patients who were observed twice: while anesthetized and while fully awake.

   In anesthetized patients, we used the extrapolation to zero-flow rate method, and in awake patients, we employed perfusion at a constant infusion speed of 0.3 μL/min, which is the infusion rate recommended by a recent consensus conference on neuromonitoring.[97] The RIs reported here provide additional support for the thresholds suggested by the most recent consensus conference

on microdialysis neuromonitoring,[97] highlighting the importance of lactate in brain energetics and raising the need to reconsider traditional definitions of metabolic disturbances observed in neurocritical patients. Our study emphasizes the importance of using different thresholds for awake patients and patients under anesthesia or deep sedation.

Based on the results of both studies, we have defined a series of patterns to be applied in a greater number of patients in order to obtain a more robust classification and to allow microdialysis to be integrated into the clinical treatment of patients with TBI.

In addition to the presented articles, during the development of this doctoral thesis, studies have been conducted to confirm or refute the hypotheses formed in the initial project. These studies are detailed in the annex of this report. The annexed document presents, on one hand, an extension of the study of normal range for brain metabolites that was conducted in order to include more patients and obtain more robust results. Also, we wanted to study the possible differences in metabolite levels between patient conditions and type of anesthesia used. On the other hand, a second study was conducted to evaluate the metabolic response after 4h of NBO in moderate or severe TBI patients. The metabolic results presented are part of a more complete study that is currently under review.

1. Brain tissue oxygen pressure ($PtiO_2$) monitoring.

Poca MA, Sahuquillo J, Sánchez-Guerrero A, Vidal-Jorge M.

Critical Care. Madrid: Marbán Libros S.L.; 2017. p. 795–801. [In press]

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Brain tissue oxygen pressure ($PtiO_2$) monitoring

Maria A. Poca, Juan Sahuquillo, Ángela Sánchez-Guerrero and Marian Vidal-Jorge

INTRODUCTION

Brain hypoxia is a frequent finding in the acute phase after a severe traumatic brain injury (TBI)[1]. Although ischemic hypoxia has been extensively studied, many experimental and clinical reports have shown that non-ischemic hypoxia can frequently exist in isolation or combined with ischemia in any organ, as in TBI associated with shock, sepsis, or other systemic or cranial phenomena[2]. Early detection of ischemic or non-ischemic brain hypoxia is one of the cornerstones of the management of severely head injured patients, especially after the discovery of the close relationship between brain hypoxia and poor clinical outcome[3]. Brain hypoxia can develop even when intracranial pressure (ICP) or jugular bulb oxygen saturation (SjO_2) are within the normal range[4]. Currently, tissue O_2 pressure can be measured directly in the brain parenchyma ($PtiO_2$). $PtiO_2$ measurement is a relatively safe, simple and low-cost technique that provides highly useful information on the availability of O_2 in tissue. In the latest update of the Brain Trauma Foundation guidelines, $PtiO_2$ values have been introduced with level III recommendation as a guide to the use of routine therapeutic measures such as hyperventilation[5].

Information provided by $PtiO_2$

$PtiO_2$ is believed to reflect the availability of free O_2 in the tissue, which is considered to be equivalent to the O_2 at the venous side of the capillary bed. $PtiO_2$ can be viewed as the mean O_2 concentration in the area monitored, which comprises arterioles, capillaries, veins and the extra- and intracellular brain tissue compartments. $PtiO_2$ readings below 15 mmHg indicate

796 brain hypoxia of any type (ischemic, shunt hypoxia, low-extractivity hypoxia, etc.) and have been related to poor functional outcome[3].

Consequently, maintaining $PtiO_2$ within the normal range should be a priority. Detection of low $PtiO_2$ can help to: 1) identify tissue hypoxia, 2) establish a differential diagnosis of the type of tissue hypoxia, 3) individually tailor therapeutic maneuvers and 4) evaluate the effectiveness and repercussions of these maneuvers. $PtiO_2$ allows changes in regional oxygenation to be detected, which could go unnoticed with global brain oxygenation monitoring systems such as SjO_2 or arterio-jugular differences of oxygen ($AVDO_2$). In addition, $PtiO_2$ provides continuous information over long periods[6].

Systems available to measure $PtiO_2$

The Licox® system (GMS, Kiel-Milkendorf, Germany) uses a modification of a Clark polarographic electrode (Fig. 96-1) and is the only system validated for $PtiO_2$ measurement, given that the Neurotrend® system is no longer commercially available. Currently, the Neurovent®–PTO

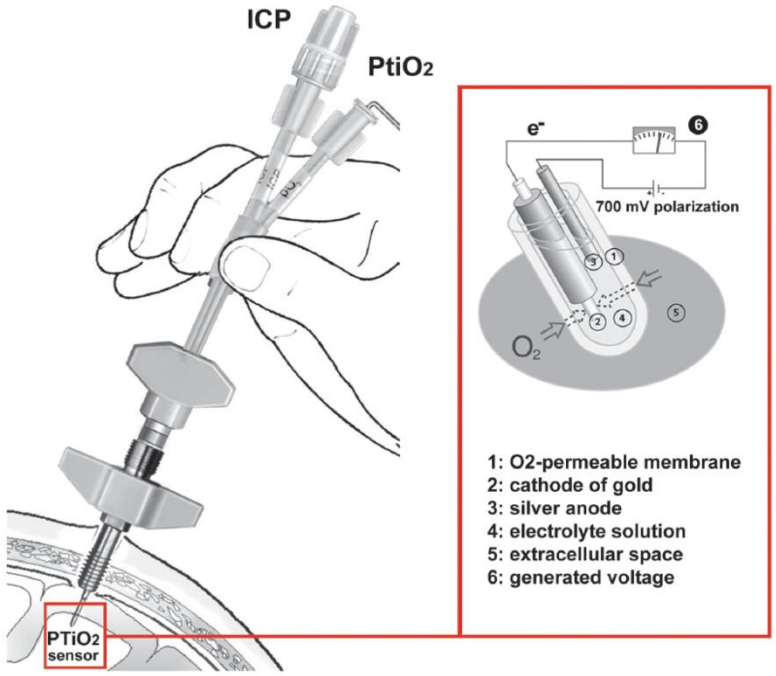

Figure 96-1. Cranial bolt with 2 lumens for simultaneous monitoring of tissue O_2 pressure ($PtiO_2$) and intracranial pressure (ICP). The images on the right shows a scheme of the $PtiO_2$ sensor tip, which will be housed in the subcortical white matter, and within is located the "revoxode" where take place the oxidation reactions that generate the electrical current that determine the $PtiO_2$ value.

sensor (Raumedic Oxygen Measurement System, Rehau, Münchberg, Germany) is also available, which is able to measure ICP, $PtiO_2$ and temperature through the same catheter. However, some discrepancies have been detected in the simultaneous use of both catheters in experimental and clinical practice, and the use of this new sensor should be validated in a larger number of patients. 797

INDICATIONS

In a recent consensus conference it was agreed that brain oxygen monitoring is indicated both in patients with a severe TBI and in those with spontaneous subarachnoid hemorrhage[6]. One of the clearest indications of this monitoring system is to guide the use of potentially harmful therapeutic maneuvers such as hyperventilation, in which $PtiO_2$ decreases below 15 mmHg should be avoided[5]. $PtiO_2$ also allows monitoring of metabolic compromise of the tissue surrounding cerebral hematomas and diagnosis of delayed vasospasm[6]. An additional indication, outside the ICU setting, is intraoperative $PtiO_2$ measurement to monitor brain regions at risk for ischemia during aneurysm surgery and during the use of temporary clipping.

KEY METHODOLOGICAL POINTS

Licox® sensors do not require pre-insertion calibration. Sensor placement is very simple and practically identical to placement of an intraparenchymal ICP sensor[7]. The technique can be performed in the ICU. However, because $PtiO_2$ is a local measurement, the location that will provide the most useful information for patient management should be considered. A recent consensus conference established that placement of $PtiO_2$ sensors in TBI patients should be individualized and depends on the information sought.

Aspects to be considered are[6]:

- The sensor should be placed in a viable tissue region (pericontusional tissue or close to a subdural hematoma) and not in the core of the lesion, which is composed of non-viable tissue.
- Eloquent cerebral areas should be avoided.
- When structurally normal tissue is monitored, the catheter should be implanted in the subcortical white matter of the most injured hemisphere.

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Normal values

Because of ethical limitations, the range of normality in awake humans has not yet been established. Normal $PtiO_2$ values measured with the polarographic system range between 15 and 30 mmHg, although some authors believe that the range in TBI patients should be higher. $PtiO_2$ values < 15 mmHg indicate tissue hypoxia[3,8], which can be moderate (between 15 and 10 mmHg)[3] or severe (< 10 mmHg)[3,8]. Consequently, one of our therapeutic goals should be to maintain $PtiO_2$ values above 20 mmHg.

TYPES OF CEREBRAL HYPOXIA

There are different causes of tissue hypoxia in the brain, and differential diagnosis is essential[2,9]. The real availability of O_2 in the tissue depends not only on partial pressure of O_2 but also on other variables such as hemoglobin concentration, the affinity between hemoglobin and O_2, cerebral blood flow, the number of functioning capillaries, O_2 diffusion through the cell membrane and the characteristics of the extracellular space. Continuous $PtiO_2$ monitoring, combined with the information provided by hemometabolic variables, can help to establish the etiology of tissue hypoxia (Table 96-1)[10].

CLINICAL MANAGEMENT WHEN $PTIO_2$ IS LOW

Table 96-2 shows the algorithm that summarizes the causes of tissue hypoxia to be sequentially excluded when the $PtiO_2$ is low and the specific therapeutic measures to be implemented. The aims of the algorithm are: 1) to exclude lesions at the sensor tip that could explain low $PtiO_2$ values, 2) to ensure correct sensor functioning, 3) to search for treatable causes that can lead to decreases in $PtiO_2$ and 4) to search for alternative causes of hypoxia (Table 96-2).

COMPLICATIONS AND ZERO-DRIFT

Complications related to the implantation of $PtiO_2$ probes are very low. In a consecutive series of 76 patients with a moderated or severe TBI studied in our ICU, there was no infections and in only 2 patients the CT scan showed the presence of blood (volume < 2 mL) in contact with the distal part of the sensor (Fig. 96-2). Although this small collec-

Table 96-1. Types of tissue hypoxia according to the Siggaard-Andersen's classification[2]

Types of hypoxia	Cause	SjO_2 (%)	$PtiO_2$ (mm Hg)	P_{50} (mm Hg)	Px (mm Hg)
Ischemic	↓ Cerebral blood flow	↓	≤ 15	24 - 29	N
Low extractibility	• Hypoxemia • Anemia • High affinity between Hb and O_2	↓ ↓ ↑	≤ 15 ≤ 15 ≤ 15	24 - 29 24 – 29 ≤ 24	↓ ↓ ↓
Shunt hypoxia	Arterio-venous Shunts	N / ↑	≤ 15	24 - 29	N
Dysperfusion	Difficulties in O_2 diffusion from Hb to the mitochondrion	N / ↑	≤ 15	24 - 29	N
Histotoxicity	Inhibition of the mitochondrial cytochromes	N / ↑	N	24 - 29	N
Uncoupling hypoxia	Uncoupling between O_2 reduction and ATP syntesis	N / ↑	N	24 - 29	N
Hypermetabolic	Increasse in metabolic requirements	↓	≤ 15	24 - 29	N

Table modified from Sahuquillo et al.[10]. Hb: hemoglobin. N: range of normality. P_{50}: O_2 pressure at which Hb is 50% saturated. Px: O_2 extraction pressure

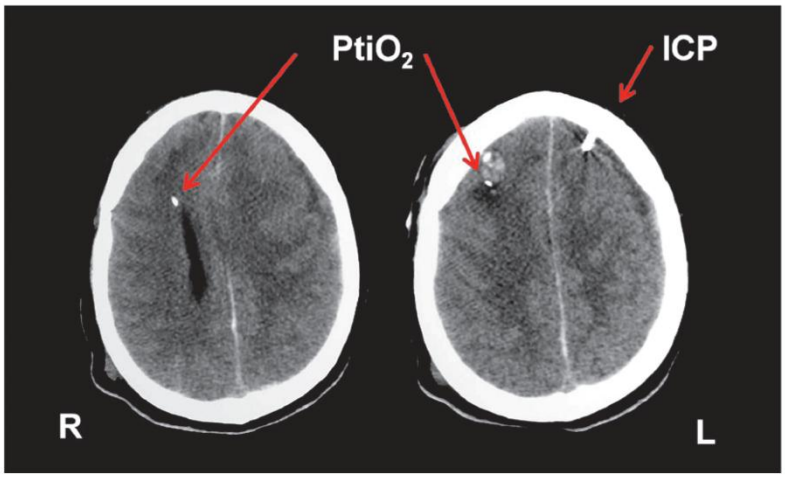

Figure 96-2. Artifact generated by the tissue O_2 pressure sensor ($PtiO_2$) in the CT scan. Note the presence of a small blood collection next to the distal part of the $PtiO_2$ sensor. This sensor must be retired and a new one inserted in a different place.

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Table 96-2. Algorithm of clinical management when $PtiO_2$ is low

	$PtiO_2$ < 15 mmHg	
1	**Is the information reliable?** →	***CT scan***
	• Look for the intracranial sensor position. • Exclude lesions at the sensor tip.	
2	**Is the $PtiO_2$ probe reliable?** →	***Sensor testing***
	• Increase FiO_2.	
3	**Search for treatable factors** →	***Exclude and treat:***
	• Intracranial hypertension. • Low CPP. • Anemia. • Systemic hypoxia. • Severe hyperventilation. • Low CBF due to vasospasm. • Cerebral hypermetabolism (seizures, fever…).	
4	**Search for alternative causes of tissue hypoxia**	
	• Shunt hypoxia. • Dysperfusion hypoxia. • Histotoxic hypoxia.	

FiO_2 = Fraction of inspired oxygen; CPP = Cerebral perfusión pressure; CBF = Cerebral blood flow

tion has not clinical relevance, its presence invalidates the sensor' information and, consequently, the sensor must be retired or located in other place.

Zero-drift associated with the Licox® sensors is also very low. After explantation, the zero-drift calculated in 125 consecutive sensors analyzed in our department was 0.4 ± 1.5 mmHg (sensors were placed in a solution without oxygen).

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2. Cerebral microdialysis in neurocritical patients.

Poca MA, Sahuquillo J, Vidal-Jorge M, Sánchez-Guerrero A.

Critical Care. Madrid: Marbán Libros S.L.; 2017. p. 830–837. [In press]

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Cerebral microdialysis in neurocritical patients

Maria A. Poca, Juan Sahuquillo, Marian Vidal-Jorge and Ángela Sánchez-Guerrero

INTRODUCTION

One of the main aims of the treatment of patients with severe traumatic brain injury (TBI) is to prevent secondary lesions, hence the importance of early detection of brain tissue ischemia. Microdialysis allows the metabolic events occurring in the brain to be determined in almost real time (the availability of substrates such as glucose and the production of several metabolites and neurotransmitters). Consequently, with some limitations, several metabolites can be continuously monitored at the bedside. Nowadays cerebral microdialysis has ceased to be simply a laboratory technique and is now applied in the clinical setting and is being progressively introduced in the monitoring of neurocritical patients. This technique provides important complementary metabolic information to that provided by routinely monitored variables (intracranial pressure [ICP], cerebral perfusion pressure, oxygen saturation in the jugular bulb…)[1]. In addition to these possibilities, the use of high resolution dialysis membranes (100 KDa) allows proteomic studies to be performed that will increase knowledge of the physiopathology of acute neurological lesions.

TECHNIQUE

Aims and principles of the technique

Microdialysis is based on the principle of solute exchange through a semi-permeable membrane, which emulates capillary function and whose main objectives are to monitor: 1) the availability of different tissue metabolites, 2) the elements released by the cells, and 3) the effects of tissue hypoxia-ischemia on the cells[2]. The semi-permeable

membrane is located at the distal end of the microdialysis catheter. Through this membrane the solutes are exchanged between a solution with a known composition and the extracellular fluid. Analysis of the recovered microdialysate allows quantification of several metabolites derived from physiological metabolic pathways or products which are generated as a result of a tissue injury. 831

Metabolites to be determined

Placement of a microdialysis catheter in the brain allows analysis and quantification of changes in several energetic metabolites such as lactate, pyruvate, adenosine, inosine and hypoxanthine. This technique also allows analysis of neurotransmitter and neuromodulator release (glutamate, aspartate, taurine, GABA…), and the release of tissue breakdown products such as glycerol[2]. However, some methodological issues need to be considered for the information to be valid. Moreover, the values obtained by brain catheters must be compared with those provided by an additional catheter placed in the subcutaneous tissue[2]. These subcutaneous values provide information about systemic metabolism (extracerebral).

Methodological considerations

Microdialysis catheters are flexible, with a small diameter (< 1 mm) and contain a double lumen with a semi-permeable membrane at the distal end (Fig. 100-1). Small solutes are able to pass freely across this membrane due to an osmotic gradient. The internal lumen of the catheter contains a metabolite-free solution (Ringer solution without lactate or an isotonic saline serum). The catheter is attached to a continuous infusion micropump, which infuses the solution at a constant and predetermined velocity. At the distal

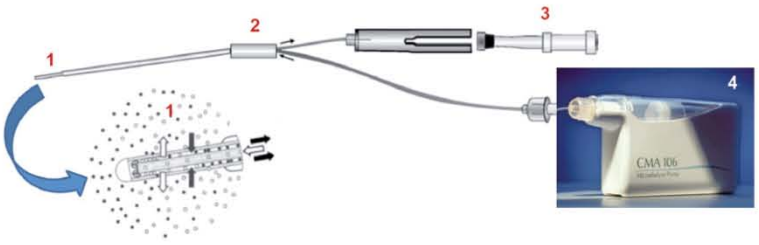

Figure 100-1. Cerebral microdialysis catheter and the basic elements for performing this technique: 1) dialysis membrane, 2) double lumen catheter, 3) microvial, and 4) infusion micropump.

832 extreme of the catheter, and through the semi-permeable membrane, there is an exchange of solutes of a particular molecular weight (< 20 KDa in standard catheters and up to 100 KDa in high-resolution catheters). The microdialysate obtained contains molecules from the extracellular space and flows through the external lumen of the catheter. This microdialysate is recovered through special microvials, which are replaced periodically. The portable analyzer analyzes the microdialysate using enzymatic techniques and quantifies changes in the composition of the initial solution (Fig. 100-2). When catheters with a dialysis membrane of 10 mm and a perfusion flow velocity of 0.3 µL/min are used, metabolite recovery is 70% of the real content of the cerebral extracellular space[3].

In most centers, cerebral microdialysis catheters are surgically implanted through a burr hole or through the cortex after a lesion have been evacuated. However, these catheters can also be implanted using a cranial screw or a percutaneous technique[4] similar to that used to implant an ICP sensor (Fig. 100-3). Like other local monitoring systems, the microdialysis catheter must be implanted in the brain region that provides the most useful information for patient management. Catheter implantation in the healthy tissue provides the possibility of monitoring the tissue with the

Figure 100-2. Analysis of the microvials performed by the nurse using the portable analyzer placed at the bedside. The different metabolites are determined using enzymatic techniques.

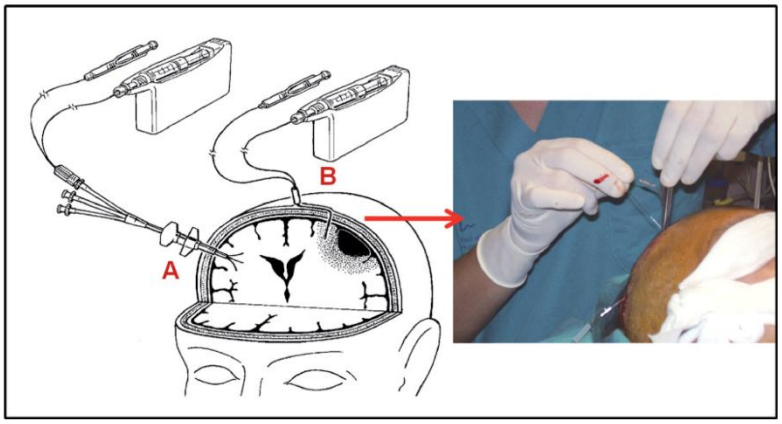

Figure 100-3. Two possible methods for implanting cerebral microdialysis catheters: **A**, a cranial screw (healthy tissue) or **B**, a percutaneous technique (traumatic penumbra).

highest probability of recovery, while implantation in areas of "penumbra" (areas surrounding focal lesions) allows monitoring of brain regions at greatest risk of ischemia (Fig. 100-3). Figure 100-4 shows the radiological artifact produced by a microdialysis catheter.

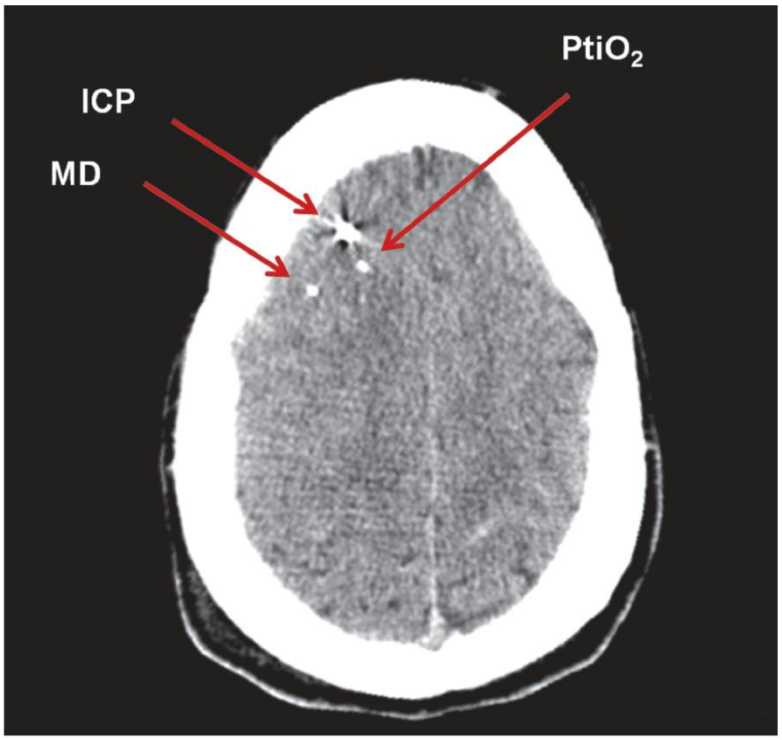

Figure 100-4. Radiological artifacts produced by a microdialysis catheter (MD), an intracranial pressure (ICP) sensor, and a brain tissue oxygen pressure ($PtiO_2$) catheter.

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Normal values

One of the key problems of the microdialysis technique is to establish normal values for the distinct metabolites. Because monitoring healthy individuals is ethically impossible, reference values must be obtained from patients with intracranial lesions, with the limitations that this implies. Table 100-1 summarizes the results of microdialysis monitoring in apparently normal tissue of patients with brain tumors[5] or subarachnoid hemorrhage (SAH)[6], using a perfusion of 0.3 μL/min. The table also includes the thresholds for ischemia of several brain metabolites.

INDICATIONS

The *Consensus statement from the 2014 International Microdialysis Forum*, a report originated on the International Microdialysis Forum held in Cambridge (UK, April 2014) and recently published, reviews the advances in the clinical use of microdialysis in neurocritical care and updates the consensus statements about its clinical use (technique, data interpretation, relationship with outcome, role in guiding therapy in neurocritical care and research applications).

In agreement with the conclusions of an expert panel meeting in the Karolinska Institute in Stockholm in 2002, published in 2004[7], the patients who will derive greatest benefit from the inclusion of cerebral microdialysis in neuromonitoring are those with a severe TBI or SAH.

In both types of patient, the aim of microdialysis monitoring is the same: the early detection of metabolic changes suggesting the development of tissue ischemia, and monitoring of the effect of the therapeutic maneuvers applied to treat the ischemia. In TBI, one or more brain catheters should be applied according to the type of lesion. In diffuse lesions, the implantation of a single brain catheter in the right frontal region is recommended. In focal lesions, two catheters are recommended, one in the macroscopically non-lesioned region and the other in the "area of penumbra" (the brain area surrounding a focal lesion, which is considered at greatest risk). The consensus conference concluded that the information that might be provided by placing an additional catheter in an established lesion does not add important information for patient management. In patients with SAH, a single brain catheter is recommended, although it should be implanted in the vas-

Table 100-1. Normal values and thresholds for ischemia of several brain metabolites

Metabolite	Normal values Reinstrup et al. [5] (mean + SD)	Normal values Schulz et al. [6] (mean + SD)	Thresholds for ischemia
Glucose (mmol/L)	1.7 ± 0.9	2.1 ± 0.2	0.1 ± 0.2
Lactate (mmol/L)	2.9 ± 0.9	3.1 ± 0.3	8.9 ± 6.5
Pyruvate (µmol/L)	166 ± 47	151 ± 12	31 ± 46
Lactate/pyruvate ratio	23 ± 4	19 ± 2	> 23 ± 4
Glycerol (µmol/L)	82 ± 44	82 ± 12	570 ± 430
Glutamate (µmol/L)	16 ± 16	14.0 ± 3.3	380 ± 240

SD: Standart desviation

cular area at greatest risk. The analytes recommended, and the relative importance of each, in both entities is as follows: a) SAH: glutamate and the lactate/pyruvate ratio and b) TBI: lactate/pyruvate ratio, glucose, glycerol and glutamate. The conclusions of the consensus conference are summarized in Table 100-2.

More recently, in the consensus conference on neuromonitoring in neurocritical patients made by the NICEM (Neuro-Intensive Care and Emergency Medicine), a sec-

Table 100-2. Conclusions of an expert panel meeting in Stockholm about cerebral microdialysis in neurocritical patients[7]

	SAH	TBI
Indications	Severe patients who require ICP and CPP monitoring	
Catheters	One catheter in vascular area at greatest risk	Diffuse lesion: One catheter Focal Lesion: Two catheters
Values	Discard values of the first hour of monitoring	
Metabolites	Glutamate Lactate-pyruvate ratio	Lactate-pyruvate ratio Glucose / Glycerol / Glutamate

SAH: Subarachnoid hemorrage. TBI: Traumatic brain injury. ICP: Intracranial pressure. CPP: Cerebral perfusion pressure

836 tion of the ESICM (European Society of Intensive Care Medicine)[8], the expert panel concluded that:

- Despite the increase in the application of cerebral microdialysis in the clinical management of patients with a severe TBI, class I evidence on the routine use of this technique is lacking.
- Microdialysis is the only technique that allows continuous monitoring of the biochemical characteristics of the extracellular space of the brain parenchyma. This information is much more reliable than any other biomarker obtained from peripheral blood [9].
- Microdialysis can help in the differential diagnosis of the distinct types of non-ischemic hypoxia.
- High-resolution cerebral microdialysis allows recovery of additional substances contained in the extracellular space, such as cytokines, interleukins and other inflammatory molecules[10], which will allow greater understanding of the physiopathology of acute neurological lesions.
- This technique is ideal to obtain direct information on the passage of drugs through the blood-brain barrier and on their metabolic repercussions, which will allow studies of neuroprotective drugs to be more rational and effective.

FINAL CONSIDERATIONS

Cerebral microdialysis is an extremely sensitive technique that can provide early metabolic information on the development of a brain lesion. The information provided by this technique is superior to that provided by any other monitoring system. Despite its undeniable current position in research, in all probability its use will become widespread in the clinical setting in the next few years, providing new knowledge on the physiopathology of neurocritical patients as well as guidance on the most effective treatment. However, the introduction of this monitoring system involves a learning curve and requires human and technical resources, which currently limits its use to certain neurocritical units.

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Further reading

PJ Hutchinson, I Jalloh, A Helmy, KL Carpenter, E Rostami, BM Bellander, et al. Consensus statement from the 2014 International Microdialysis Forum. *Intensive Care Med* 2015;41:1517-28.

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3. Lactate and the lactate-to-pyruvate molar ratio cannot be used as independent biomarkers for monitoring brain energetic metabolism: a microdialysis study in patients with traumatic brain injuries.

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Lactate and the Lactate-to-Pyruvate Molar Ratio Cannot Be Used as Independent Biomarkers for Monitoring Brain Energetic Metabolism: A Microdialysis Study in Patients with Traumatic Brain Injuries

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Abstract

Background: For decades, lactate has been considered an excellent biomarker for oxygen limitation and therefore of organ ischemia. The aim of the present study was to evaluate the frequency of increased brain lactate levels and the LP ratio (LPR) in a cohort of patients with severe or moderate traumatic brain injury (TBI) subjected to brain microdialysis monitoring to analyze the agreement between these two biomarkers and to indicate brain energy metabolism dysfunction.

Methods: Forty-six patients with an admission Glasgow coma scale score of ≤13 after resuscitation admitted to a dedicated 10-bed Neurotraumatology Intensive Care Unit were included, and 5305 verified samples of good microdialysis data were analyzed.

Results: Lactate levels were above 2.5 mmol/L in 56.9% of the samples. The relationships between lactate and the LPR could not be adequately modeled by any linear or non-linear model. Neither Cohen's kappa nor Gwet's statistic showed an acceptable agreement between both biomarkers to classify the samples in regard to normal or abnormal metabolism. The dataset was divided into four patterns defined by the lactate concentrations and the LPR. A potential interpretation for these patterns is suggested and discussed. Pattern 4 (low pyruvate levels) was found in 10.7% of the samples and was characterized by a significantly low concentration of brain glucose compared with the other groups.

Conclusions: Our study shows that metabolic abnormalities are frequent in the macroscopically normal brain in patients with traumatic brain injuries and a very poor agreement between lactate and the LPR when classifying metabolism. The concentration of lactate in the dialysates must be interpreted while taking into consideration the LPR to distinguish between anaerobic metabolism and aerobic hyperglycolysis.

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Introduction

High intracranial pressure (ICP) and brain ischemia have traditionally been considered the main secondary intracranial insults, and have thus been the subject of most traumatic brain injury (TBI) research. However, evidence accumulated in the last decade indicates that ischemia (ischemic brain hypoxia) is the least frequent etiologic factor in TBI-related energetic disturbances [1,2]. Several studies have shown that disturbances in energy metabolism pathways are frequent in experimental models and clinical studies of TBI. A better understanding of the metabolic events that occur in the acute and subacute phases of TBI is essential for detecting these abnormalities at the bedside and to be better able to reverse or modulate such changes. Microdialysis (MD) probes, alone or in combination with brain oxygen monitoring, allow for the direct assessment of brain energetic

metabolism via the detection of quasi-real-time changes in the concentration of the brain's energetic substrate (i.e., glucose), the intermediate product of glycolysis (i.e., pyruvate) and the product of both aerobic and anaerobic metabolism (i.e., lactate).

For decades, lactate has been considered an excellent biomarker for oxygen limitation and therefore of organ ischemia; as a result lactate is often regarded as an essential biomarker for managing circulatory failure. Most clinical research in hypoperfused organs, including the brain, is based on the "anaerobic threshold" concept introduced by Wasserman and McIlroy in 1964 [3]. According to the traditional paradigm, increase in lactate in any organ or in blood, even during muscular contraction or exercise, is a direct consequence of tissue hypoxia. Under conditions of oxygen limitation, the inhibition of the respiratory chain increases the mitochondrial and cytosolic pools of reduced nicotinamide adenine dinucleotide (NADH) and as a consequence lactate production increases [4].

In accordance with conventional theory, in patients with acute brain injuries, an increase in brain extracellular lactate levels above a somewhat arbitrary and variable threshold (i.e., 2.0–4.0 mmol/L) has been considered an indicator of increased anaerobic glycolysis and brain hypoxia [1,5–8]. However, the lactate-to-pyruvate ratio (LPR) is considered a more robust indicator of anaerobic metabolism and the redox status of the tissue, and has been found to be an independent predictor of mortality and unfavourable outcome in a multivariate analysis of the largest cohort of TBI patients monitored with MD [7]. In TBI clinical research, lactate and the LPR have been used as equivalent indicators of ischemic and non-ischemic brain hypoxia. This has confounded the discussion regarding brain metabolism impairment and the potential benefit of some therapies. Normobaric hyperoxia could be used as a potential treatment to improve brain oxygenation and consequently the metabolic disorders resulting from TBI. However, contradictory results have raised significant controversy regarding this treatment, in part due to the diverse metabolic criteria used to evaluate the brain's response to the hyperoxic challenge, mostly based in brain lactate levels without taking into consideration changes in pyruvate and in the LPR [8–13].

The conventional interpretation of high brain lactate levels was first challenged by the studies of Vespa et al. which showed that in TBI patients, increases in lactate may indicate hyperglycolysis or metabolic crisis and not necessarily ischemia [2]. These studies in the acute brain injuries aligns with subsequent evidence indicating that lactate is a non-specific biomarker of an increased glycolytic flux, but that the increase in glycolysis can have multiple etiologic factors other than tissue hypoxia [14]. The resting brain releases a small amount of lactate that increases by 3- to 4-fold during brain activation and there is growing evidence that lactate may be a fuel used by neurons under aerobic conditions in agreement with the hypothesis of the astrocyte-neuron lactate shuttle suggested by Pellerin and Magistretti [15]. For a comprehensive review of the recent controversies regarding theory of lactate as a fuel and signaling molecule we recommend the recently published Dienel's review [16].

In a pilot study published by our group we found a high prevalence of increased lactate and a striking discordance between the concentration of lactate and the LPR [17]. The present study aimed to evaluate the frequency of increased brain lactate levels and the LPR in a larger cohort of TBI patients and to analyze the agreement between these two biomarkers, thus determining whether they could be used independently to indicate brain energy metabolism dysfunction.

Materials and Methods

Subject selection and study design

Between September 1999 and March 2013, 188 patients with moderate/severe TBI were monitored using brain MD. Moderate/severe TBI was defined as an admission Glasgow coma scale score $\leq$13 after resuscitation and in the absence of paralytic agents or sedation. The Traumatic Coma Data Bank (TCDB) classification [18] was used to stratify patients. All patients were admitted to a dedicated 10-bed Neurotraumatology Intensive Care Unit (NICU) at Vall d'Hebron University Hospital, a tertiary center dedicated to the management of acute traumatic brain injuries. For all patients, clinical information, such as ICP monitoring data and systemic data, which were collected at least hourly, was stored in an in-house specially dedicated database in which demographic, monitoring and outcome variables are routinely recorded by research fellows upon discharge of patients from the NICU. All patients were treated according to the Brain Trauma Foundation guidelines for adult TBI patients, which were incorporated into our routine management protocols in 1996 [19,20].

A randomly generated list of numbers was used to select a random sample of 54 patients from this cohort. This strategy allowed us to confirm the quality of the neuromonitoring data and review the MD data; we could thus verify that patients had at least 12 hours of complete monitoring, reject artifacts and verify the correct placement of the MD probe. To avoid variability in selection criteria, a single person (MAM) was responsible for conducting this analysis. Of the selected 54 patients, 8 were excluded because data concerning energetic metabolites (i.e., lactate, pyruvate and glucose) were missing. The remaining 46 patients with complete metabolic data were included in this study.

This cohort yielded 6530 samples. After verification, the data set was reduced to 5305 samples (81% of the total samples) that had good quality data, in which predefined artifacts and doubtful measurements were excluded. Six months after injury, outcomes were assessed by an independent neuropsychologist using the Extended Glasgow Outcome Scale (GOSE). The obtained scores were sorted into two categories: bad outcome (i.e., GOSE: 1–4) and good outcome (i.e., GOSE: 5–8).

Ethics statement

This study received institutional approval (protocol number PI-030153). The Institutional Review Board waived the need for informed consent because MD is routinely performed in all TBI patients admitted to our Neurotraumatology Intensive Care Unit.

ICP monitoring and general management

Continuous ICP monitoring was performed in all patients using a Camino Model 110-4B intraparenchymatous ICP sensor (Integra Neurosciences, Plainsboro, NJ, USA). Our complete ICP monitoring protocol in neurocritical patients has been previously published [21]. Due to the significant interhemispheric ICP gradients in patients with focal lesions [22], the ICP sensor was always implanted in the "worst" hemisphere, which was defined as the hemisphere with the most evident lesion, in the precoronal region, 11 cm from the nasion and 3 cm from the midline. End-hour ICP readings were recorded manually by patient nurses. All patients received standard treatment that aimed to maintain the following therapeutic targets: a cerebral perfusion pressure above 60 mmHg and an ICP below 20 mmHg. No clinical decisions were based on MD data, except for decisions to screen for potential secondary intra- or extracranial insults that may induce significant changes in MD trends.

Brain microdialysis monitoring

Most patients who require ICP monitoring and lack contraindications for multimodality neuromonitoring are routinely monitored using MD probes. Brain microdialysis catheters are inserted into non-injured brain tissue according to a previously described methodology [23]. A CMA-70 brain microdialysis catheter with a 20-kDa cut-off membrane (MDialysis AB, Stockholm, Sweden) was used in 16 patients, and a CMA-71 catheter with a 100-kDa cut-off membrane (MDialysis AB, Stockholm, Sweden) was used in 30 patients. The position of the catheter was confirmed by a control CT scan in which the gold-tip of the catheter is always visible. In addition, all patients were monitored using a CMA-60 microdialysis catheter (MDialysis AB, Stockholm, Sweden) inserted into the subcutaneous adipose tissue of the abdominal region, and data was collected hourly and used as a systemic reference.

Cerebral catheters were perfused with sterile isotonic fluid containing 147 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 2.7 mmol/L KCl, and 0.85 mmol/L $MgCl_2$ at a fixed flow rate of 0.30 μL/min using a CMA-106 pump (MDialysis AB, Stockholm, Sweden), and the dialysates were collected in capped microvials specially designed to collect micro-volumetric samples and minimize evaporation. The microdialysate samples were collected hourly by patient nurses.

For the purpose of the analysis, data from the first two samples were always excluded. Lactate, pyruvate and glucose levels were routinely monitored using a CMA-600 microdialysis analyzer (MDialysis AB, Stockholm, Sweden), with glutamate, urea, or glycerol chosen as the fourth analyte, depending on the patient. The CMA detection intervals, as provided by the manufacturer, are 0.1–25 mmol/L for glucose, 0.1–12 mmol/L for lactate, and 10–1500 μmol/L for pyruvate. Concentrations below the level of detection were replaced by a value corresponding to the lower level of detection of the analyte divided by two, as suggested by other authors [24,25]. The microvials were placed in a microvial rack after analysis, which was designed to seal the microvials and prevent evaporation (MDialysis AB, Stockholm, Sweden). The microvial rack was stored at 4°C at the bedside before transfer to −80°C for long-term storage.

Metabolic thresholds

The range of normality for brain lactate, the LP ratio and the "anaerobic" threshold is still quite arbitrary and usually extrapolated from animal studies, studies in other organs or from brain MD conducted in patients who underwent neurosurgical operations for posterior fossa lesions or epilepsy [5,26,27]. The best estimate we have for the upper lactate and LPR thresholds are from studies of CSF and from a few studies conducted in patients in whom brain MD were monitored under general anesthesia (using different anesthetic management and/or operated on neurosurgical procedures). In these studies the upper range of normality in normal brain tissue for lactate is extremely variable and ranges from 1.50 to 5.10 [7,14,27]. A recent study for the reference ranges in the CSF established an upper 95% percentile at 2.6 mmol/L [28]. Reinstrup et al. measured energetic metabolism in nine awake and anesthetized patients using the same probes (10 mm length and 20 kDa cut-off) and flow rate that were used in our study (0.3 μL/min), and that are used by most groups in the clinical setting [5].

Perfusion speed is known to influence the relative recovery of any extracelular fluid (ECF) metabolite, whereas the LPR remains stable and is less prone to be affected by methodological issues. In most studies that have provided reference ranges the LPR has a more consistent upper threshold ranging from 15 to 25 [7,29]. For the purpose of this study we selected a 2.5 mmol/L threshold for lactate (corresponding to an actual brain tissue lactate of around 3.0 mmol/L) and a LPR of 25. To compare our data with recently published studies we also conducted the analysis with the proposed thresholds for lactate of 4.0 mmol/L [6,7].

Statistical analysis

Descriptive statistics were obtained for each variable. For continuous variables, summary statistics were the mean, median, range and the standard deviation. Percentages and sample sizes were used to summarize categorical variables. The Shapiro-Wilks test and inverse probability plot were used to test whether data followed a normal distribution. To correlate two continuous variables the most conservative Kendall's tau (when data did not follow a normal distribution) or Pearson correlation test (for data following a normal distribution) was used. To model the dependence of the LPR on lactate simple linear regression and the Ordinary Least Squares (OLS) method were used with lactate as the independent variable. Adjusted R^2 were calculated for all models to test whether linear or non-linear models adequately explained the relationships between both variables.

Statistical analyses were carried out with R (version 3.0.1) [30] and the integrated development environment R Studio (v0.97.551) [31]. The car package was used for regression analysis [32]. For evaluating agreement between lactate and the LP ratio the overall proportion of agreement, the unweigthed Cohen's Kappa and Gwet's AC1 [33] were used to calculate chance-corrected inter-rater reliability coefficients using AgreeStat v2011.3 (Advanced Analytics, Gaithersburg, MD, USA). Gwet's AC1 statistic is more robust than Cohen's Kappa in avoiding what has been called the "Kappa paradox", the situation in which Kappa values are low despite a high percentage of agreement [33]. We established a Kappa and Gwet's AC1 of at least 0.61 as the minimum acceptable degree of agreement to consider both lactate and the LPR as interchangeable in the clinical setting [34].

Results

Forty-six (36 men and 10 women) patients with a median initial Glasgow coma sale score of 6 were included in the study. Mean age of the group was 35±15 years. We included 26 patients with a diffuse type II injury, 5 patients with a diffuse type III injury, 5 patients with a diffuse type IV injury, 8 patients with an evacuated mass lesion (V) and 2 patients with a non-evacuated mass lesion (VI). Demographic and clinical characteristics of this cohort are summarized in **Table 1**. Because of the changes in the CT scan pattern, we verified that the MD probe was always placed in a macroscopically normal brain in all patients. Six-month mortality rate was 17.4% (8 patients). Five patients could not be contacted for long-term follow-up and thus it was not possible to assess the six-month GOSE. Of the remaining 33 patients, 15 presented a good outcome and 18 a bad outcome.

Lactate, pyruvate and glucose extracellular concentrations

The 46-patient group yielded 5305 verified samples of good data in which predefined artefacts and uncertain or incomplete measurements had been excluded. **Figure 1** presents a summary of the glucose, lactate and pyruvate data. None of the variables were normally-distributed and were highly skewed to the right. The median for glucose was 1.42 (min: 0.05, max: 11.64), 2.80 for lactate (min: 0.10, max: 12.00) and 0.117 for pyruvate (min: 0.011, max: 0.644), all in mmol/L. The median for the LPR was 23.9 (min: 2.0, max: 545.5). In 28 microvials (0.53%) LP ratio was above 150. In all cases, the extreme LPR values were due to a

Table 1. Demographic and clinical characteristics of the patients.

Sex[a]		
Man	36	(78%)
Woman	10	(22%)
Age[b]	35	(±15)
Initial GCS[c]	6	(5,8)
Initial CT classification[a]		
II	26	(56.5%)
III	5	(10.9%)
IV	5	(10.9%)
V	8	(17.4%)
VI	2	(4.3%)
GOSE (6 months)[a]		
Good outcome	15	(32.6%)
Bad outcome[d]	18	(39.1%)
Dead[e]	8	(17.4%)
Lost to 6-months follow-up	5	(10.9%)

GOSE, Extended Glasgow Outcome Scale; GCS, Glasgow. Coma Scale.
[a]Number of cases (percentage).
[b]Mean (standard deviation).
[c]Median (first quartile, third quartile).
[d]Patients with upper or lower severe disability or vegetative state.
[e]Mortality at hospital discharge.
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remarkably low pyruvate (0.01 to 0.06 mmol/L). Lactate was above 2.5 mmol/L in 3018 readings (56.9%) (**Table 2**). If the lactate threshold was increased to 3.0 mmol/L, 2457 determinations (46.3%) were above this threshold, while 1496 (28.2%) would be above 4.0 mmol/L threshold. The LPR was above the predefined threshold of 25 in 2309 determinations (43.5%).

Correlation between lactate and the LP ratio

A scatter plot of these two variables is shown in **Figure 2**. When all the data were considered, no linear relationship between lactate and the LP ratio was found (adjusted $R^2 = 0.15$). When all outliers with an LP ratio >150 were excluded (28 samples, 0.53%), a very modest linear regression model was fit and the variables were found to be correlated (adjusted $R^2 = 0.22$). However, the plotted residuals showed that the relationships between lactate and the LP ratio could not be adequately modeled by any linear or non-linear model. This fact suggests that the variations in pyruvate were very influential but that they could not be used as an independent predictor of the LPR (data not shown). The same analysis was conducted between lactate (predictor) and the lactate-to-glucose ratio (outcome), but we could not fit a linear or non-linear model to these data either ($R^2 = 0.03$).

Agreement between lactate and the LP ratio

To calculate the agreement between lactate and the LPR in classifying hourly microdialysates, we assumed that all 5305 readings were rated independently by the two biomarkers where the ratings were based on a dichotomous scale consisting of 1) normal metabolism or 2) abnormal metabolism. The thresholds of 2.5 mmol/L for lactate and 25 for the LP ratio were used to sort the patients. From the generated contingency tables **(Table 2)**, the overall proportion of agreement, the un-weighted Cohen's kappa, and Gwet's AC1 statistic were used to calculate chance-corrected inter-rater reliability coefficients. The percentage of agreement was 65%, however Cohen's kappa was 0.32 (95% CI: 0.29–0.34) and Gwet's AC1 was 0.31 (95% CI: 0.28–0.33). None of the statistics showed an acceptable chance-corrected agreement in classifying patients with normal or abnormal metabolisms. When using a contingency table with a higher threshold for lactate (4.0 mmol/L) and the value of 25 for the LPR used by other authors [6,7], neither Cohen's kappa (0.27, 95% CI: 0.25–0.30) nor Gwet's AC1 (0.36, 95% CI: 0.34–0.39) were above what is considered moderate agreement [34].

Metabolic patterns

In an attempt to simplify the data analysis, we divided the entire dataset into the four metabolic patterns defined in **Figure 3**:1) **Pattern 1**: lactate normal (≤2.5 mmol/L) and LPR below 25, 2) **Pattern 2**: high lactate (>2.5 mmol/L) and a concurrent LPR ≤ 25, 3) **Pattern 3:** increased lactate (>2.5 mmol/L) and increased LPR (>25), 4) **Pattern 4:** normal lactate (≤2.5 mmol/L) and increased LPR (>25). The frequency of each pattern and the descriptive statistics are summarized in **Table 3**.

Pattern 4 ("low pyruvate") was observed the least frequently (10.7%) and was analyzed in depth because it challenged our understanding of energetic metabolism. The most striking difference in this pattern was that the median concentration of glucose in the brain ECF was significantly lower than the brain glucose in the other three groups (Kruskal Wallis, chi-squared = 471.4, df = 3, $p<0.001$). Of the 565 samples included in this profile ("low pyruvate"), we had good subcutaneous MD data for 435 of them (77%). In this subset, we defined a threshold of 3.6 mmol/L (65 mg/dL) to define low subcutaneous glucose and potential hypoglycemia. An excellent correlation has been shown in diabetic patients by continuous monitoring of glucose by vascular and subcutaneous MD [35]. Furthermore, in neurocri-

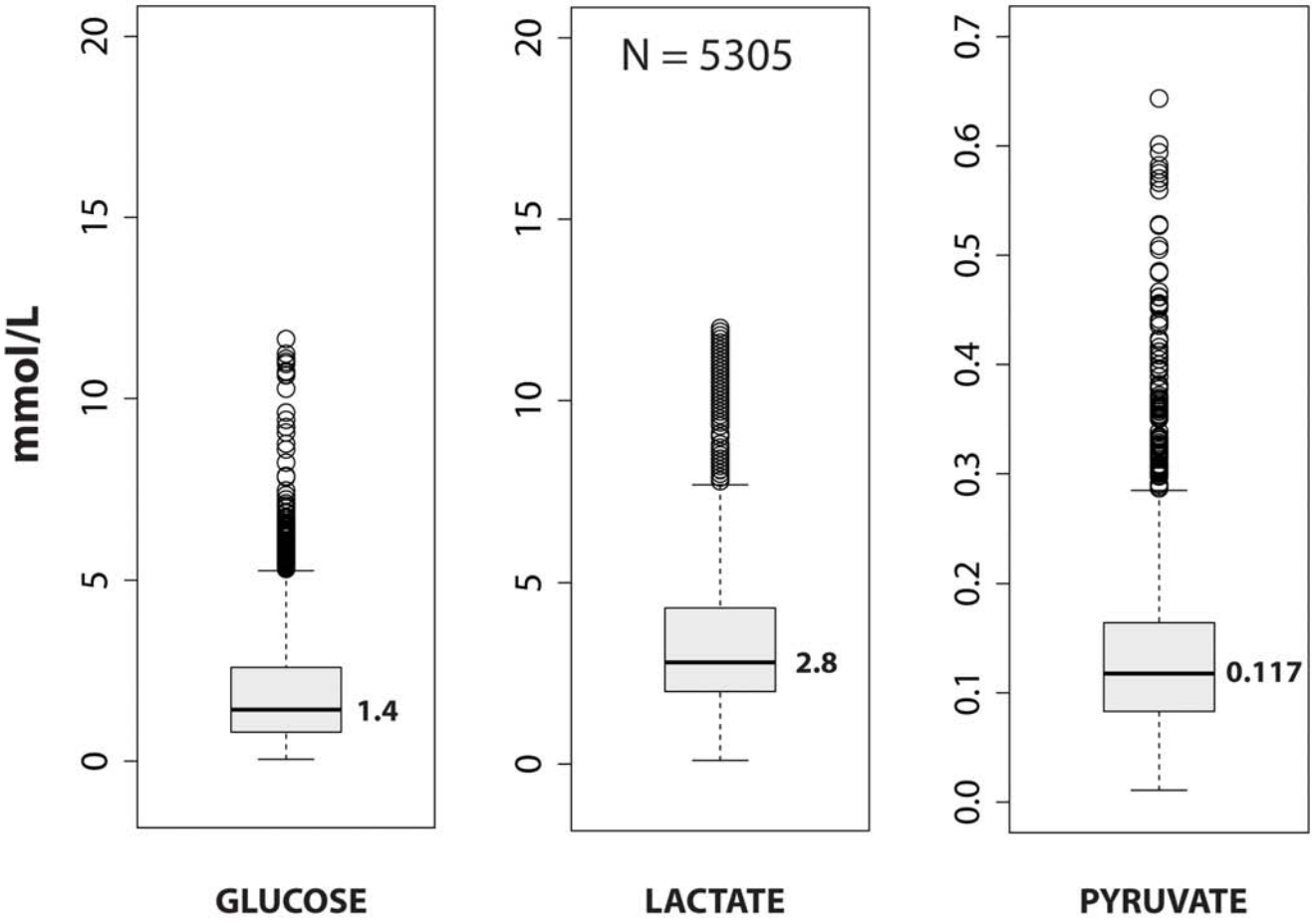

Figure 1. Box-and-whisker plots summarizing the distribution of data for glucose, lactate, and pyruvate. The rectangular box spans the first to the third quartile of data and the median, marked in the center of the box, is labeled with its value to the right. Whiskers extend to the furthest observations within ±150% of the interquartile range. Observations outside 150% of the interquartile range are marked as outliers with dots. N = 5305 represents all valid matched data for the three variables. All variables were highly skewed to the right. For an explanation, see the text in the results section.
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tical patients, a good correlation has been shown between blood glucose and subcutaneous glucose six hours after insertion of the MD catheter [36]. Therefore, we considered subcutaneous MD glucose an acceptable surrogate for hypoglycemia. According to this threshold, only 69 subcutaneous samples (15.8%) showed a low subcutaneous glucose concentration.

Table 2. Intermethod agreement in the classification of dialysate samples by lactate and the LPR.

			Lactate		
			Normal metabolism	*Abnormal metabolism*	*Total*
			L ≤2.5	*L >2.5*	
Lactate-to-pyruvate ratio	*Normal metabolism*	*N*	1722	1274	2996
	LP ratio ≤25	*%*	32.5%	24.0%	56.5%
	Abnormal metabolism	*N*	565	1744	2309
	LP ratio >25	*%*	10.7%	32.9%	43.5%
	TOTAL	*N*	2287	3018	**5305**
		%	43.1%	56.9%	**100%**

LP, lactate-to-pyruvate; L, lactate; >, greater than; ≤, less than or equal to; N, number of readings; %, percentage.
doi:10.1371/journal.pone.0102540.t002

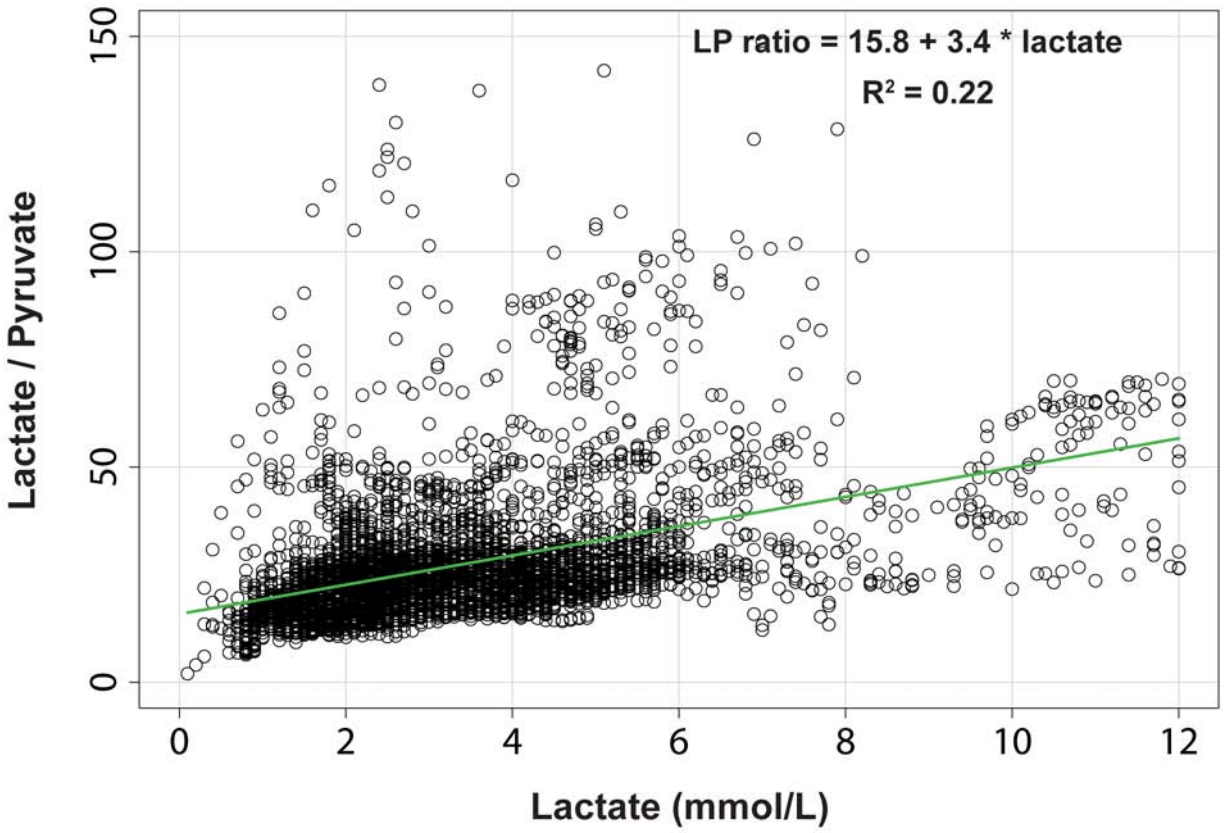

Figure 2. Scatter plot of the relationship between lactate and the lactate-to-pyruvate ratio. The best-fit straight line using an ordinary least squares method is included. In this plot, outliers with a lactate-to-pyruvate ratio >150 were excluded. The plotted residuals and other regression diagnostics show that the relationships between lactate and the lactate-to-pyruvate ratio could not be adequately modeled by any linear or non-linear model.
doi:10.1371/journal.pone.0102540.g002

Discussion

Brain MD allow for continuous neurochemical monitoring and the unique opportunity to explore the disturbances in metabolism in neurocritical patients. However, MD is not yet a routine neuromonitoring tool and the interpretation of the results is still plagued by methodological problems, disagreement in reference ranges and thus in diagnostic thresholds, and uncertainties in the interpretation of the results. Our data showed poor agreement between the two most commonly used biomarkers for impaired energetic metabolism and highlighted the need for a common ground for future comparisons of results across studies.

The ongoing problem of the reference ranges

One of the crucial steps for any biomarker is determining its reference range (i.e., its normal range) in a cohort of disease-free patients. For brain MD, this approach is limited by its invasiveness and by ethical issues. In MD, technical issues increase the complexity of determining appropriate reference intervals. The infusion flow rate, the length of the dialyzing membrane, and the fluid used as a perfusate modify the amount of the recovered analyte. The concentration of the analyte in the dialysate reflects a variable–and sometimes unknown–percentage of the real tissue concentration [37]. To minimize this problem, most centers use a standardized method with 10 mm length membranes, mock CSF as the perfusate, and a flow rate of 0.3 μL/min.

The most commonly used reference range was taken from the study of Reinstrup et al. conducted on nine awake and anesthetized patients operated on for posterior fossa tumors in whom a ventricular drainage was inserted [5]. In the awake patients, the mean lactate was 2.9±0.9 mmol/L and the LPR was 23±4. Applying the conventional rule–using 1.96 standard deviation– for calculating the upper thresholds, gives an upper threshold for lactate of 4.6 mmol/L and of 31 for the LPR. However, due to the small sample size, the anesthetic conditions –isoflurane was used in some of them– and the changes in perfusion speed, using the traditional method for calculating the upper and lower threshold from this cohort is misleading. In addition, volatile anesthetics may induce a significant increase in lactate due to reversible mitochondrial dysfunction [38,39] and the awake patients were studied immediately after surgery and therefore metabolic stress or even hyperglycolysis could be a confounding factor.

Most papers in the last decade that studied patients with TBI and aneurysmatic subarachnoid hemorrhage used an upper threshold of 4.0 mmol/L, a threshold that Timofeev et al. showed was useful in predicting poor outcomes in TBI patients [7]. However, we believe that such a high threshold is difficult to justify, especially when much lower thresholds are used in blood or other organs [40,41]. In our study, we used an upper threshold of 2.5 mmol/L for lactate corresponding to a real ECF lactate of around 3.7 mmol/L according to lactate's relative recovery of 67% calculated by Hutchinson et al. using the same methodology as our study [42]. For the LPR, we selected the threshold of 25 used in most studies because this ratio is robust to changes in fluid recovery [2,6–8,26,43].

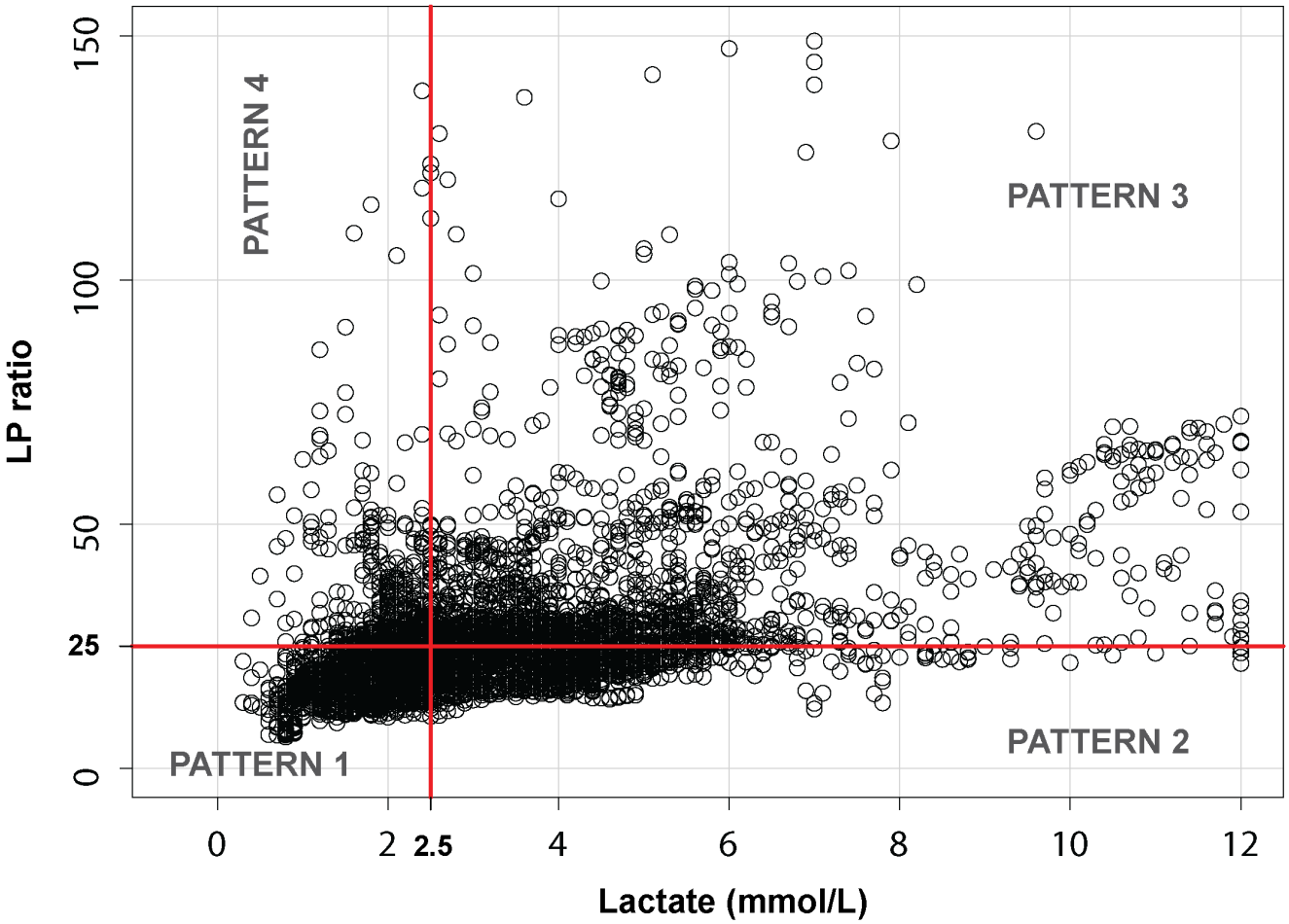

Figure 3. Scatter plot for lactate and the lactate-to-pyruvate ratio. The entire dataset is divided in the four patterns described in the text. We propose the following terminology for the four patterns: Pattern 1 (normal metabolism), Pattern 2 (aerobic hyperglycolysis), Pattern 3 (anaerobic metabolism), and Pattern 4 (low pyruvate). For an explanation, see the text.
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Lactate and the LP ratio need to be combined as screening tools

Moderate and severe TBIs cause frequent alterations in brain energy metabolism even in macroscopically normal brain tissue. At the predefined threshold of 2.5 mmol/L, we found an increase in brain lactate in 56.9% of the samples analyzed (**Table 2**). In 1496 samples (28.2%), lactate was above 4.0 mmol/L. Our data are in agreement with a recent study from our group in which the effects of normobaric hyperoxia on brain energy metabolism were evaluated and 45% of the patients had lactate levels above 3 mmol/L despite the probe placement being in macroscopically non-injured brain tissue and with a $PtiO_2$ within the normal range in most of them [8]. If probes had been placed in traumatic penumbra and not in brain that appeared normal, the frequency of increased lactate could have been even higher. However, despite the high prevalence of elevated lactate, concurrent elevation of lactate and the LPR (>25) was observed in only 33% of the microdialysates, suggesting that a true anaerobic pattern was detected in one third of the samples.

We found a remarkable disagreement between lactate and the LP ratio in classifying the brain energy state in a simple dichotomized scale (normal versus abnormal/anaerobic). Methods used to evaluate inter-rater agreement showed a non-acceptable agreement between lactate and the LPR in classifying samples. Therefore, the conventional approach of using lactate or the LPR alone–at any threshold– needs to be abandoned for metabolic profiling both in clinical settings and for reporting clinical research. Any attempt to classify the metabolic status as normal or abnormal (i.e., "anaerobic") using only lactate can lead to an overdiagnosis of anaerobic metabolism when not combined with pyruvate and the LPR. Hovda's group has shown the major flaws of using this approach in different studies [2,44].

The meaning of high lactate in the brain has been extrapolated from research in shock and sepsis and from experimental models of brain ischemia. An increase in lactate in any organ has been traditionally associated with low tissue perfusion and anaerobic metabolism [29]. In addition, hyperlactatemia has been consistently associated with an increase risk of death in patients with various types of shock [45]. However, in critical care research, pyruvate and consequently the LPR have been rarely used because of the complex handling of pyruvate that is required and the lack of analytical tools to assay it at the bedside [29]. The availability of portable MD bedside analyzers has changed this scenario, allowing the systematic use of pyruvate. The incorporation of pyruvate into neurocritical care has made it increasingly clear that the production and clearance of lactate in any tissue is multifactorial and far more complex than expected [2,14]. In brief, lactate concentration is a highly sensitive indicator of

Table 3. Summary of metabolites for each metabolic pattern.

Metabolic pattern	Thresholds	Suggested terminology	N	%	Glucose (mmol/L)[a]	Pyruvate (mmol/L) [a]	Lactate (mmol/L) [a]	Lactate-pyruvate ratio [a]
1	L ≤2.5	Normal metabolism	1722	32.5	1.43 (0.05–7.1)	0.097 (0.01–0.236)	1.8 (0.1–2.5)	18.0 (2.0–25.0)
	LPR ≤25							
2	L >2.5	Aerobic hyperglycolysis	1274	24.0	2.27(0.11–11.6)	0.185 (0.10–0.643)	3.8 (2.6–12.0)	21.8 (10.8–25.0)
	LPR ≤25							
3	L >2.5	Anaerobic metabolism	1744	32.9	1.28 (0.05–6.5)	0.128 (0.01–0.455)	4.4 (2.6–12.0)	31.3 (25–545.5)
	LPR >25							
4	L ≤2.5	Low pyruvate	565	10.7	0.93 (0.05–7.1)	0.069 (0.01–0.098)	2.1 (0.4–2.5)	28.8 (25–195.3)
	LPR >25							
TOTAL			5305	100	1.42 (0.05–11.6)	0.117 (0.01–0.644)	2.8 (0.1–12.0)	23.9 (2.0–545.5)

L, lactate; LPR, lactate-to-pyruvate ratio; N, number of readings; %, percentage.
[a]Median (minimum-maximum).
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upregulated glycolytic flux but pyruvate levels are necessary in order to differentiate whether the upregulation of the glycolytic flux is anaerobic (i.e., hypoxic) or simply an indicator of an increased use of the glycolysis under aerobic conditions.

Metabolic patterns

We confirmed that some sort of metabolic impairment was present in 57% of the samples analyzed when we used a lactate threshold as a criterion. In the analysis of our data, we found it useful to partition the entire data set in the four patterns described in **Figure 3**. According to our data, only one third of the samples were compatible with a normal metabolism. An increase in ECF levels of lactate above 4.0 mmol/L and a high LPR (>40) have been consistently shown by independent groups to be highly sensitive predictors of poor outcomes and even of pronounced neural loss and brain atrophy [2,7]. In an attempt to interpret the patterns we suggested the following working terminology: Normal metabolism for pattern 1, Aerobic hyperglycolysis for pattern 2, Anaerobic metabolism for pattern 3 and Low pyruvate for pattern 4. The frequency of each pattern and the descriptive statistics are summarized in **Table 3**. We believe this interpretation may be controversial and for a better understanding of these patterns there is need to combine MD data with brain oxygen monitoring data to verify or refute patterns 2 (“aerobic hyperglycolysis”) and 3 (“anaerobic metabolism”) and to prove whether our interpretation is reasonable.

Hypermetabolism was considered in the past a form of “hypoxia”. In a pivotal paper by Siggaard-Andersen et al., “hypermetabolic hypoxia” was defined as a situation in which ATP hydrolysis was not balanced by an increase in oxidative ATP synthesis. As a consequence, glycolysis was activated and lactate increased [46]. However, strictly speaking, we know that this pattern is present when oxygen delivery and utilization is within the normal range; therefore, this pattern has to be differentiated from any other form of tissue hypoxia. The hyperglycolytic pattern was defined in TBI by a pivotal study from Hovda’s group [2,47] and emerges only when both lactate and the LP ratio are used. Bergsneider et al. showed hyperglycolysis in 56% of severe TBI patients studied using PET scanning within one week of injury and in patients with sufficient brain oxygen [47]. This pattern has also been reported in the brain of patients with aSAH [6,26,48]. In a pivotal paper, Hutchinson et al. in 17 patients with severe TBI used both MD and positron emission tomography (PET) parameters– using the glucose analogue [F^{18}]-fluorodeoxyglucose (FDG) – to correlate the extracelullar levels of glucose, lactate and pyruvate with the regional cerebral metabolic rate for glucose (CMRglc) in the same region of interest [49]. The most relevant finding of this study is that there was a linear relationship between CMRglc and the MD levels of lactate and pyruvate but not with the LPR [49]. These data confirm the general view that the increase in CMRglc in these patients is often indicative of hyperglycolysis and not of a shift towards anaerobic metabolism.

The main difference between hyperglycolysis and an anaerobic profile is that the LPR is significantly increased in the latter. It has been postulated that glycolysis upregulation in an injured brain indicates a hypermetabolic state directed toward restoring perturbed ionic homeostasis or the reuptake of high extracellular levels of glutamate. Hypermetabolism following TBI occurs because oxidative phosphorylation normally runs at near maximal capacity and, consequently, an increased energy demand should be supplied by and increased in glycolysis [26]. Different authors have shown that a hyperglycolytic pattern is a predictor of good neurological outcomes in patients with aSAH compared with patients with an anaerobic pattern [6,26].

The anaerobic pattern (high lactate/high LP ratio) was observed in one third of the analyzed samples. This pattern is well consolidated and it has been the only pattern detected in studies that have used only lactate for profiling energy metabolism. The LPR reflects the equilibrium between product and substrate of the reaction catalyzed by lactate dehydrogenase and the LPR is a good surrogate for the cytosolic oxido-reduction status [50]. In patients with aSAH, an anaerobic pattern is related to poor neurological outcomes [6,26]. Cesarini et al. showed that when this pattern is associated with ischemia in a sample of patients with aSAH, it is also linked to concurrent low brain glucose concentrations [26]. However, although reduced ECF concentration of glucose is a hallmark of hypoxic ischemia, its concentration may be normal (or even high) in non-ischemic forms of hypoxia (anemia, hypoxemia, high-affinity hypoxia, etc.). This pattern is the most important in clinical settings; when detected, it requires a systematic approach to rule out all the classes of brain hypoxia.

The low pyruvate pattern detected in 10.7% of our samples was quite unexpected, given that this profile was already described by Hlatky et al. and confirmed by others [24,44,49,51]. Hillered et al. called this pattern a type 2 elevation of the LP ratio [44,51]. This pattern, in which lactate is within the normal range, is misleading and, if not detected, can be easily considered an indicator of anaerobic metabolism and thus indicative of brain hypoxia when it is not. In our series, this pattern was associated with a low brain ECF glucose concentration, as was described by Marcoux et al. [44] Because the brain is an obligate glucose consumer, its fuel depends on the glucose plasma levels. With all the limitations of our study (lack of matched oxygen data for this cohort), a low ECF glucose was associated with the drop in pyruvate concentrations and the increase in the LP ratio. However, hypoglycemia was infrequent. In all the samples in which we had good quality subcutaneous data, only 16% presented a low subcutaneous concentration of glucose; hypoglycemia might therefore have been the cause. Marcoux et al. suggested that a low pyruvate and a high LPR may be secondary to mitochondrial dysfunction [44]. However, when mitochondrial dysfunction has been reproduced in experimental models, a high lactate level, together with a high LPR has been consistently found [39,52]. A third alternative to explain this pattern and the concomitant low levels of glucose is the shunting of glucose-6-phosphate toward alternative metabolic pathways, specifically the pentose phosphate pathway. This process has been observed in both experimental models and in clinical studies of TBI [53,54]. Hutchinson et al. in their combined MD/PET-FDG study suggested that the low levels of glucose in the ECF of head-injured patients are a consequence of an increase in substrate demand rather than inadequate substrate delivery [49]. However, a better understanding of this pattern requires additional studies with simultaneous measurements of plasmatic glucose, lactate, pyruvate, and brain tissue oxygen. When mitochondrial dysfunction is present, as hypothethised by some, the tissue oxygen is not used and the amount of dissolved oxygen is significantly increased [39,52].

Metabolic impairment and brain hypoxia

Evidence accumulated in the last two decades shows that non-ischemic causes of brain hypoxia are frequent. The most comprehensive classification of tissue hypoxia was developed by Siggaard-Andersen et al. in 1995 [46,55]. However, so far, this classification has been rarely used in neurocritical care. The brain is a highly aerobic organ that requires a sufficient supply of oxygen (O_2) to the mitochondria to maintain adequate ATP production. The supply of O_2 to the brain is multifactorial and depends on cerebral blood flow (CBF), the ability of the blood to transport O_2, hemoglobin (Hb) oxygen affinity, Hb characteristics, O_2 diffusive conductance from arterial capillaries to the cells, and the arterial oxygen pressure gradient between the capillaries and the intracellular compartment [56]. It is thus obvious that the potential causes of hypoxia are multiple and not limited to ischemia (i.e., ischemic hypoxia), a term that should be reserved to describe brain hypoxia caused by a reduction in CBF uncoupled to brain metabolism. In a recent brain MD study conducted by Nelson et al. on TBI patients, highly impaired energy metabolism was very prevalent [57]. However, the relationships between MD and either intracranial pressure and/or cerebral perfusion pressure were very weak and did not explain the observed energetic disturbances. These findings suggest that other factors besides pressure and/or flow may be the main cause of metabolic perturbations in these patients [57].

The discussion about lactate has been plagued by the same problems as that of hypoxia. For many years, the primary causes of lactate production by any tissue were thought to be either low levels of blood flow (ischemia) or low levels of blood oxygen content (hypoxemia). However, this oversimplification is misleading. In the Siggaard-Andersen classification, nine types of tissue hypoxia were described. If ''hypermetabolic hypoxia'' is excluded and the uncoupling hypoxia is merged with hystotoxic hypoxia (i.e., mitochondrial dysfunction), seven profiles remain that are very useful as a theoretical framework [46,55]. In the original Siggaard-Andersen classification, histotoxic hypoxia is a term that is equivalent to mitochondrial dysfunction and reflects the situation in which oxygen delivery is sufficient but the respiratory chain cannot utilize it. This situation stimulates glycolysis but without a decrease in pyruvate. There is increasing experimental and indirect clinical evidence suggesting that severe TBI may be associated with mitochondrial dysfunction. In the presence of adequate oxygenation, the only available BMs that indicate mitochondrial dysfunction are lactate and the LP ratio. In the last decade, different groups have provided important experimental data that point to mitochondria as a cause of many metabolic disturbances and therefore the potential therapeutic target [58,59].

Study limitations

Because the main goal of our study was to evaluate the frequency and agreement between lactate and the LPR, hourly data were pooled from the entire cohort of patients. Therefore, we cannot analyze the influence of the metabolic profile in the clinical evolution and short- or long-term patient's outcome and caution needs to be exercised in the interpretation of our findings. In addition, our study focused on the metabolic disturbances found in brain tissue that appeared normal. Therefore, although this sample is probably representative of most of the non-injured brain, further studies need to be conducted to clarify whether brain metabolism in such areas is representative of the energetic metabolism in the whole brain or at least of the hemisphere where the probe is implanted.

A third limitation is that patients may show different patterns at different time points after injury. This fact was not explored in our study, nor was the coexistence of different metabolic patterns in the same patient. An additional limitation is that our study did not consider the potential causes of specific profiles, nor we did correlate them with the more common variables that are routinely monitored in TBI patients (intracranial pressure, cerebral perfusion pressure, brain oxygenation, etc.).

Conclusions and Future Directions

Brain MD allows for the screening of disorders in energy metabolism in patients with acute brain injuries. Our study showed that metabolic abnormalities are frequent in the macroscopically normal brain of patients in the acute phase of TBI. These disorders by their nature are clinically silent until their late stages, at which point patients may suffer significant irreversible brain damage; early detection is important. A very poor agreement between lactate and the LPR was found when classifying normal or abnormal metabolism. Our data suggest that the concentration of lactate in MD should always be interpreted taking into consideration the LPR to distinguish between anaerobic metabolism and hypermetabolism. The use of both lactate and the LPR can be used at the bedside to classify the metabolic profiles in four patterns that can be useful for further exploring the characteristics, their causes, and their prognostic values. Whether or not a normal metabolic pattern has any influence in functional outcome needs to be explored in further studies with a different design and a bigger sample size in which summary measures –the area under the curve above certain thresholds, the percentage of time above a threshold, etc.– are incorporated.

The pattern of low pyruvate was associated with a low concentration of ECF glucose but not hypoglycemia. This pattern and its clinical significance need further clarification, especially because even in situations of normoglycemia the transport of glucose across the blood-brain barrier might be impaired in these patients. Despite the discussed limitations, we believe that the classification provided here can be a starting point for further research in the impairment of energetic metabolism. The definition and better characterization of different metabolic patterns provides an opportunity to study their causes, modulate them, and potentially influence the outcomes of patients with acute brain injuries.

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Author Contributions

Conceived and designed the experiments: JS MM. Performed the experiments: AS FA MV TM AR MR. Analyzed the data: JS MM MP. Contributed to the writing of the manuscript: JS MM MP.

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4. Reappraisal of the reference levels for energy metabolites in the extracellular fluid of the human brain.

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Reappraisal of the reference levels for energy metabolites in the extracellular fluid of the human brain

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Abstract

Cerebral microdialysis is widely used in neurocritical care units. The goal of this study was to establish the reference interval for the interstitial fluid concentrations of energy metabolites and glycerol by using the extrapolation to zero-flow methodology in anesthetized patients and by constant perfusion at 0.3 μL/min in awake patients. A CMA-71 probe was implanted during surgery in normal white matter of patients with posterior fossa or supratentorial lesions, and the perfusion flow rate was randomized to 0.1, 0.3, 0.6, 1.2, and 2.4 μL/min. Within 24 h of surgery, perfusion was restarted at a constant 0.3 μL/min in fully awake patients. The actual interstitial fluid metabolite concentrations were calculated using the zero-flow methodology. In vitro experiments were also conducted to evaluate the reproducibility of the in vivo methodology. Nineteen patients (seven males) with a median age of 44 years (range: 21–69) were included in the in vivo study. The median (lower–upper) reference interval values were 1.57 (1.15–4.13 mmol/L) for glucose, 2.01 (1.30–5.31 mmol/L) for lactate, 80.0 (54.4–197.0 μmol/L) for pyruvate, and 49.9 (23.6–227.3 μmol/L) for glycerol. The reference intervals reported raises the need to reconsider traditional definitions of brain metabolic disturbances and emphasize the importance of using different thresholds for awake patients and patients under anesthesia.

Keywords

Brain metabolism, cerebral microdialysis, lactate, reference intervals, traumatic brain injury

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Introduction

Cerebral microdialysis (MD) was introduced by Bito et al.[1] for in vivo dialysis of the canine brain and is now used for an extensive array of applications that explore the regional chemistry of the human brain. The first known application of cerebral MD in humans was reported by Meyerson et al.,[2] who implanted microdialysis probes during thalamotomy procedures in patients with Parkinson's disease. Since that time, cerebral MD has been increasingly used as a neuromonitoring technique in neurocritical patients with traumatic brain injury (TBI), middle cerebral artery infarction, and spontaneous subarachnoid hemorrhage (SAH) to monitor cerebral energy metabolism during the acute phase after injury or stroke.[3]

One problem inherent to microdialysis is the fact that the dialysate concentration of any substance is never a perfect mirror of the interstitial levels but instead represents a variable fraction of the actual values.[3–6] Since its introduction, a recurrent problem with cerebral MD has been the lack of a reliable in vivo calibration method, which prevents accurate

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in vivo concentrations from being established.[7] The approach most frequently used to evaluate MD efficiency for any substance is to use the in vitro relative recovery (RR), which is defined as the ratio of the concentration in the dialysate to the true in vitro concentration of a given substance.[8] In clinical practice, the true substance concentration in the interstitial fluid (ISF) is calculated by measuring the concentration in the dialysate and dividing that concentration by the in vitro RR expressed as a fraction of 1.[5] The validity of this approach rests on the principle that the in vitro and in vivo conditions are similar; however, this assumption is far from true.[5] The RR in vitro depends primarily on the physical properties of the membrane, the temperature of the medium, the perfusion flow rate, the characteristics of the sample matrix, and the diffusion coefficient of the substance of interest.[3–5,8] However, these results are difficult to extrapolate to the brain, due to the effect of the tissue properties on the diffusion of analytes. The ISF space in any organ is an inhomogeneous and tortuous medium; therefore, the in vitro RR can only provide a rough estimate of the efficiency of MD probes in vivo.[5,9]

The main study reporting the reference intervals (RIs) for analytes involved in monitoring brain energy metabolism in neurocritical patients was conducted by Reinstrup et al.[10] in patients who underwent neurosurgical procedures under general anesthesia. Other authors have reported brain metabolite levels in the normal brains of patients with central nervous system tumors,[11] in awake epileptic patients,[12,13] and in patients with spontaneous SAH.[14] However, in most of these studies, the true ISF concentrations were unknown, and the reference limits were estimated from the concentrations in the dialysate and from the in vitro RR for the specific metabolite. Consequently, the normal ranges for brain lactate, the lactate-to-pyruvate (LP) ratio, and what has been considered the ''anaerobic threshold,'' are still quite arbitrary. In human studies, the upper reference limit for lactate in normal brain tissue is unexpectedly variable and ranges from 1.50 to 5.1 mmol/L.[11,13,15–17] This variability remains an important obstacle for adequately evaluating metabolic findings in neurocritical patients, as well as for making physiopathological interpretations and assessing clinical relevance.

To overcome the limitations of in vitro studies, three different methods have been proposed to estimate the true ISF concentration of any analyte of interest. These methods are (1) the extrapolation to zero-flow rate method (ZFM),[18] (2) the no-net flux method, and (3) the slow perfusion rate.[7,19] All three methods are considered reliable and yield similar results, as shown in the experimental studies conducted by Menacherry et al.[7] Here, we present the results of a human study in which the reference limits for analytes involved in energy metabolism (i.e., glucose, lactate, and pyruvate) and for glycerol (a biomarker of cell damage) were determined in a cohort of patients who were observed twice: while anesthetized and while fully awake.

In anesthetized patients, we used the extrapolation to ZFM, and in awake patients we employed perfusion at a constant infusion speed of 0.3 μL/min, the infusion rate recommended by a recent consensus conference on neuromonitoring.[20] The RIs reported here provide additional support for the thresholds suggested by the most recent consensus conference on microdialysis neuromonitoring,[20] highlighting the importance of lactate in brain energetics and raising the need to reconsider traditional definitions of metabolic disturbances observed in neurocritical patients. Our study emphasizes the importance of using different thresholds for awake patients and patients under anesthesia or deep sedation.

Materials and methods

The present study was divided into three separate sections: (1) preliminary in vitro experiments to confirm the RR of the CMA-71 probes (M Dialysis AB, Stockholm, Sweden) for the analytes of interest, (2) an in vitro evaluation of the reproducibility of the ''ZFM'' used in the in vivo setting to calculate the brain tissue concentrations of the metabolites, and (3) a study of the brain ISF concentrations of glucose, lactate, pyruvate, and glycerol—and their reference limits—in patients who underwent neurosurgical procedures both under general anesthesia and while fully awake after surgery.

In vitro RR experiments

In vitro RR experiments were conducted to confirm the efficient recovery of CMA-71 probes that have been previously described by others.[9,21] The experiments were conducted using an in vitro setup that was described by our group elsewhere.[22] In brief, the experiments involved placing each CMA-71 catheter into two different matrix solutions (MA-1 and MA-2), which had different concentrations of glucose, lactate, pyruvate, and glycerol (Sigma-Aldrich, St Louis, MO, USA). The study matrices composition is described in Table S1 in Supplementary information. A total volume of 2.5 mL of the matrix solution was placed in a 5-mL glass tube (BD Vacutainer®) in a dry bath (Labnet International, Edison, NJ, USA) set at a temperature of 37°C.

The tips of the CMA-71 probes were placed in the matrix, and the microdialysis (MD) catheters were connected to a CMA-402 pump (M Dialysis AB) and perfused at 0.3 μL/min (18 μL/h) with an isotonic solution

that had the same ionic composition as the matrix but contained an additional 3% albumin. Our in vitro experiments were conducted without stirring. The main reason to avoid stirring was that it produced significant variability in the volume recovered in the microvial (see Supplementary information).

When steady-state conditions were achieved, the experiments were initiated by perfusing the in vitro probes at a constant flow rate of 0.3 μL/min and collecting one microvial per hour for six consecutive hours; six microvials were obtained for each matrix in each catheter. The matrix solution was analyzed before and resampled after each microvial collection to control for all of the variables that can modify the initial analyte concentration in the medium, such as evaporation, solute depletion by the dialysis procedure, etc. A total volume of 140 μL was removed from the matrix at the end of the 6-h experiment. Both dialysate and matrix samples were analyzed in the ISCUSflex analyzer (M Dialysis AB). The RR was calculated using the following equation:

$$RR = (C_{md}/C_{matrix}) \times 100 \quad (1)$$

where C_{md} and C_{matrix} are the analyte concentrations in the microdialysate and in the matrix, respectively.

In vitro extrapolation to zero-flow rate

To test in vitro the extrapolation to ZFM that was used during surgery in the included patients, a second experiment was conducted in which CMA-71 probes pertaining to two different lots (M Dialysis AB, lots #T23301 and #T24612) were placed in a matrix with the same solute concentrations described for MA-2 in the previous section. The catheter was connected to a 1-mL disposable syringe (Luer-Lok™, New Jersey, USA) placed in a microinfusion pump (CMA-402, M Dialysis AB). The CMA-402 is a variable flow rate pump that enables the adjustment of the infusion rate between 0.1 and 20 μL/min. For these experiments, the same isotonic solution used in the in vitro RR experiments was infused. For each catheter, the flow rate was changed randomly to the following rates: 0.1, 0.3, 0.6, 1.2, and 2.4 μL/min. Each flow rate was maintained for 1 h, except for the 0.1 μL/min flow rate, which was maintained for 2 h to compensate for the low recovery volume. Microvials were collected every 30 min for the 2.4–0.3 μL/min infusion rates and hourly for the 0.1 μL/min flow rate. Each catheter experiment yielded a total of 10 determinations (two for each infusion rate). The metabolite concentrations in the matrix were analyzed at the beginning and at the end of each experiment. All analyses were conducted in the ISCUSflex analyzer.

The actual concentrations of matrix metabolites were estimated using the ZFM described by Jacobson et al.[18] This method determines the analyte concentration through the determination of the relation between the perfusion flow rate (F), the probe's membrane area, and the transference coefficient. This relation is defined by the following equation:

$$C_{dial} = C_0 - [C_0 \exp(-rA/F)] \quad (2)$$

where C_{dial} is the analyte concentration in the dialysate, C_0 the matrix concentration of the analyte of interest, r the average mass transfer coefficient, A the surface of the microdialysis membrane, and F the infusion flow rate. The external concentration (C_0) can be estimated by fitting a nonlinear regression model to the dialysate concentrations obtained at different perfusion rates.[7,18] According to this method, at a theoretical flow rate of 0 μL/min, the metabolite concentration of the dialysate is considered to be equal to the true concentration in the matrix.[18,23] Because the first dialysate sample at each flow rate is heavily influenced by the previous flow rate (due to the dead liquid volume of the outlet tube [~5.1 μL]), the first sample was always discarded and was not included in the calculations; therefore, at any flow rate, only the second determination was used to fit the curve. The catheters used in these experiments were never infused for more than 96 h.

Determination of the brain tissue metabolites concentrations

Patient selection and study design. A prospective study was conducted in patients aged above 18 years who underwent surgical treatment under standard general anesthesia to treat posterior fossa and supratentorial lesions at Vall d'Hebron University Hospital (VHUH), between November 2012 and January 2016. To enroll patients in the study, the inclusion criteria were: (1) posterior fossa or supratentorial lesions requiring the implantation of an external ventricular catheter for the drainage of cerebrospinal fluid (CSF); (2) no neuroradiological abnormalities in the white/gray matter in the supratentorial compartment where the MD probe will be implanted in magnetic resonance imaging (MRI) sequences evaluated in the following MRI sequences: T1W, T2W, and FLAIR; (3) a normal ventricular size defined as an Evan Index below or equal to 0.30 or moderate ventricular enlargement without clinical symptoms of intracranial hypertension;[24] and (4) written informed consent signed by the patient or the next-of-kin. The study was approved by the Institutional Ethics Committee of the VHUH (protocol number approval PR/AG-140-2011). All provisions of the Declaration of Helsinki were followed.

Surgical procedure. According to the manufacturer's specifications, the CMA-71 probe has a nominal cut-off of "around 100 kDa".[25] CMA-71 probes were placed with the patient under general anesthesia at the same time the external ventricular drainage was placed, through a small hole in the duramater at ~3 mm from the ventriculostomy entry. The position of the probe in the normal white matter was confirmed by a control computed tomography (CT) scan conducted within the first 24 h after probe implantation (Figure S1 in the Supplement). Patients with any hemorrhagic or hypodense lesion around the probe detected in the control CT were excluded from analysis. The MD probe was connected through a 150-cm length extension line, with 1.58 mL residual volume (Prolonsend® PA-150, Sendal, Cáceres, Spain) primed with CNS perfusion fluid (M Dialysis AB), to a 1-mL disposable syringe (Luer-Lok™) placed in the microinfusion pump CMA-402 and perfused at 0.3 μL/min during 1 h with CNS perfusion fluid (M Dialysis AB). The first sample from the first hour was always discarded to allow stabilization of the system. Then, for each patient, the perfusion flow rate was varied randomly, and the same methodology described in the in vitro section was applied except in the first four patients, where microvials were collected hourly for all infusion rates and just one microvial was collected for each infusion rate. Once all the experiment was completed, and if the duration of the surgery allowed it, the perfusion rate was changed to 0.3 μL/min rate, and microvials were collected each hour until the surgery was completed. At the end of the surgical procedure, the perfusion of the cerebral catheter was interrupted until the patient was fully awake in the reanimation unit.

During the surgical procedure, blood and CSF samples were also collected periodically. Blood gas analysis was performed in a Co-oximeter (1200 RAPIDLab Systems, Siemens, Munich, Germany). The CSF samples were centrifuged at 4000 rpm during 10 min, and the supernatant was analyzed in the ISCUSflex analyzer.

Microdialysis in the awake patient. Within 24 h after surgery and when the patient was extubated and fully conscious, the MD probe was reconnected to a CMA-106 microinfusion pump (M Dialysis AB), and the perfusion was restarted. In this second part of the study, the flow rate was maintained at a constant rate of 0.3 μL/min, and the samples were collected hourly. Blood and CSF samples were also collected and processed using the same methodology previously described. All monitoring data obtained during the study were collected in a Microsoft Excel spread sheet (Microsoft, Redmond, Washington, USA).

Statistical analysis

Statistical analyses were performed with R software v3.2.2 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) and the integrated development environment R Studio v0.99.491 (RStudio, Inc., Boston, MA, USA; http://www.rstudio.com). Unless otherwise specified, differences were considered statistically significant for $p \leq 0.05$.

For estimating the true concentrations in the matrices (in vitro experiments) and in the brain ISF (in vivo experiments) with the ZFM, all the flow rate-concentration pairs were plotted in a scatter plot and analyzed by using the function and the R packages "nlstools" and "minpack.lm."[26,27] The R code (available on request) used the original equation described by Jacobson et al.[18] The goodness of fit for the exponential model with the total data was evaluated based on visual inspection of the residuals calculated from the fit and testing the normality of the residuals with the Shapiro–Wilk test and the randomness of residuals with the runs test.

RI calculation. The Horn's algorithm[28] implemented in the package "referenceIntervals" was applied to detect outliers. Each detected outlier was reviewed, and if the patient or the data were considered doubtful, the case was eliminated of the RIs calculation. To calculate the brain upper and lower RI limits for each analyte, we used the distribution-free nonparametric method described in the NCCLS and Clinical and Laboratory Standards Institute (CLSI) guidelines C28-A3 for estimating percentiles intervals[29,30] by using the package "referenceIntervals" for R.[31]

Results

In vitro RR of metabolites

Four catheters were used in these experiments. We observed minimal differences between the initial and the final concentrations of the matrix metabolites, with a mean reduction of approximately 5.5%. Although these differences were statistically significant, we considered them methodologically irrelevant. The denominator used for the RR calculation (equation (1)) was always the mean concentration of the matrix sampled before and after each microvial change. The four catheters included in the analysis yielded a total of 48 samples. The RR for each metabolite is summarized in Table 1. The mean RR for all metabolites was approximately 95%, similar to the findings published by others who used the CMA-71 probe.[32] We did not find any significant difference between the two matrices studied (see Table 1); furthermore, the RR inter-catheter agreement was excellent, with differences always below 5%.

Table 1. In vitro relative recoveries.

Metabolite	Mean ± SD	Median (min–max)	*p* value
Glucose (mmol/L)	91 ± 5	91 (80–103)	
MA-1	92 ± 4	91 (85–101)	0.39
MA-2	91 ± 5	92 (80–103)	
Lactate (mmol/L)	95 ± 6	95 (83–109)	
MA-1	95 ± 5	95 (85–109)	0.92
MA-2	94 ± 6	96 (83–107)	
Pyruvate (μmol/L)	104 ± 17	100 (83–150)	
MA-1	109 ± 20	97 (89–150)	0.13
MA-2	100 ± 13	101 (83–128)	
Glycerol (μmol/L)	90 ± 6	91 (73–105)	
MA-1	92 ± 4	93 (82–99)	0.09
MA-2	89 ± 7	90 (73–105)	

Means and medians are expressed as percent recovery, as explained in the text (equation (1)). Comparisons between matrices were made using the Kruskal–Wallis test; no statistically significant differences were observed.

In vitro extrapolation to zero-flow

Seven catheters were included in this experiment. For each catheter, the metabolite concentrations at a theoretical flow rate of 0 μL/min were calculated using a nonlinear regression model. The matrix concentration (C_0 in equation (2)) was calculated as the *y*-intercept, as described. A *k* value (where $k = rA$ in equation (2)) was also determined for each metabolite. Figure 1[18] shows examples of the ZFM applied both in vitro and in vivo. The goodness of fit for the exponential model in vitro was excellent for all metabolites. The concentrations estimated at 0 μL/min with the nonlinear regression model were compared with the measured concentrations in the matrices (Table 2). To minimize errors, the matrix concentration used in the analysis was the mean concentration for each metabolite at the start and the end of the experiment. In all metabolites and for all catheters, we found a high agreement between the estimated concentrations and the true concentrations in the medium. The differences between both concentrations were not statistically significant (see Table 2) (Wilcoxon test for paired samples).

Descriptive data of included patients

A total of 19 patients were included, 16 with a posterior fossa lesion and 3 with a supratentorial lesion. All presented normal-appearing gray and white matter in the MRI. The 19-patient cohort included 7 males and 12 females with a median age of 44 (range: 21–69 years) and a median Evans index of 0.28 (min: 0.20, max: 0.33). Table 3 shows a summary of the clinical data.

Table 2. In vitro estimated concentrations using the extrapolation to zero-flow methodology against true concentrations.

	Estimated concentration	Measured concentration	*p* value
Glucose (mmol/L)	1.97 (1.59–2.19)	2.06 (1.93–2.25)	0.16
Lactate (mmol/L)	2.01 (1.71–2.44)	2.15 (2.05–2.37)	0.09
Pyruvate (μmol/L)	106.7 (87.3–143.9)	119.5 (108.5–127.5)	0.38
Glycerol (μmol/L)	242.1 (160.6–284.5)	231.6 (200.1–295.0)	0.11

In these experiments, seven catheters were used. Data summarized correspond to the data for each metabolite in all experiments. Data are expressed as median (min–max). Comparisons between estimated and measured concentrations were made using the Wilcoxon test for paired samples; no statistically significant differences were observed.

Brain tissue concentrations in anesthetized patients

In 3 of the 19 patients, the intraoperative data were discarded; for one, the surgery was too short to obtain data for all the perfusion flow rates, and for the other two patients, the volume recovered was not correct. The remaining 16 patients had valid intraoperative data, which were included in the analysis. For each patient, the extracellular fluid concentration was determined with the ZFM, as already described. In some patients, the non-linear curve fitting to the equation did not result in a correct estimated C_0 value because the iterative procedure of the "nls" function did not successfully converge to a certain value. This unsuccessful convergence was because the equation is too complex for the amount of data we had. In order to simplify the equation, we obtained a fixed *k* value for each metabolite by merging the data for all patients. We used the obtained *k* to estimate the C_0 by nonlinear curve fitting for each individual patient.

Of these 16 patients, the data from the first three patients were excluded from the RIs calculation at 0.3 μL/min because the microdialysis sampling methodology used during surgery was modified after the fourth patient, so as to avoid generating inaccurate values from the dead volume inside the outlet tube (~5.1 μL). The data for the analyzed metabolites calculated by the ZFM and those measured at a flow rate of 0.3 μL/min in anesthetized patients are summarized in Table 4 and Figures 2 and 3. Median plasma levels for glucose and lactate were 7.13 (min, max: 4.99–9.88 mmol/L) and 2.03 (min, max: 1.24–5.04 mmol/L), respectively.

The in vivo RR at a perfusion flow rate of 0.3 μL/min was calculated by taking into account the estimated tissue concentration for each analyte obtained with the ZFM. The in vivo median RR values were 80% for glucose (min: 56, max: 96), 75% for lactate (min: 59, max: 92), 83% for pyruvate (min: 66, max: 100), and 89% for glycerol (min: 69, max: 103).

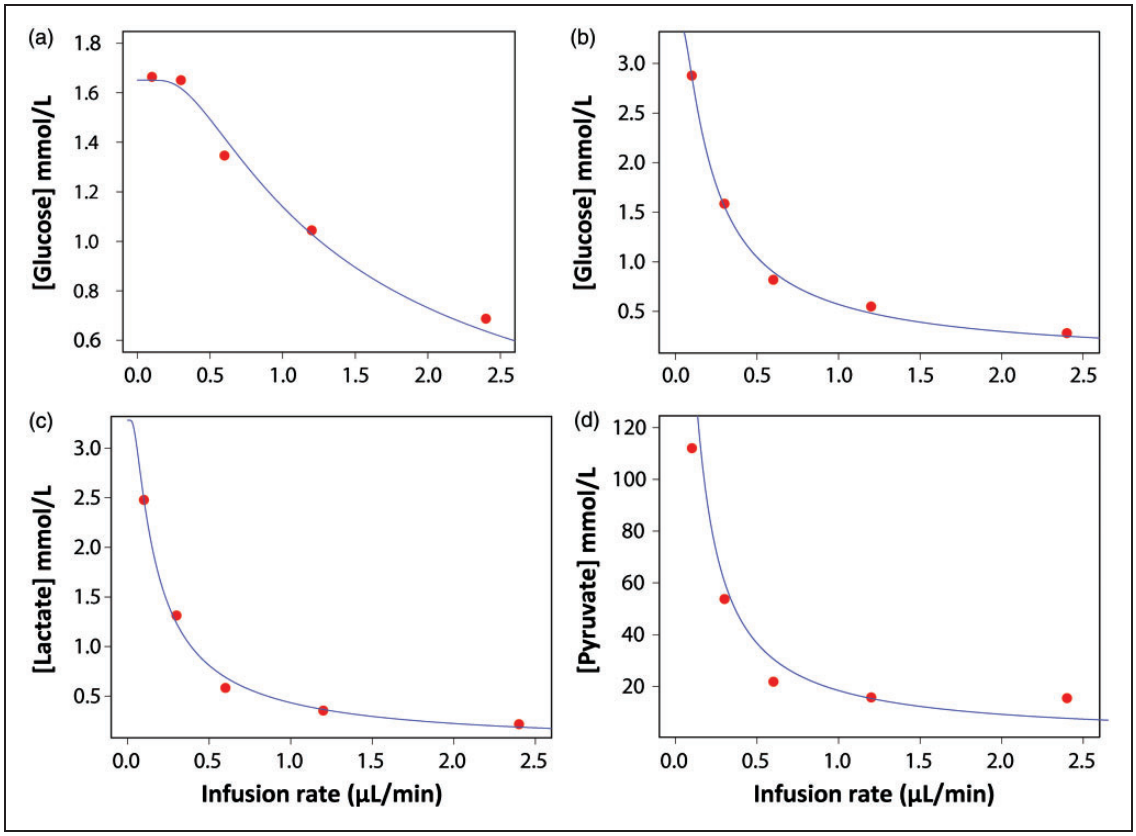

Figure 1. Example of the graphical representation of the extrapolation to zero-flow method. Using non-linear curve fitting to the equation described by Jacobson et al.[18] (see equation (2) in the manuscript), the concentration in the matrix and in the extracellular fluid (C_0 in equation (2)) corresponds to the *y*-intercept. (a) In vitro extrapolation of the glucose data for catheter # 5. The estimated C_0 was 1.65 mmol/L, the value of *k* (where $k = rA$ in equation (2)) was 1.17 mm/min, and the measured C_0 for glucose was 2.06 mmol/L. (b) In vivo extrapolation of the glucose data for patient # 12 ($C_0 = 3.43$ mmol/L, $k = 0.18$ mm/min). (c) In vivo extrapolation of the lactate data for patient # 12 ($C_0 = 3.28$ mmol/L, $k = 0.14$ mm/min). (d) In vivo extrapolation of the pyruvate data for patient #12 ($C_0 = 169$ mmol/L, $k = 0.11$ mm/min).

Brain tissue concentrations in awake patients

In 2 of the 19 patients, data could not be obtained due to a malfunction in the microdialysis probe. The median values for the 17 remaining patients at a fixed perfusion flow of 0.3 µL/min are summarized in Table 4 and are also shown in the box plots presented in Figures 2 and 3. Table 4 shows the upper and lower reference limits found by the nonparametric method. Levels at a perfusion flow rate of 0.3 µL/min were significantly higher for lactate, pyruvate, and lactate-to-glucose (LG) ratio levels when the patient was fully awake, as compared to the values obtained when the patient was under anesthesia. However, we did not find statistically significant differences between the glucose, glycerol, and LP ratio levels of awake versus anesthetized patients (Figures 2 and 3). Median plasma levels for glucose and lactate were 7.72 (min, max: 5.11–8.22 mmol/L) and 1.40 (min, max: 0.55–3.80 mmol/L), respectively.

Discussion

Cerebral MD is not yet used as a routine neuromonitoring tool, but it is a mature powerful research tool that is widely used in many neurocritical care units worldwide. However, unless probes are calibrated in vivo, the microdialysate does not give the absolute brain concentration of the analytes of interest, but only provides an estimate.[3] In vivo calibration methods are time-consuming, cannot be routinely conducted at the bedside and present ethical constraints. Therefore, in clinical practice, the true concentrations are estimated from the absolute values given by the analyzer and the known in vitro RR for the analyte of interest, although in vitro conditions are rarely reproduced in vivo. In clinical practice, thresholds that were determined for a fixed flow perfusion rate of 0.3 µL/min are typically used because this is the flow rate recommended by the 2014 consensus statement and is the most commonly used rate in the cerebral MD literature.[20] A direct

Table 3. Demographic and clinical characteristics of the patients included in the study.

Case No.	Age (years)/sex	Diagnosis of pathology	Evans index	Duration of microdialysis on surgery (h)	Duration of microdialysis on awakeness (h)	Anesthesia
1	38/F	Cerebellar tonsil lesión	0.28	4.67	7.42	Inhalation
2	32/F	Ependymoma	0.28	8.00	11.90	TIVA
3	61/F	Atypical fibrous meningioma	0.33	6.83	NA	Inhalation
4	21/F	Hemangioblastoma	0.29	3.30	5.17	Inhalation
5	43/F	Chiari malformation	0.27	4.93	13.17	Inhalation
6	22/M	Classic medulloblastoma	0.27	7.72	7.42	Inhalation
7	34/F	Cerebellar metastasis	0.24	10.83	5.08	Inhalation
8	60/F	Vestibular Schwannoma	0.30	6.53	15.83	Inhalation
9	65/M	Cerebellar metastasis	0.31	5.00	5.33	Inhalation
10	65/F	Hemangioblastoma	0.28	5.00	9.33	Inhalation
11	66/M	Cerebellar metastasis	0.28	8.00	NA	Inhalation
12	55/F	Anaplasic glioma	0.30	5.83	7.67	Inhalation
13	42/M	Transitional meningioma	0.24	6.83	39.00	Inhalation
14	31/M	Vestibular schwannoma	0.21	6.50	14.00	Inhalation
15	69/F	Mixed meningioma	0.27	6.25	10.00	TIVA
16	32/F	Central neurocytoma	0.30	5.08	13.42	TIVA
17	65/F	Low grade glioma	0.20	7.10	14.00	TIVA
18	66/M	Cerebellar metastasis	0.29	7.17	13.17	TIVA
19	44/M	Epidermoid cyst	0.30	7.17	12.67	TIVA
Median	44/M		0.28	6.53	11.90	

M: male; F: female; TIVA: total intravenous anesthesia; NA: not applicable.

consequence of these variable approaches is that researchers have used different cut-off levels for the RIs of energy metabolites, introducing significant variability and biases when explaining cerebral MD data and interpreting findings observed in neurocritical patients. These implications are especially relevant for the definition of brain hypoxia/ischemia and hyperglycolysis. In addition, ambiguous RIs in any neuromonitoring tool create significant uncertainty among clinicians about correct patient management. In our study, we enrolled a cohort of homogeneous patients who were studied twice: once while under general anesthesia and once when they were fully awake. In this cohort, we determined the actual brain concentrations for each analyte by using the Jacobson et al.[18] methodology. In addition, we followed the statistical methods recommended by the latest version of the CLSI to identify outliers and to perform the calculations necessary to set valid biological RIs.[33]

Brain glucose thresholds

Reinstrup et al.[10] established the traditional clinical upper threshold for MD brain glucose at 3.5 mmol/L. This upper limit was similar to the values found in our awake and anesthetized patients at the same perfusion rate. This threshold corresponds to a true ISF glucose of ~4 mmol/L, similar to the upper reference limit calculated for $[Glu]_{brain}$ in our anesthetized patients (Table 4). This cut-off agrees with the upper limit determined in awake epileptic patients (3.1 mmol/L),[12,13] and to the upper threshold in the CSF determined for adults.[34] Therefore, taking together all these studies, it can be safely assumed that in patients under general anesthesia or heavy sedation, an upper limit of 3.5 mmol/L is appropriate when using the standard flow rate of 0.3 µL/min. Our lower $[Glu]_{brain}$ was 0.43 mmol/L in the absence of hypoglycemia and with normal or moderately increased glucose plasma levels, a threshold significantly lower than the lower limit reported for the CSF (2.8 mmol/L).[34] Based on our data, $[Glu]_{brain} < 0.43$ mmol/L should be indicative of substrate limitation or ischemia, which reinforces the reference limits recommended by the 2014 cerebral microdialysis consensus statement.[20] Our data also support the findings of Abi-Saab et al.[13], who showed that $[Glu]_{brain}$ levels are substantially lower than $[Glu]_{plasma}$.

All metabolites in our study presented wide reference ranges, which demonstrate the variability in metabolite levels between different subjects and under different

Table 4. Brain tissue concentrations of metabolites.

Condition	Glucose (mmol/L)		Lactate (mmol/L)		Pyruvate (μmol/L)		Glycerol (μmol/L)		LP ratio		LG ratio	
	Median (min–max)	RI[a]	Median (min–max)	RI[a]	Median (min–max)	RI[a]	Median (min–max)	RI[a]	Median (min–max)	RI[a]	Median (min–max)	RI[a]
ZFM ($n = 16$)	1.57 (1.12–4.70)	1.15–4.13	2.01 (1.37–5.44)	1.3–5.31	80.0 (53.9–223.3)	54.4–197.0	49.9 (21.7–228.7)	23.6–227.3	27.5 (14.9–39.5)	15.6–39.2	1.33 (0.46–3.64)	0.49–3.47
Anesthetized (0.3 μL/min) ($n = 15$)	1.25 (0.64–3.53)	0.68–3.11	1.40 (1.10–3.84)	1.11–3.63	73.8 (36.6–149.7)	39.0–137.1	53.8 (24.4–205.1)	25.3–202.9	23.3 (12.8–34.5)	13.1–34.3	1.42 (0.50–2.78)	0.52–2.57
Awake (0.3 μL/min) ($n = 17$)	1.55 (0.29–3.01)	0.43–2.94	3.41 (1.56–5.62)	1.64–5.50	137.1 (85.0–192.0)	86.1–188.7	79.8 (29.3–346.4)	31.7–338.7	24.9 (16.9–35.1)	18.3–33.5	2.32 (0.62–15.0)	0.65–11.3

ZFM: zero-flow method; RI: reference interval; LP: lactate-to-pyruvate ratio; LG: lactate-to-glucose ratio. [a]2.5% and 97.5% percentile values, as lower and upper reference interval limits, respectively. All data were recorded from white matter.

conditions (under general anesthesia or awake). Our data highlight that no single metabolic value can be interpreted outside the context of the patient. The importance of monitoring systemic parameters in parallel to brain MD must be emphasized. In patients with SAH, Schlenk et al.[35] showed that hyperglycemic episodes were not reflected in the levels of $[Glu]_{brain}$ but episodes of $[Glu]_{brain} < 0.6$ mmol/L induced metabolic derangements in the brain and a significant increase in $[Gly]_{brain}$ and in the LPR. In the case of lactate, we reported previously on the misleading interpretation of an isolated increase in $[Lac]_{brain}$ and the low level of agreement between $[Lac]_{brain}$ and the LPR in indicating brain metabolic dysfunction.[36] In monitoring brain metabolism, integrating all data is essential and, in the case of ischemia, the use of multiparametric monitoring may help in interpreting them. The use of a regional method for monitoring rCBF or $PtiO_2$ close to the MD probe may aid interpretation of the absolute and/or the observed values. This is crucial for attributing any metabolic abnormality to either ischemic or non-ischemic episodes by considering both the $PtiO_2$ values and brain glucose levels. A reduced $PtiO_2$ and/or reduced $[Glu]_{brain}$, the latter in the absence of hypoglycemia, is a clear indication of ischemia.

The anaerobic threshold revisited

As we discussed in a previous paper, the best available estimate for upper brain lactate levels derives from studies of CSF and from a few studies of patients operated on in neurosurgical procedures in whom brain MD were monitored under general anesthesia and using different anesthetic management techniques.[36] These studies and others—conducted in severe TBI and patients with SAH—found that certain lactate thresholds were related to poor clinical outcomes, and the upper limit for brain lactate most widely used in neurocritical patients is extremely variable (range: 1.50 to 5.10).[11,13,16,17,37] The 2014 cerebral microdialysis consensus statement recommended 4 mmol/L as the upper reference limit.[20] Reinstrup et al. found a mean lactate of 1.2 ± 0.6 mmol/L (in anesthetized patients and at a perfusion rate of 1 μL/min), which translates to 2.82 mmol/L at 0.3 μL/min assuming the in vivo RR of 32% observed in our study at such perfusion rate. The recommended 4 mmol/L upper reference limit is based on the Reinstrup et al. study, and three additional studies conducted in patients with spontaneous SAH (i.e., comatose or requiring external ventricular drainage) or severe TBI.[16,37] Indeed, Oddo et al. used a threshold of 4 mmol/L, based on findings in patients with severe TBI and a poor outcome.[16]

Our data suggest that the upper lactate reference limit in anesthetized patients, when used independently

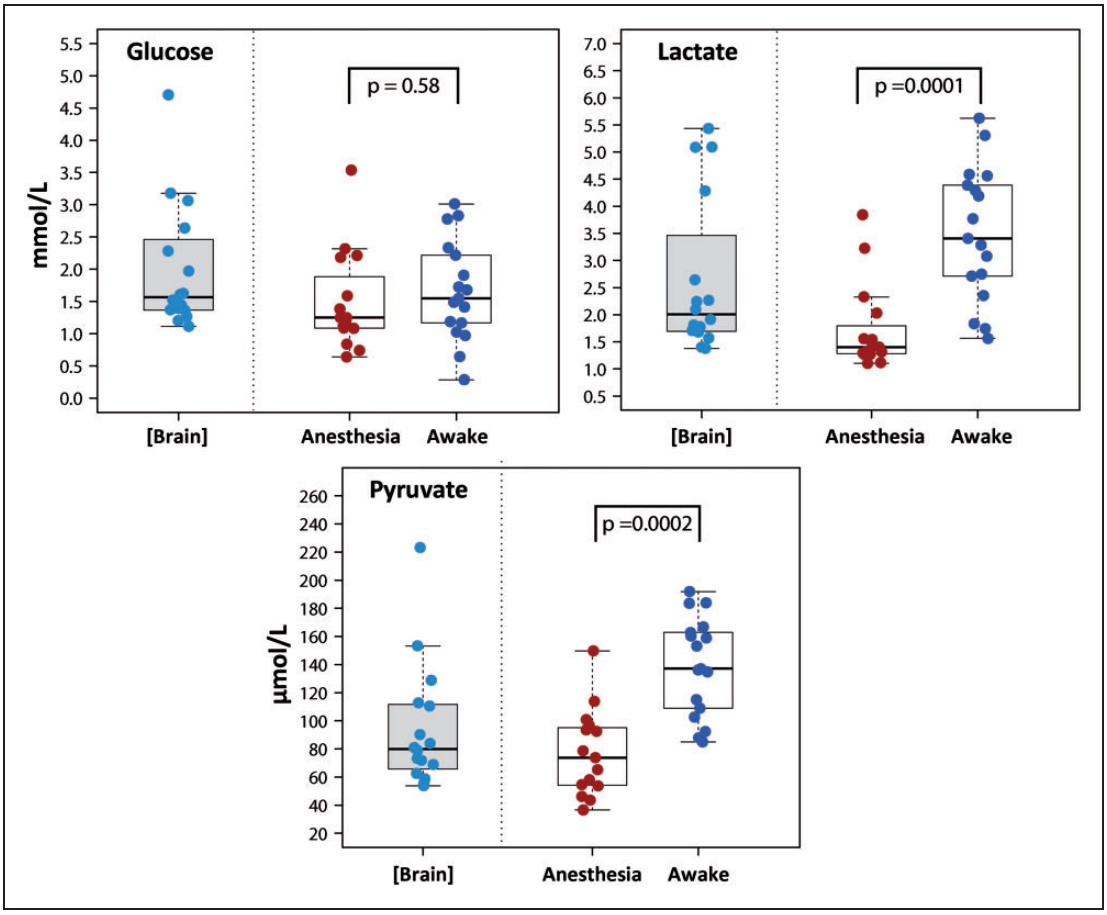

Figure 2. Box plots showing the concentrations of glucose, lactate, and pyruvate obtained with the extrapolation to zero-flow method [Brain] and at a perfusion flow of 0.3 µL/min under different conditions: anesthesia and awake. A summary of the data is shown in Table 4. All data were recorded from white matter. Anesthetized values were compared with awake values by the Wilcoxon signed-rank test. Lactate and pyruvate concentrations were significantly higher when the patients were awake. No significant differences were observed in glucose concentration.
NS: statistically non-significant.

of the LP ratio, should be reduced to ~3.5 mmol/L when using a fixed perfusion rate of 0.3 µL/min. This corresponds to a true ISF lactate of ~5 mmol/L, as shown in our cohort by using the ZFM. However, values above 3.0 mmol/L were unusual (13%), and most patients had an ISF lactate ≤ 3 mmol/L (Figure 2). Differences in the depth of anesthesia or anesthetic agents used might account for these differences. In awake patients, the upper lactate level should be raised significantly such that an upper level of 5.5 mmol/L falls within the normal range.

This recommendation is consistent with prior findings where a true ISF upper lactate limit of 5.7 mmol/L was observed in the non-epileptic cortex and hippocampus of 38 awake epileptic patients during the interictal period.[12] Also, other studies suggest that lactate increases in the brain during physiological stimulation, due to a transient boost of glycolysis that occurs as a normal response to physiologic stimulation.[38] A second relevant finding in our study was that the $[Lac]_{brain}$ is usually higher than expected in both the awake and anesthetized patient. Our findings support the 1994 astrocyte-neuron lactate model suggested by Pellegrin and Magistretti, which suggests that lactate is an effective fuel for the brain.[39] Therefore, lactate formed within the brain parenchyma from glycolysis in astrocytes may be used by activated neurons that take lactate from the ISF by MCT-2.[39] Our data support the notion that an increase in lactate by itself does not indicate ischemia if the LP ratio is below 35 and may only indicate active aerobic metabolism with increased glycolysis.

Thresholds for pyruvate and the lactate–pyruvate ratio

Increases in lactate can be indicative of either hyperglycolysis or hypoxia/ischemia. In patients with spontaneous SAH, Oddo et al.[37] found that brain lactate

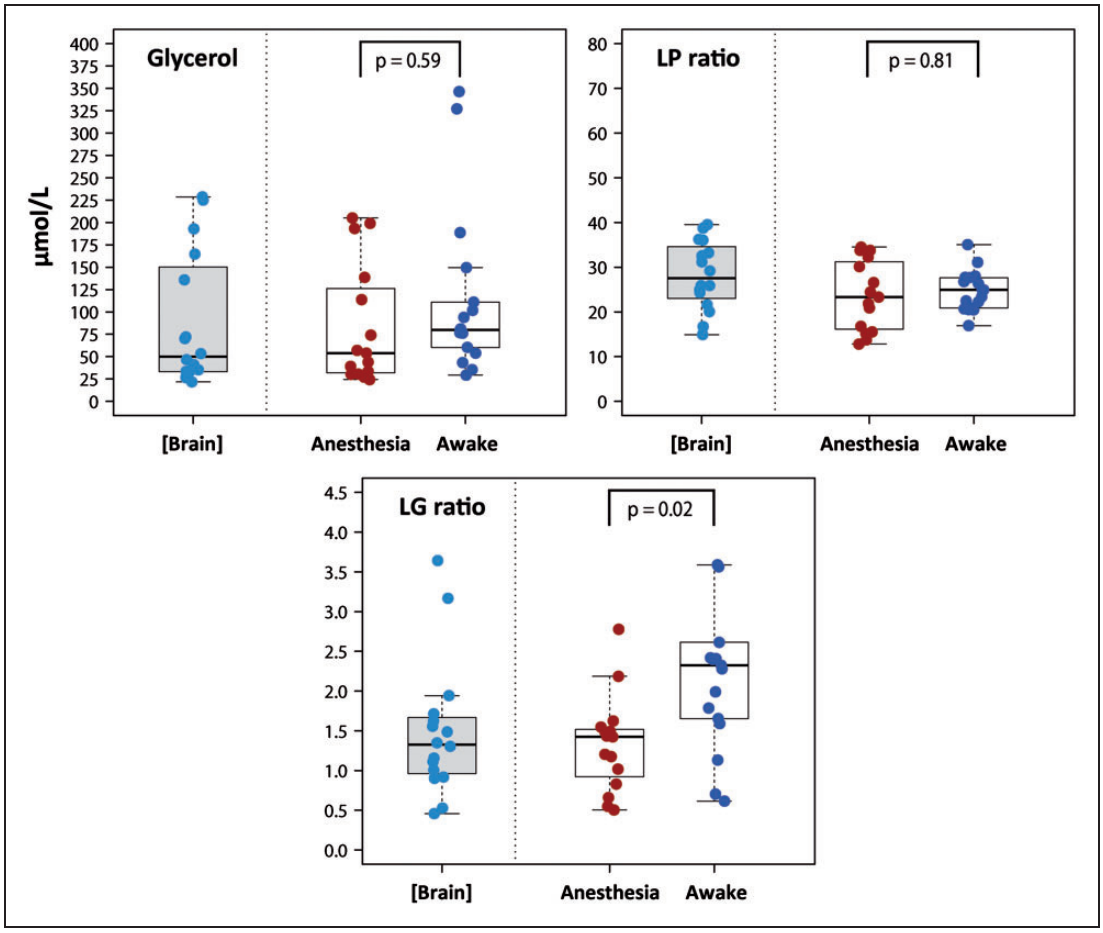

Figure 3. Box plots showing the concentrations of glycerol, LP ratios, and LG ratios, all of which were obtained with the extrapolation to zero-flow method [Brain] and at a perfusion flow of 0.3 µL/min under different conditions: anesthesia and awake. All data were recorded from white matter. A summary of the data is shown in Table 4. Anesthetized values were compared with awake values by the Wilcoxon signed-rank test. No significant differences were observed in glycerol concentration and in the LP ratio. The LG ratio was significantly higher in patients who were awake compared with patients under anesthesia.
NS: statistically non-significant; LP: lactate-to-pyruvate; LG: lactate-to-glucose.

elevations (>4 mmol/L) were more often caused by cerebral hyperglycolysis than by brain hypoxia and that hypoxic lactate was associated with increased mortality whereas hyperglycolysis was a predictor of good outcomes.[37] LP ratio is generally thought to be a more reliable indicator of anaerobic metabolism than lactate alone and furthermore is unaffected by changes in the probes' recovery. When oxygen is present, pyruvate is converted into acetyl-CoA in order to enter the citric acid cycle. At 0.3 µL/min, we found a lower pyruvate limit of 39 µmol/L in anesthetized patients, corresponding to a true $[Pyr]_{brain}$ of ~55 µmol/L. This value was similar to the lower limit obtained by Reinstrup et al.[10]

Our upper limit for the LP ratio was 34.2 at a perfusion rate of 0.3 µL/min, and it was 39.2 when using the ZFM data (Table 4); this corresponds to a LP ratio that is above 35–40, as suggested by the 2014 microdialysis consensus conference.[20] Therefore, the limit of 25 we suggested in a previous paper is likely too low to indicate ischemic or non-ischemic brain hypoxia or mitochondrial dysfunction.[36] As additional data are not available, a pragmatic upper limit for the LP ratio in both awake and anesthetized patients appears to be 35, and this threshold should be used in future classifications of the metabolic disturbances in neurocritical patients.

Glycerol

Glycerol (Gly) is an end product of phospholipid degradation. Some studies have used it as a biomarker for cell membrane deterioration (and therefore cell destruction) in TBI injury and other acute brain injuries.[40,41] In a small cohort of TBI patients, Peerdeman et al.[40] found that values of $[Gly]_{brain} > 150$ µmol/L in the normal-appearing regions of the brain had a positive

predictive value of 100% for an unfavorable outcome. In a previous paper, where we studied the ionic profile of normal and injured brains, we found a significant increase in $[Gly]_{brain}$ in both the ischemic and traumatic core,[42] but the $[Gly]_{brain}$ levels were always below the upper reference threshold in both the normal-appearing brain and the traumatic penumbra. Our findings indicate that a $[Gly]_{brain} < 209\ \mu mol/L$ is a good predictor for tissue viability, with high sensitivity (99.4%) but modest specificity (52.0%). In our study, the upper reference limit for $[Gly]_{brain}$ at a perfusion rate of $0.3\ \mu L/min$ was $203\ \mu mol/L$, corresponding to a $[Gly]_{brain}$ of $227\ \mu mol/L$. In awake patients, two cases had $[Gly]_{brain}$ above $300\ \mu mol/L$ without any abnormalities in the control CT scan around the tip of the MD probe.

The effects of anesthesia on brain metabolism

We obtained energy metabolites values in the same patients under two conditions: while anesthetized and while fully awake. We consistently found that upper reference limits were significantly higher in awake patients for pyruvate, lactate, and the LG ratio (Figures 2 and 3), but remained unchanged for glucose, the LP ratio, and glycerol. Although our data need further verification in larger cohorts, they suggest that anesthesia depresses the glycolytic pathway and that brain lactate levels increase when the patient is awake. However, increased lactate was never accompanied by an increase in the LPR, indicating a normal redox status. In humans, positron emission tomography studies have shown that both isoflurane and propofol may reduce whole brain glucose metabolism by ~50%.[43–45] Volatile anesthetics also reduce CMR and energy consumption, as well as protect the brain against short-term ischemia. Similar neuroprotective effects have been shown for isoflurane, sevoflurane, or desflurane.[46] Similar to propofol, volatile anesthetics induce a strong concentration-dependent suppression of electroencephalographic (EEG) activity and therefore of the brain's functional metabolism.

Following the lactate-shuttle hypothesis,[39] a drug-induced reduction in synaptic activity during anesthesia may cause a reduction in aerobic glycolysis and therefore in $[Lac]_{brain}$. Our data suggest that reference limits for neurocritical patients—in whom both propofol and midazolam are widely used—should be identical to those obtained in patients under general anesthesia.

Recent experimental models in rats showed that brain lactate was fivefold higher for isoflurane compared with propofol anesthesia and that this increase was independent of blood lactate levels.[47,48] Horn and Klein[48] concluded that volatile anesthetics like isoflurane, halothane, and sevoflurane, but not intravenous anesthetics, caused a specific, dose-dependent rise in extracellular lactate and pyruvate levels in mice brains. We obtained data during anesthesia in 14 patients (nine anesthetized with desflurane and five with continuous infusion of propofol); no statistically significant difference in lactate or ISF glucose levels between both groups was observed (data not shown). Our data suggest that the changes in the metabolomic profile shown with volatile anesthetics can be species-specific and must be verified in the human brain before any conclusion can be reached.

Study limitations and future directions

RI estimation methodology has significantly evolved over the past few decades. Once RIs are established, the immediate clinical consequence is that any value outside this range is flagged.[30] Although our cohort was accurately selected, our samples were not representative of healthy individuals, which limits the generalizability of our findings. In addition, NCCLS guidelines recommend the use of nonparametric RIs and that sample sizes consist of at least 120 values.[30] For obvious reasons, to include 120 patients in a single-center study is difficult, and therefore, a multicenter study is needed. The ZFM described by Jacobson in 1985 relies on the fact that the brain concentration stays constant during the study.[6] This assumption could be challenged in our study, as the median experiment time in our cohort was 6.5 h. However, we believe that, under general anesthesia and when surgery was uneventful and with no intraoperative adverse events, the margin of error introduced by the length of the experiment is acceptable and that the randomization of the perfusion rates that we performed reduced the risk of bias. The differences in the depth of anesthesia or the different anesthetic agents used might account for some of the differences among studies. The reliability of future studies could be significantly improved if a measure of brain activity suppression (EEG or bispectral index) was included in routine monitoring.

In our in vitro experiments, we did not use stirring because it produced significant and unpredictable changes in volume recovery, as explained in the in vitro experiments section. However, in non-agitated solutions, changes in solute concentration can occur at the surface of any porous semipermeable membrane, as long as the membrane shows different permeability for the various components of the solution. This phenomenon is known as polarization by concentration, and its immediate consequence is that concentrations at the membrane surface are not the same as in the bulk fluid (matrix) and can produce overestimates or underestimates of the true concentration (see Supplementary information).[49,50] In our in vitro experiments, we cannot disregard the possibility that this phenomenon

was the cause of underestimating the true concentrations in the matrices. Therefore, we believe this may affect all concentrations and the true in vitro concentrations calculated by the ZFM. However, the main goal of these experiments was to replicate Jacobson's finding and to show that this method can be applied in vivo. This phenomenon could also justify the approximately 5% difference in RR found for all metabolites in our in vitro studies and the statistically non-significant underestimation shown in Table 2 between the estimated in vitro concentrations and the real concentrations in the matrices (except for glycerol).

An additional limitation of the Jacobson method, when used in vivo, is that it inherently assumes that the unperturbed tissue next to the probe is representative of tissue far from the probe. However, it has been shown that the physical insertion of any probe into the brain causes a local injury that initiates a progressive inflammatory tissue response that alters the microenvironment and the function of the tissue from which metabolites are sampled (see Supplementary information).[51] In addition, our studies were limited in time and therefore only the more acute changes induced by inserting a probe need to be taken into consideration. We excluded all patients with any CT scan abnormality after insertion, and therefore, we minimized the odds of obvious bleeding or edema around the probe.

MD has—with its present methodology—some intrinsic and unavoidable limitations. Probably the most important one is that it only provides an estimate of the time-averaged metabolic profile of the time—30–60 min—before the microvials are collected. Therefore, MD is quite insensitive to rapid changes in metabolite levels. However, trends are still very useful as a method for identifying brain tissue at risk for patients in neurocritical care, detecting changes in metabolic profiles, and detecting ischemia. The future of the technique is real-time online techniques already introduced into clinical research. Rogers et al.[52] reported on the use of continuous online MD by using microfluidic sensors to study glucose, lactate, and potassium in cortical spreading depolarization.[53] Ideally, when commercially available and approved for human use, continuous MD might improve its temporal resolution and therefore will be able to detect metabolic changes that are short in duration and thus missed with conventional MD.

We believe one of the next steps in neurocritical care is to establish a clear classification of the metabolic disturbances found in patients who suffer acute brain damage and to clarify its pathophysiology. Ideally, both cerebral MD and $PtiO_2$ monitoring should be conducted in order to define hypoxic-induced metabolic crisis from aerobic hyperglycolysis or mitochondrial dysfunction, in which oxygen limitation is not the cause. A better characterization of the metabolic response of the brain to injury and its pathophysiology is an essential step for implementing better and more targeted therapeutic strategies for managing patients with acute brain damage.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

Authors' contributions to the study and manuscript preparation include the following: Conception and design: JS and ASG. Acquisition of data: IC, EC, GMB, MAP, ASG, MVJ, and DGS. Analysis and interpretation of data: JS, MAP, and ASG. Statistical analysis: JS, KM, and ASG. Drafting the article: JS and ASG. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: JS. Study supervision: JS.

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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RESULTS AND DISCUSSION

In this section, the results of the 2 published articles are explained in the first 2 separated sub-sections. The discussion of the results obtained from both articles, which is complemented with the theory revised in the initial introduction and the 2 textbook chapters included in this thesis, is developed in a third sub-section. Finally, the limitations of the studies and the future directions are detailed in the last sub-section.

1. LACTATE AND THE LACTATE-TO-PYRUVATE MOLAR RATIO IN TRAUMATIC BRAIN INJURY PATIENTS

In a pilot study published by our group we found a high prevalence of increased lactate and a striking discordance between the concentration of lactate and the LPR.[37] The present study aimed to evaluate the frequency of increased brain lactate levels and the LPR in a larger cohort of patients with moderate or severe traumatic brain injury to analyze the agreement between these two biomarkers, thus determining whether they could be used independently to indicate brain energy metabolism dysfunction.

1.1. Study group

Between September 1999 and March 2013, 188 patients with moderate/severe TBI were monitored using brain MD. Moderate/severe TBI was defined as an admission Glasgow coma scale (GCS) score ≤13 after resuscitation and in the absence of paralytic agents or sedation. The Traumatic Coma Data Bank (TCDB) classification[122] was used to stratify patients. All patients were admitted to a dedicated 10-bed Neurotraumatology Intensive Care Unit at Vall d'Hebron University Hospital.

A randomly generated list of numbers was used to select a random sample of 54 patients from this cohort. Of the selected 54 patients, 8 were excluded because data concerning energetic metabolites (i.e., lactate, pyruvate and glucose) were missing. The remaining 46 patients with complete metabolic data were included in this study. Demographic and clinical characteristics of this cohort are summarized in **Table 3**. We verified that the MD probe was always placed in a macroscopically normal brain in all patients.

Table 3. Demographic and clinical characteristics of the patients

Sex[a]	
Man	36 (78%)
Woman	10 (22%)
Age[b]	35 (±15)
Initial GCS[c]	6 (5,8)
Initial CT classification[a]	
II	26 (56.5%)
III	5 (10.9%)
IV	5 (10.9%)
V	8 (17.4%)
VI	2 (4.3%)
GOSE (6 months) [a]	
Good outcome	15 (32.6%)
Bad outcome[d]	18 (39.1%)
Dead[e]	8 (17.4%)
Lost to 6-months follow-up	5 (10.9%)

GOSE: extended glasgow outcome scale; GCS: glasgow coma scale.
[a] Number of cases (percentage)
[b] Mean (standard deviation)
[c] Median (first quartile, third quartile)
[d] Patients with upper or lower severe disability or vegetative state
[e] Mortality at hospital discharge

1.2. Metabolic thresholds

As mentioned in the introduction section, the ranges of normality for brain lactate, the LPR and the "anaerobic" threshold are still quite arbitrary and usually extrapolated from animal studies, studies in other organs or from brain MD conducted in patients who underwent neurosurgical operations for posterior fossa lesions or epilepsy.[49,110,114] The best estimate we have for the upper lactate and LPR thresholds are from studies of CSF and from a few studies conducted in patients in whom brain MD were monitored under general anesthesia (using different anesthetic management and/or operated on neurosurgical procedures). In these studies the upper range of normality in normal brain tissue for lactate is extremely variable and ranges from 1.50 to 5.10.[50–52,110,111] A recent study established an upper 95% percentile for lactate in CSF at 2.6 mmol/L,[115] and Reinstrup et al.[49] results allowed to calculate an upper threshold for lactate of 4.6 mmol/L and of 31 for the LPR in awake patients. In most studies that have provided RIs the LPR has a more consistent upper threshold ranging from 15 to 25.[51,116] For the purpose of this study, we decided to use an upper threshold of 2.5 mmol/L for lactate (corresponding to an actual brain tissue lactate of around 3.7 mmol/L according to lactate's relative recovery of 67% calculated by Hutchinson et al.[109]). For the LPR, we selected the threshold of 25 used in most studies because this ratio is robust to changes in fluid recovery.[22,50–52,114,123] To compare our data with recently published studies we also conducted the analysis with the proposed thresholds for lactate of 4.0 mmol/L.[50,51]

1.3. Lactate, pyruvate and glucose extracellular concentrations

The 46-patient group yielded 6530 samples. After verification, the data set was reduced to 5305 samples (81% of the total samples) that had good quality data, in which predefined artifacts and doubtful measurements were excluded.

Figure 6 presents a summary of the glucose, lactate and pyruvate data. None of the variables were normally-distributed and were highly skewed to the right. The median for glucose was 1.42 (min: 0.05, max: 11.64), 2.80 for lactate (min: 0.10, max: 12.00) and 0.117 for pyruvate (min: 0.011, max: 0.644), all in mmol/L. The median for the LPR was 23.9 (min: 2.0, max: 545.5). In 28 microvials (0.53%) LPR was above 150.

In all cases, the extreme LPR values were due to a remarkably low pyruvate (0.01 to 0.06 mmol/L).

Lactate was above 2.5 mmol/L in 3018 readings (56.9%) (**Table 4**). If the lactate threshold was increased to 3.0 mmol/L, 2457 determinations (46.3%) were above this threshold, while 1496 (28.2%) would be above 4.0 mmol/L threshold. The LPR was above the predefined threshold of 25 in 2309 determinations (43.5%).

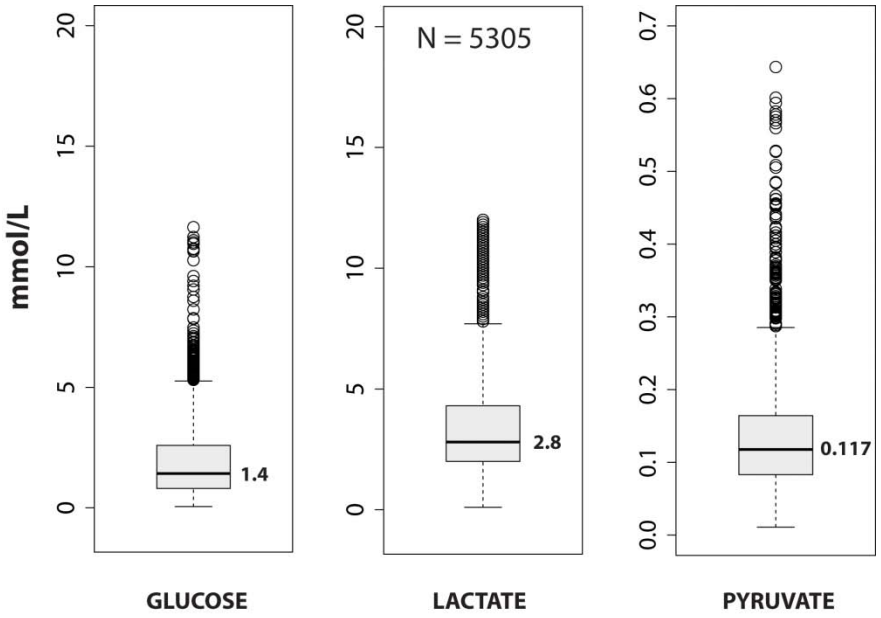

Figure 6.Box-and-whisker plots summarizing the distribution of data for glucose, lactate and pyruvate. The rectangular box spans the first to the third quartile of data and the median, marked in the center of the box, is labeled with its value to the right. Whiskers extend to the furthest observations within ±150% of the interquartile range. Observations outside 150% of the interquartile range are marked as outliers with dots. N= 5305 represents all valid matched data for the three variables.

1.4. Correlation between lactate and the LPR

A scatter plot of these two variables is shown in **Figure 7**. When all the data were considered, no linear relationship between lactate and the LPR was found (adjusted R^2 = 0.15). When all outliers with an LPR> 150 were excluded (28 samples, 0.53%), a very modest linear regression model was fit and the variables were found to be correlated (adjusted R^2 = 0.22). However, the plotted residuals showed that the

relationship between lactate and the LPR could not be adequately modeled by any linear or non-linear model. This fact suggests that the variations in pyruvate were very influential but that they could not be used as an independent predictor of the LPR (data not shown). The same analysis was conducted between lactate (predictor) and the lactate-to-glucose ratio (outcome), but we could not fit a linear or non-linear model to these data either ($R^2 = 0.03$).

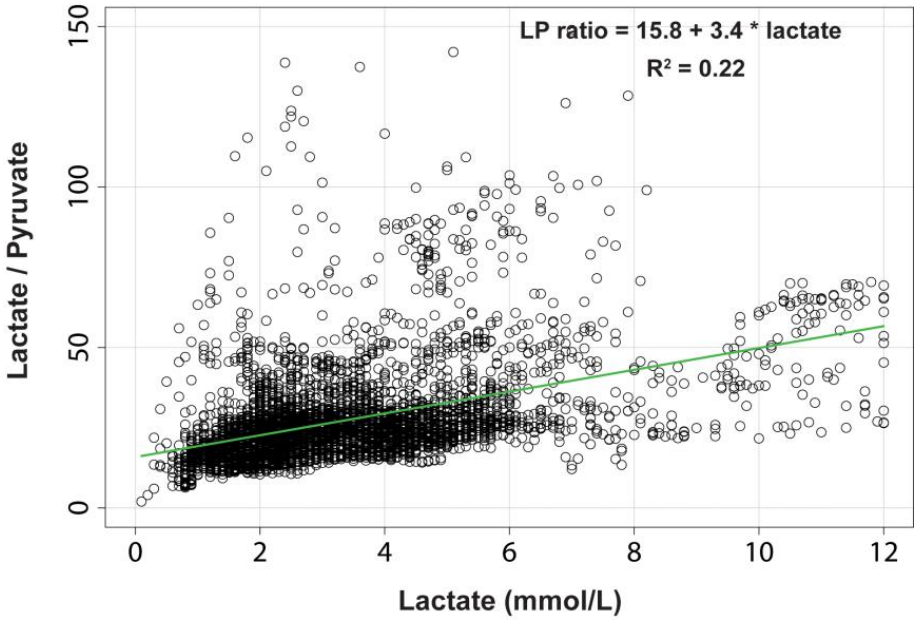

Figure 7. Scatter plot of the relationship between lactate and the lactate-to-pyruvate ratio. The best-fit straight line using an ordinary least squares method is included. In this plot, outliers with a lactate-to-pyruvate ratio > 150 were excluded. The plotted residuals and other regression diagnostics show that the relationship between lactate and the lactate-to-pyruvate ratio could not be adequately modeled by any linear or non-linear model.

1.5. Agreement between lactate and the LPR

To calculate the agreement between lactate and the LPR in classifying hourly microdialysates, we assumed that all 5305 readings were rated independently by the two biomarkers where the ratings were based on a dichotomous scale consisting of 1) normal metabolism or 2) abnormal metabolism. The thresholds of 2.5 mmol/L for lactate and 25 for the LPR were used to sort the patients. For evaluating agreement between lactate and the LPR from the generated contingency table (**Table 4**), the overall proportion of agreement, the un-weigthed Cohen's Kappa and Gwet's AC1

statistic[124] were used to calculate chance-corrected inter-rater reliability coefficients using AgreeStat v2011.3 (Advanced Analytics, Gaithersburg, MD, USA). Gwet's AC1 statistic is more robust than Cohen's Kappa in avoiding what has been called the "Kappa paradox", the situation in which Kappa values are low despite a high percentage of agreement. We established a Kappa and Gwet's AC1 of at least 0.61 as the minimum acceptable degree of agreement to consider both lactate and the LPR as interchangeable in the clinical setting.[125]

Table 4. Intermethod agreement in the classification of dialysate samples by lactate and the LPR

			Lactate		
			Normal metabolism *L ≤ 2.5*	***Abnormal metabolism*** *L > 2.5*	***Total***
Lactate-to-pyruvate ratio	***Normal metabolism*** *LPR ≤ 25*	***N***	1722	1274	2996
		%	32.5%	24.0%	56.5%
	Abnormal metabolism *LPR> 25*	***N***	565	1744	2309
		%	10.7%	32.9%	43.5%
	TOTAL	***N***	2287	3018	**5305**
		%	43.1%	56.9%	**100%**

LPR: lactate-to-pyruvate ratio; L: lactate; >: greater than; ≤: less than or equal to; N: number of readings; %: percentage.

The percentage of agreement was 65%, however Cohen's kappa was 0.32 (95% CI: 0.29-0.34) and Gwet's AC1 was 0.31 (95% CI: 0.28-0.33). None of the statistics showed an acceptable chance-corrected agreement in classifying patients with normal or abnormal metabolisms. When using a contingency table with a higher threshold for lactate (4.0 mmol/L) and the value of 25 for the LPR used by other authors,[50,51] neither Cohen's kappa (0.27, 95% CI: 0.25-0.30) nor Gwet's AC1 (0.36, 95% CI: 0.34-0.39) were above what is considered moderate agreement.[125]

1.6. Metabolic patterns

In an attempt to simplify the data analysis, we divided the entire dataset into the four metabolic patterns defined in **Figure 8**: 1) **Pattern 1**: lactate normal (≤ 2.5 mmol/L)

and LPR below 25, 2) **Pattern 2**: high lactate (> 2.5 mmol/L) and a concurrent LPR ≤ 25, 3) **Pattern 3**: increased lactate (> 2.5 mmol/L) and increased LPR (> 25), 4) **Pattern 4**: normal lactate (≤ 2.5 mmol/L) and increased LPR (> 25). The frequency of each pattern and the descriptive statistics are summarized in **Table 5**.

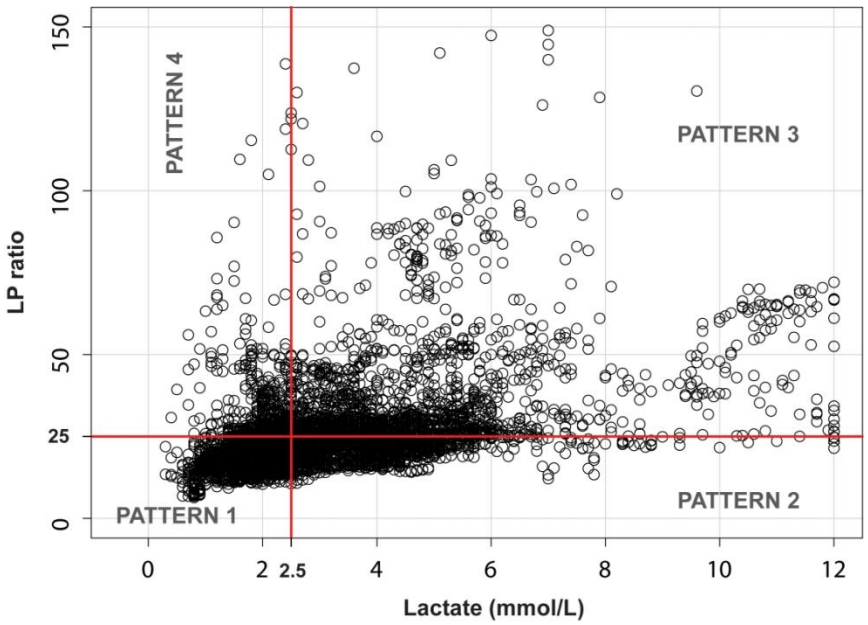

Figure 8. Scatter plot for lactate and the lactate-to-pyruvate ratio. The entire dataset is divided in the four patterns described in the text. We propose the following terminology for the four patterns: Pattern 1 (normal metabolism), Pattern 2 (aerobic hyperglycolysis), Pattern 3 (anaerobic metabolism), and Pattern 4 (low pyruvate).

Pattern 4 ("low pyruvate") was observed the least frequently (10.7%) and was analyzed in depth because it challenged our understanding of energetic metabolism. The most striking difference in this pattern was that the median concentration of glucose in the brain ECF was significantly lower than the brain glucose in the other three groups (Kruskal Wallis, chi-squared = 471.4, df = 3, $p < 0.001$). Of the 565 samples included in this profile ("low pyruvate"), we had good subcutaneous MD data for 435 of them (77%). In this subset, we defined a threshold of 3.6 mmol/L (65 mg/dL) to define low subcutaneous glucose and potential hypoglycemia. An excellent correlation has been shown in diabetic patients by continuous monitoring of glucose by vascular and subcutaneous MD.[126] Furthermore, in neurocritical patients, a good correlation has been shown between blood glucose and subcutaneous glucose six hours

after insertion of the MD catheter.[127] Therefore, we considered subcutaneous MD glucose an acceptable surrogate for hypoglycemia. According to this threshold, only 69 subcutaneous samples (15.8%) showed a low subcutaneous glucose concentration.

Table 5. Summary of metabolites for each metabolic pattern

Metabolic pattern	Thresholds	Suggested terminology	N	%	Glucose (mmol/L)[a]	Pyruvate (mmol/L)[a]	Lactate (mmol/L)[a]	LPR[a]
1	L ≤ 2.5 LPR ≤ 25	Normal metabolism	1722	32.5	1.43 (0.05-7.1)	0.097 (0.01-0.236)	1.8 (0.1-2.5)	18.0 (2.0-25.0)
2	L > 2.5 LPR ≤ 25	Aerobic hyperglycolysis	1274	24.0	2.27 (0.11-11.6)	0.185 (0.10-0.643)	3.8 (2.6-12.0)	21.8 (10.8-25.0)
3	L > 2.5 LPR > 25	Anaerobic metabolism	1744	32.9	1.28 (0.05-6.5)	0.128 (0.01-0.455)	4.4 (2.6-12.0)	31.3 (25-545.5)
4	L ≤ 2.5 LPR > 25	Low pyruvate	565	10.7	0.93 (0.05-7.1)	0.069 (0.01-0.098)	2.1 (0.4-2.5)	28.8 (25-195.3)
TOTAL			5305	100	1.42 (0.05-11.6)	0.117 (0.01-0.644)	2.8 (0.1-12.0)	23.9 (2.0-545.5)

L: lactate; LPR: lactate-to-pyruvate ratio; N: number of readings; %: percentage.
[a] Median (minimum-maximum).

2. NORMAL RANGE FOR BRAIN METABOLITES

Cerebral microdialysis is widely used in neurocritical care units. The normal ranges for brain metabolites are still quite arbitrary. This variability remains an important obstacle for adequately evaluating metabolic findings in neurocritical patients and creates significant uncertainty among clinicians about correct patient management. The goal of this study was to establish the RI for the ECF concentrations of energy metabolites (glucose, lactate and pyruvate) and glycerol in the normal brain by using microdialysis and the extrapolation to zero-flow rate method in anesthetized patients and a constant perfusion at 0.3 μL/min in awake patients.

The present study was divided into three separate sections: 1) preliminary *in vitro* experiments to confirm the RR of the CMA-71 probes (M Dialysis AB) for the analytes of interest, 2) an *in vitro* evaluation of the reproducibility of the 'zero-flow methodology' used in the *in vivo* setting to calculate the brain tissue concentrations of the metabolites, and 3) a study of the brain ECF concentrations of glucose, lactate, pyruvate and glycerol —and their reference limits— in patients who underwent neurosurgical procedures both under general anesthesia and while fully awake after surgery.

2.1. *In vitro* relative recovery experiments

In vitro RR experiments were conducted to confirm the efficient recovery of CMA-71 probes that had been previously described by others.[104,109] The experiments were conducted using an *in vitro* setup that was described by our group elsewhere.[128] A CMA-71 catheter was placed into two different matrix solutions (MA-1 and MA2), which were prepared with the following components: 1) Milli-Q water, 2) the CNS fluid that is used as a perfusate in patients (CNS fluid, M Dialysis AB) containing 147 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 2.7 mmol/L KCl and 0.85 mmol/L $MgCl_2$, 3) different concentrations of the metabolites of interest (Sigma-Aldrich), and 4) a constant concentration of 200 mg/L of albumin (Grifols®, Barcelona, Spain), which is within the reference range for the albumin concentration in normal human CSF (**Table 6)**.[129,130] The catheter was perfused at 0.3 μL/min (18 μL/hr) with an isotonic solution

that had the same ionic composition as the matrix but contained an additional 3% albumin. Albumin was added to the perfusate as an osmotic agent to compensate for the osmotic pressure difference between the matrix and the dialysate and to facilitate fluid recovery. In each experiment, minor adjustments were made during the initial phase to correct for small differences in hydrostatic pressures and to obtain a zero-fluid loss and a fluid recovery close to the expected theoretical volume (18 µL/hr). Our *in vitro* experiments were conducted without stirring. In summary, 6 microvials were obtained for each matrix in each catheter. Matrix solution was also sampled before and after each microvial collection. Both dialysate and matrix samples were analyzed in the ISCUS Flex analyzer (M Dialysis AB). The RR was calculated using the equation: $RR = (C_{md}/C_{matrix}) \times 100$ (**Equation 1**).

Table 6. Composition of the matrices used on *in vitro* relative recovery experiments

	MA-1	MA-2
Metabolites		
Pyruvate (µmol/L)	250	125
Lactate (mmol/L)	5.0	2.5
Glycerol (µmol/L)	500	250
Glucose (mmol/L)	5.0	2.5
Ions		
Na^+ (mmol/L)	147	147
Ca^{2+} (mmol/L)	1.2	1.2
K^+ (mmol/L)	2.7	2.7
Mg^{2+} (mmol/L)	0.85	0.85
Cl^- (mmol/L)	153.8	153.8
Albumin (mg/L)	200	200
Osmolarity (mOsm/L)	302	294

Four catheters were used in these experiments. We observed minimal differences between the initial and the final concentrations of the matrix metabolites, with a mean reduction of approximately 5.5%. Although these differences were statistically significant we considered them methodologically irrelevant. The denominator used for the RR calculation (**Equation 1**) was always the mean concentration of the matrix sampled before and after each microvial change. The 4 catheters included in the analysis yielded a total of 48 samples. The RR for each metabolite is summarized in

Table 7. The mean RR for all metabolites was approximately 95%, similar to the findings published by others who used the CMA-71 probe.[131] We did not find any significant difference between the two matrices studied (see **Table 7**); furthermore, the RR inter-catheter agreement was excellent, with differences always below 5%.

Table 7. *In vitro* Relative Recoveries

Metabolite	Mean ± SD	Median (min-max)	p-value
Glucose (mmol/L)	91 ± 5	91 (80-103)	
MA-1	92 ± 4	91 (85-101)	0.39
MA-2	91 ± 5	92 (80-103)	
Lactate (mmol/L)	95 ± 6	95 (83-109)	
MA-1	95 ± 5	95 (85-109)	0.92
MA-2	94 ± 6	96 (83-107)	
Pyruvate (µmol/L)	104 ± 17	100 (83-150)	
MA-1	109 ± 20	97 (89-150)	0.13
MA-2	100 ± 13	101 (83-128)	
Glycerol (µmol/L)	90 ± 6	91 (73-105)	
MA-1	92 ± 4	93 (82-99)	0.09
MA-2	89 ± 7	90 (73-105)	

Means and medians are expressed as percent recovery, as explained in the text (Equation 1). Comparisons between matrices were made using the Kruskal-Wallis test; no statistically significant differences were observed.

2.2. *In vitro* extrapolation to zero-flow rate

To test *in vitro* the extrapolation to zero-flow rate method that was used during surgery in the included patients, a second experiment was conducted in which CMA-71 probes were placed in a matrix with the same solute concentrations of MA-2 used in the RR experiments of the previous section. The same isotonic solution used in the *in vitro* RR experiments was infused. For each catheter, the flow rate was changed randomly to the following rates: 0.1, 0.3, 0.6, 1.2 and 2.4 µL/min. Each flow rate was maintained for one hour, except for the 0.1 µL/min flow rate, which was maintained for 2 hours to compensate for the low recovery volume. Microvials were collected every 30 minutes for the 2.4 - 0.3 µL/min infusion rates and hourly for the 0.1 µL/min flow rate. Each catheter experiment yielded a total of 10 determinations (2 for each infusion rate). The metabolite concentrations in the matrix were analyzed at the beginning and at the end of each experiment. All analyses were conducted in the ISCUS Flex analyzer.

The actual concentrations of matrix metabolites were estimated using the ZFM,[106] which has already been explained in the subsection "Extrapolation to zero-flow rate method" under the "Introduction" section of the present doctoral thesis. Because the first dialysate sample at each flow rate is heavily influenced by the previous flow rate (due to the dead liquid volume of the outlet tube [≈5.1 µL]), the first sample was always discarded and was not included in the calculations; therefore, at any flow rate, only the second determination was used to fit the curve.

Seven catheters were included in this experiment. For each catheter, the metabolite concentrations at a theoretical flow rate of 0 µL/min were calculated using a nonlinear regression model fitted to the following equation: $C_{dial} = C_0 - C_0 [\exp(-rA/F)]$ (**Equation 2**). The matrix concentration (C_0 in **Equation 2**) was calculated as the y-intercept, as described. A *k* value (where *k*=rA in **Equation 2**) was also determined for each metabolite. **Figure 9** shows an example of the ZFM applied for glucose data from the catheter #5. The goodness of fit for the exponential model *in vitro* was excellent for all metabolites. The concentrations estimated at 0 µL/min with the nonlinear regression model were compared with the measured concentrations in the matrices (**Table 8**). To minimize errors, the matrix concentration used in the analysis was the mean concentration for each metabolite at the start and the end of the experiment. In all metabolites and for all catheters, we found a high agreement between the estimated concentrations and the true concentrations in the medium. The differences between both concentrations were not statistically significant (see **Table 8**) (Wilcoxon test for paired samples).

Table 8. *In vitro* estimated concentrations using the extrapolation to zero-flow rate methodology against true concentrations

	Estimated concentration	Measured concentration	p-value
Glucose (mmol/L)	1.97 (1.59-2.19)	2.06 (1.93-2.25)	0.16
Lactate (mmol/L)	2.01 (1.71-2.44)	2.15 (2.05-2.37)	0.09
Pyruvate (µmol/L)	106.7 (87.3-143.9)	119.5 (108.5-127.5)	0.38
Glycerol (µmol/L)	242.1 (160.6-284.5)	231.6 (200.1-295.0)	0.11

In these experiments, seven catheters were used. Data summarized correspond to the data for each metabolite in all experiments. Data are expressed as median (min-max). Comparisons between estimated and measured concentrations were made using the Wilcoxon test for paired samples; no statistically significant differences were observed.

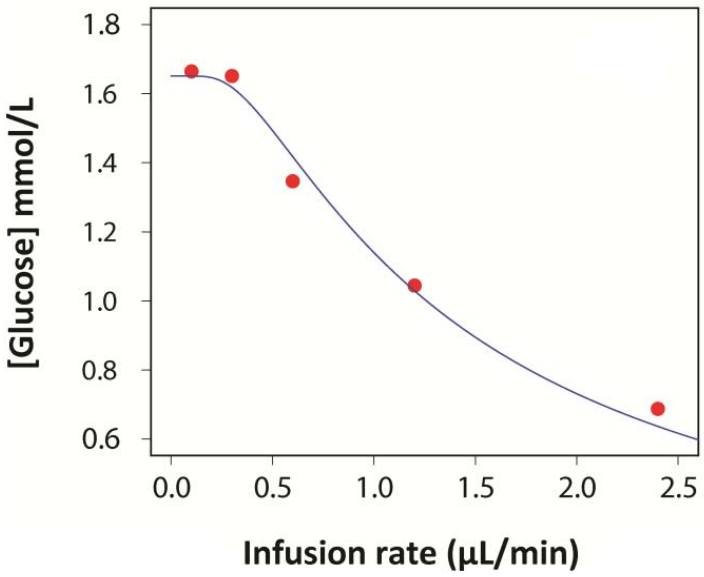

Figure 9. Graphical representation of the *in vitro* extrapolation to zero-flow rate method of glucose concentration for catheter #5. Using non-linear curve fitting to the equation described by Jacobson et al.[106] (see Eq 2 in the main text), the concentration in the matrix (C_0 in Eq 2) corresponds to the y-intercept. The estimated C_0 was 1.65 mmol/L, the value of k (where k = rA in Eq 2) was 1.17mm/min, and the measured C_0 for glucose was 2.06 mmol/L.

2.3. *In vivo* determination of the brain tissue metabolites concentrations

2.3.1. Study group

A prospective study was conducted in patients aged above 18 years who underwent surgical treatment under standard general anesthesia to treat posterior fossa and supratentorial lesions at Vall d'Hebron University Hospital, between November 2012 and January 2016. To enroll patients in the study, the inclusion criteria were: 1) posterior fossa or supratentorial lesions requiring the implantation of an external ventricular catheter for the drainage of CSF; 2) no neuroradiological abnormalities in the white/gray matter in the supratentorial compartment where the MD probe will be implanted in magnetic resonance imaging (MRI) sequences: T1W, T2W and FLAIR; 3) a normal ventricular size defined as an Evans Index below or equal to 0.30 or moderate ventricular enlargement without clinical symptoms of intracranial hypertension;[132] and 4) written informed consent signed by the patient or the next-of-kin.

Of the 19 patients included in the analysis, 16 had a posterior fossa lesion and 3 a supratentorial lesion. All presented normal-appearing grey and white matter in the MRI. The 19-patient cohort included 7 males and 12 females with a median age of 44 (range: 21-69 years) and a median Evans index of 0.28 (min: 0.20, max: 0.33). **Table 9** shows a summary of the demographic and clinical data.

Table 9. Demographic and clinical characteristics of the patients included in the study

Case No.	Age (yrs)/ Sex	Diagnosis of pathology	Evans index	Duration of microdialysis on surgery (h)	Duration of microdialysis on awakeness (h)	Anesthesia
1	38/F	Cerebellar tonsil lesion	0.28	4.67	7.42	Inhalation
2	32/F	Ependymoma	0.28	8.00	11.90	TIVA
3	61/F	Atypical fibrous meningioma	0.33	6.83	NA	Inhalation
4	21/F	Hemangioblastoma	0.29	3.30	5.17	Inhalation
5	43/F	Chiari malformation	0.27	4.93	13.17	Inhalation
6	22/M	Classic medulloblastoma	0.27	7.72	7.42	Inhalation
7	34/F	Cerebellar metastasis	0.24	10.83	5.08	Inhalation
8	60/F	Vestibular Schwannoma	0.30	6.53	15.83	Inhalation
9	65/M	Cerebellar metastasis	0.31	5.00	5.33	Inhalation
10	65/F	Hemangioblastoma	0.28	5.00	9.33	Inhalation
11	66/M	Cerebellar metastasis	0.28	8.00	NA	Inhalation
12	55/F	Anaplasic glioma	0.30	5.83	7.67	Inhalation
13	42/M	Transitional meningioma	0.24	6.83	39.00	Inhalation
14	31/M	Vestibular schwannoma	0.21	6.50	14.00	Inhalation
15	69/F	Mixed meningioma	0.27	6.25	10.00	TIVA
16	32/F	Central neurocytoma	0.30	5.08	13.42	TIVA
17	65/F	Low grade glioma	0.20	7.10	14.00	TIVA
18	66/M	Cerebellar metastasis	0.29	7.17	13.17	TIVA
19	44/M	Epidermoid cyst	0.30	7.17	12.67	TIVA
Median	44/F		0.28	6.53	11.90	

M: male; F: female; TIVA: total intravenous anesthesia; NA: not applicable.

2.3.2. *In vivo* extrapolation to zero-flow rate

During surgery, CMA-71 probes were placed with the patient under general anesthesia at the same time the external ventricular drainage was placed, through a small hole in the duramater at ≈3 mm from the ventriculostomy entry. The position of the probe in the normal white matter was confirmed by a control CT scan conducted within the first

24 hrs after probe implantation (**Figure 10**). Patients with any hemorrhagic or hypodense lesion around the probe detected in the control CT were excluded from analysis. The same methodology described in the above section "*In vitro* extrapolation to zero-flow rate" was applied, except in the first 4 patients, where microvials were collected hourly for all infusion rates and just one microvial was collected for each infusion rate.

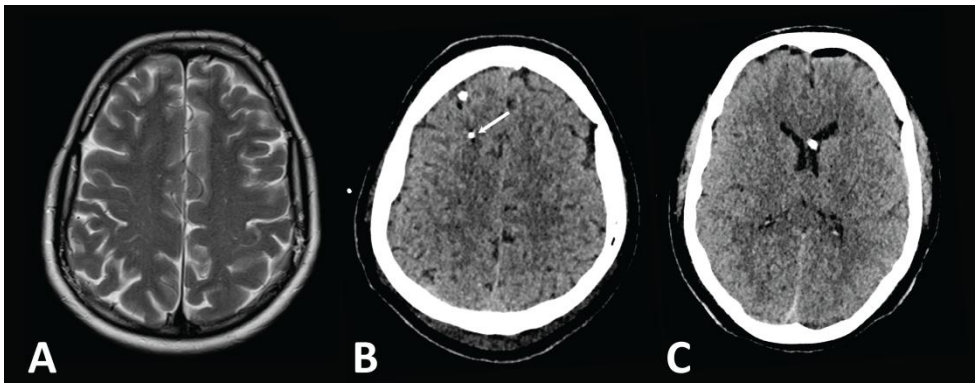

Figure 10. Example of the placement of a cerebral microdialysis MD probe and an external ventricular catheter. The MD catheter has a gold tip that makes it visible on a computed tomography (CT) scan and thus allowing the determination of the exact position of the probe (arrow). A 38-year-old female received an operation for a glial cyst in the right cerebellar tonsil. **A.** Preoperative T2W MRI slice shows a normal signal in the zone in which the MD probe was inserted. **B.** Control CT scan performed the day after surgery. The tip of the MD probe is shown (arrow). **C.** The ventricular catheter is seen entering into the left frontal horn in the control CT scan.

During the surgical procedure, blood and CSF samples were also collected periodically. Blood gas analysis were performed in a Co-oximeter (1200 RAPIDLab Systems, Siemens, Munich, Germany). The CSF samples were centrifuged at 4000 rpm during 10 minutes and the supernatant analyzed in the ISCUS Flex analyzer. At the end of the surgical procedure, the subcutaneous catheter was removed and the perfusion of the cerebral catheter was interrupted until the patient was fully awake in the reanimation unit.

In 3 of the 19 patients the intraoperative data were discarded; for one, the surgery was too short to obtain data for all the perfusion flow rates, and for the other two patients the volume recovered was not correct. The remaining 16 patients had valid

intraoperative data, which were included in the analysis. For each patient, the ECF concentration was determined with the ZFM, as already described. In some patients, the non-linear curve fitting to the equation did not result in a correct estimated C_0 value because the iterative procedure of the 'nls' function did not successfully converge to a certain value. This unsuccessful convergence was because the equation is too complex for the amount of data we had. In order to simplify the equation, we obtained a fixed *k* value for each metabolite by merging the data for all patients. We used the obtained *k* to estimate the C_0 by non-linear curve fitting for each individual patient. **Figure 11** shows an example of the ZFM applied *in vivo* for glucose, lactate and pyruvate data from patient #12.

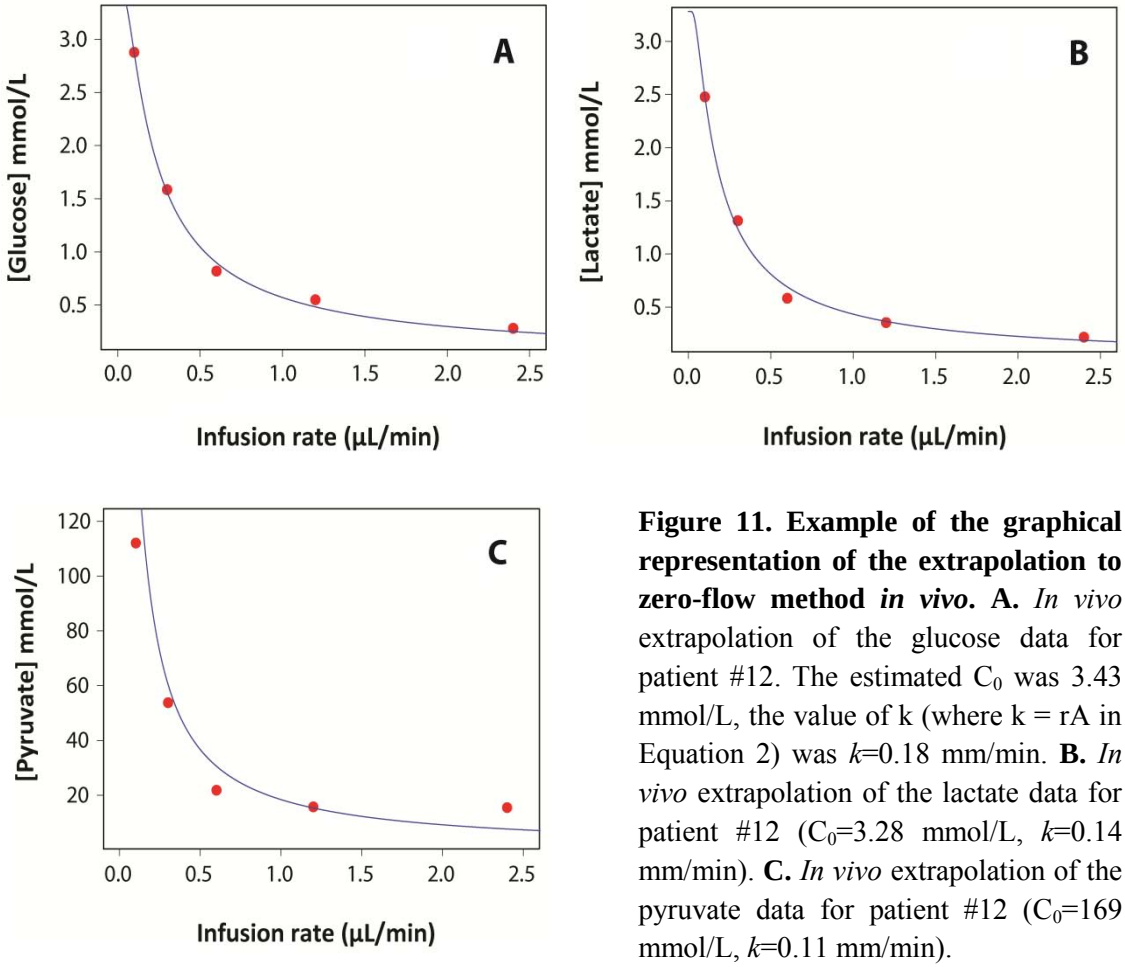

Figure 11. Example of the graphical representation of the extrapolation to zero-flow method *in vivo*. A. *In vivo* extrapolation of the glucose data for patient #12. The estimated C_0 was 3.43 mmol/L, the value of k (where k = rA in Equation 2) was *k*=0.18 mm/min. **B.** *In vivo* extrapolation of the lactate data for patient #12 (C_0=3.28 mmol/L, *k*=0.14 mm/min). **C.** *In vivo* extrapolation of the pyruvate data for patient #12 (C_0=169 mmol/L, *k*=0.11 mm/min).

The data for the analyzed metabolites calculated by the ZFM in anesthetized patients are summarized in **Table 10** and **Figure 12**.

The *in vivo* RR at a perfusion flow rate of 0.3 µL/min was calculated by taking into account the estimated tissue concentration for each analyte obtained with the ZFM. The *in vivo* median RR values were 80% for glucose (min: 56, max: 96), 75% for lactate (min: 59, max: 92), 83% for pyruvate (min: 66, max: 100), and 89% for glycerol (min: 69, max: 103).

2.3.3. Brain tissue concentrations in anesthetized patients

Metabolite concentrations at 0.3 µL/min in the anesthetized patient were calculated using samples recovered at the mentioned flow rate during the extrapolation to zero-flow rate method *in vivo*.

As explained above, the microdialysis sampling methodology used during surgery was modified after the fourth patient. Therefore, the dialysate concentrations obtained in the first patients were influenced by the previous flow rate. In order to avoid generating inaccurate values from the dead volume inside the outlet tube (≈5.1 µL), data from 3 patients were excluded from the RIs calculation at 0.3 µL/min. A fourth patient was discarded because no sample at 0.3 µL/min was collected. The metabolite concentrations at 0.3 µL/min in the anesthetized patient, that was calculated with data of 15 patients, are summarized in **Table 10** and **Figure 12**.

2.3.4. Brain tissue concentrations in awake patients

Within 24 hours after surgery and when the patient was extubated and fully conscious, the MD probe was reconnected to a CMA-106 microinfusion pump (M Dialysis AB) and the perfusion was restarted. In this second part of the study, the flow rate was maintained at a constant rate of 0.3 µL/min and the samples were collected hourly.

In two of the 19 patients data could not be obtained due to a malfunction in the microdialysis probe. The median values for the 17 remaining patients at a fixed perfusion flow of 0.3 µL/min are summarized in **Table 10** and are also shown in the box plots presented in **Figure 12**. **Table 10** shows the upper and lower reference limits

found by the nonparametric method. Levels at a perfusion flow rate of 0.3 μL/min were significantly higher for lactate, pyruvate, and LGR when the patient was fully awake, as compared to the values obtained when the patient was under anaesthesia. However, we did not find statistically significant differences between the glucose, glycerol, and LPR levels of awake versus anesthetized patients (**Figure 12**).

Subcutaneous, CSF and arterial metabolite levels, obtained in patients under general anesthesia and when they were fully awake, are summarized in **Table 11**.

Table 11. Subcutaneous microdialysis (MD), cerebrospinal fluid (CSF) and arterial blood gases (ABG) concentrations

Variable	**Surgery**	**Awake**
Subcutaneous MD		
Glucose (mmol/L)	2.32 (0.24-4.12)	
Lactate (mmol/L)	1.01 (0.13-6.84)	
Pyruvate (μmol/L)	89.5 (12.6-327.0)	NA
Glycerol (μmol/L)	241.8 (100.0-404.0)	
LPR	12.3 (4.64-110.2)	
LGR	0.64 (0.32-2.95)	
CSF		
Glucose (mmol/L)	3.52 (1.74-5.33)	4.44 (2.63-7.40)
Lactate (mmol/L)	1.88 (0.92-2.49)	2.07 (1.23-3.75)
Pyruvate (μmol/L)	131.7 (22.4-217.5)	140.5 (86.5-380.1)
Glycerol (μmol/L)	12.3 (4.28-22.3)	25.6 (7.45-52.5)
LPR	13.9 (10.8-56.9)	16.7 (4.99-20.5)
LGR	0.54 (0.35-0.89)	0.53 (0.28-0.89)
ABG		
Glucose (mmol/L)	7.13 (4.99-9.88)	7.72 (5.11-8.22)
Lactate (mmol/L)	2.03 (1.24-5.04)	1.40 (0.55-3.80)

Data are expressed as median (min-max).
LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio; NA: not applicable.

Table 10. Brain tissue concentrations of metabolites

Condition	Glucose (mmol/L)		Lactate (mmol/L)		Pyruvate (µmol/L)		Glycerol (µmol/L)		LPR		LGR	
	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*
ZFM (n = 16)	**1.57** (1.12-4.70)	1.15-4.13	**2.01** (1.37-5.44)	1.3-5.31	**80.0** (53.9-223.3)	54.4-197.0	**49.9** (21.7-228.7)	23.6-227.3	**27.5** (14.9-39.5)	15.6-39.2	**1.33** (0.46-3.64)	0.49-3.47
Anesthetized (0.3µL/min) (n = 15)	**1.25** (0.64-3.53)	0.68-3.11	**1.40** (1.10-3.84)	1.11-3.63	**73.8** (36.6-149.7)	39.0-137.1	**53.8** (24.4-205.1)	25.3-202.9	**23.3** (12.8-34.5)	13.1-34.3	**1.42** (0.50-2.78)	0.52-2.57
Awake (0.3µL/min) (n = 17)	**1.55** (0.29-3.01)	0.43-2.94	**3.41** (1.56-5.62)	1.64-5.50	**137.1** (85.0-192.0)	86.1-188.7	**79.8** (29.3-346.4)	31.7-338.7	**24.9** (16.9-35.1)	18.3-33.5	**2.32** (0.62-15.0)	0.65-11.3

ZFM: zero-flow method; RI: reference interval; LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio. *2.5% and 97.5% percentile values, as lower and upper reference interval limits, respectively.

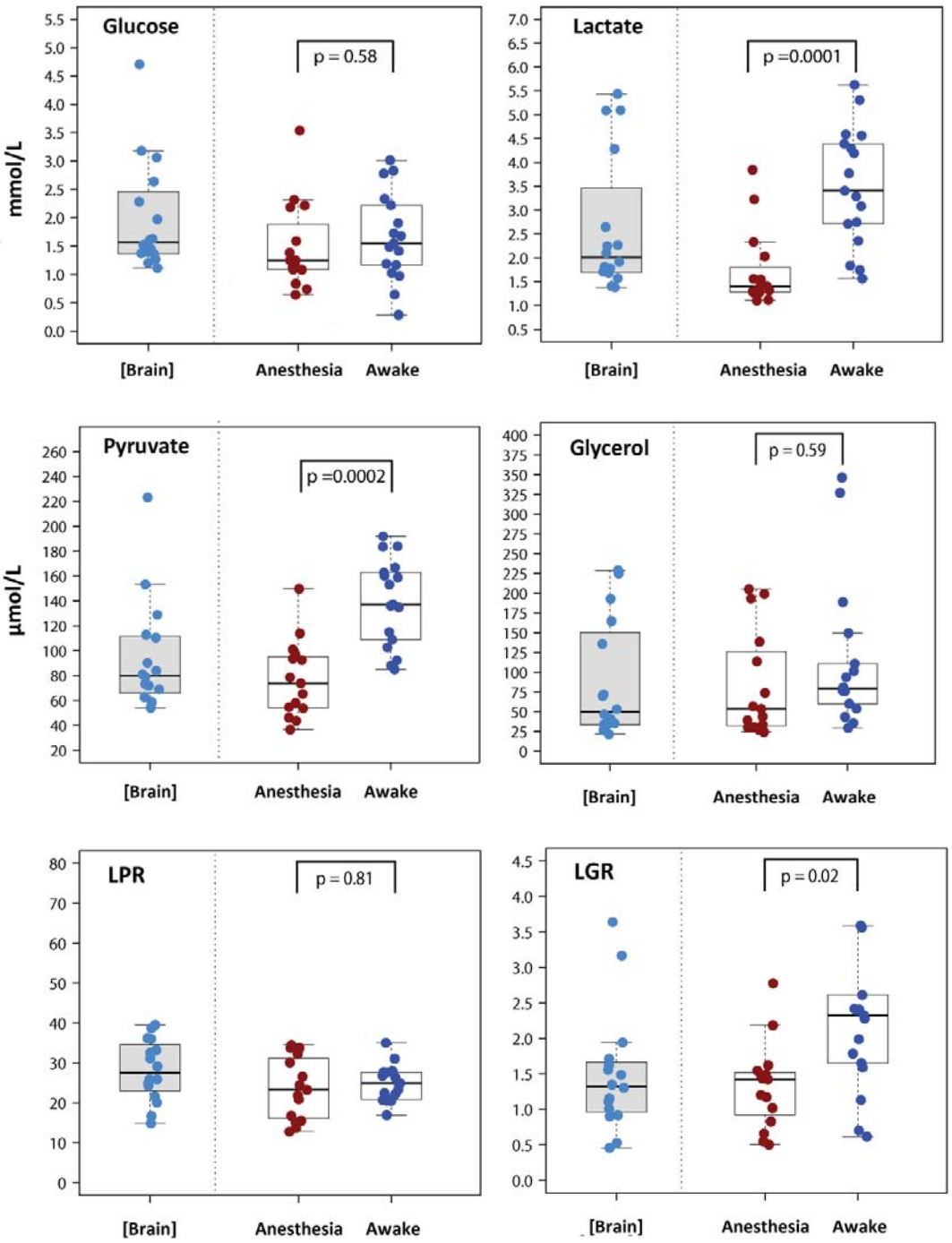

Figure 12. Box plots showing the concentrations of glucose, lactate, pyruvate, glycerol, lactate-to-pyruvate ratio (LPR) and lactate-to-glucose ratio (LGR) obtained with the extrapolation to zero-flow rate method ([Brain]) and under different conditions at a 0.3 µL/min: anesthesia and awake. A summary of the data is shown in **Table 10**. Anesthetized values were compared with awake values by the Wilcoxon signed-rank test. Lactate, pyruvate and LGR values were significantly higher when the patients were awake. No significant differences were observed in glucose, glycerol and LPR values. NS: statistically non-significant.

3. DISCUSSION

Brain microdialysis allows for continuous neurochemical monitoring and the unique opportunity to explore the disturbances of metabolism in neurocritical patients. However, MD is not yet a routine neuromonitoring tool, and the interpretation of the results is still plagued by methodological problems, disagreement in reference ranges and thus in the diagnostic thresholds and uncertainties in the interpretation of the results.

The data obtained in the study of lactate and LPR showed poor agreement between these 2 most commonly used biomarkers for diagnosing impaired energetic metabolism and highlighted the need for a common ground for future comparisons of results across studies.

In the second study, we enrolled a cohort of homogeneous patients that were studied twice—once while under general anesthesia and once when they were fully awake. In this cohort, we determined the actual brain concentrations for each analyte by using the Jacobson et al. methodology.[106] In addition, we followed the statistical methods recommended by the latest version of the Clinical and Laboratory Standards Institute to identify outliers and to perform the calculations necessary to set valid biological RIs.[133]

The presented studies allowed us to study the role of lactate and LPR biomarkers in the classification of metabolic patterns present in TBI patients and to define the RIs for energy metabolites in order to propose a more robust classification of these patterns. Both studies will help to apply microdialysis to clinical decisions in neurocritical care units worldwide.

3.1. The ongoing problem of the reference ranges

One of the crucial steps for any biomarker is determining its reference range (i.e., its normal range) in a cohort of disease-free patients. For brain MD, this approach is limited by its invasiveness and by ethical issues. In MD, technical issues increase the complexity of determining appropriate RIs. The infusion flow rate, the length of the

dialyzing membrane, and the fluid used as a perfusate modify the amount of the recovered analyte. The concentration of the analyte in the dialysate reflects a variable—and sometimes unknown—percentage of the real tissue concentration.[100] To minimize this problem, most centers use a standardized method with 10mm length membranes, mock CSF as the perfusate, and a flow rate of 0.3 μL/min.

The most commonly used reference range was taken from the study of Reinstrup et al.[49] who obtained a mean lactate of 2.9 ± 0.9 mmol/L and a LPR of 23 ± 4 during microdialysis sampling performed on 9 awake patients at 0.3μL/min. Applying the conventional rule—using 1.96 standard deviation— for calculating the upper thresholds, gives an upper threshold for lactate of 4.6 mmol/L and of 31 for the LPR. However, due to the small sample size, the anesthetic conditions —isoflurane was used in some of them— and the changes in perfusion speed, using the traditional method for calculating the upper and lower threshold from this cohort is misleading. In addition, volatile anesthetics may induce a significant increase in lactate due to reversible mitochondrial dysfunction[134,135] and the awake patients were studied immediately after surgery and therefore metabolic stress or even hyperglycolysis could be a confounding factor.

The main difficulty in estimating the *in vivo* concentration is that substances diffuse across the probes because of the concentration gradient on both sides of the membrane, as remarked by Menacherry et al.,[105] thus the analytes of interest are carried away by the perfusion medium. A potential solution for this drawback is to use an *in vivo* calibration method.[105] *In vivo* calibration methods are time-consuming, cannot be routinely conducted at the bedside and present ethical constraints. Therefore, in clinical practice, the true concentrations are estimated from the absolute values given by the analyzer and the known *in vitro* RR for the analyte of interest, although *in vitro* conditions are rarely reproduced *in vivo*. In clinical practice, thresholds that were determined for a fixed flow perfusion rate of 0.3 μL/min are typically used because this is the flow rate recommended by the 2014 consensus statement and is the most commonly used rate in the cerebral MD literature.[97] A direct consequence of these variable approaches is that researchers have used different cut-off levels for the RIs of energy metabolites, introducing significant variability and biases when explaining cerebral MD data and interpreting findings observed in neurocritical patients. These

implications are especially relevant for the definition of brain hypoxia/ischemia and hyperglycolysis. In addition, ambiguous RIs in any neuromonitoring tool create significant uncertainty among clinicians about correct patient management. Thus, some patients are exposed to the risks of treatment without any obvious clinical benefit, while for others relevant secondary events might be missed or warning signs disregarded.

3.2. Lactate and the LPR need to be combined as screening tools

Moderate and severe TBIs cause frequent alterations in brain energy metabolism even in macroscopically normal brain tissue. At the predefined threshold of 2.5 mmol/L, we found an increase in brain lactate in 56.9% of the samples analyzed (**Table 4**). In 1496 samples (28.2%), lactate was above 4.0 mmol/L. Our data are in agreement with a recent study from our group in which the effects of normobaric hyperoxia on brain energy metabolism were evaluated and 45% of the patients had lactate levels above 3 mmol/L despite the probe placement being in macroscopically non-injured brain tissue and with a $PtiO_2$ within the normal range in most of them.[52] If probes had been placed in traumatic penumbra and not in brain that appeared normal, the frequency of increased lactate could have been even higher. However, despite the high prevalence of elevated lactate, concurrent elevation of lactate and the LPR (> 25) was observed in only 33% of the microdialysates, suggesting that a true anaerobic pattern was detected in one third of the samples.

We found a remarkable disagreement between lactate and the LPR in classifying the brain energy state in a simple dichotomized scale (normal versus abnormal/anaerobic). Methods used to evaluate inter-rater agreement showed a non-acceptable agreement between lactate and the LPR in classifying samples. Therefore, the conventional approach of using lactate or the LPR alone—at any threshold— needs to be abandoned for metabolic profiling both in clinical settings and for reporting clinical research. Any attempt to classify the metabolic status as normal or abnormal (i.e., “anaerobic”) using only lactate can lead to an overdiagnosis of anaerobic metabolism when not combined with pyruvate and the LPR. Hovda’s group has shown the major flaws of using this approach in different studies.[22,136]

The meaning of high lactate in the brain has been extrapolated from research in shock and sepsis and from experimental models of brain ischemia. An increase in lactate in any organ has been traditionally associated with low tissue perfusion and anaerobic metabolism.[116] In addition, hyperlactatemia has been consistently associated with an increase risk of death in patients with various types of shock.[137] However, in critical care research, pyruvate and consequently the LPR have been rarely used because of the complex handling of pyruvate that is required and the lack of analytical tools to assay it at the bedside.[116] The availability of portable MD bedside analyzers has changed this scenario, allowing the systematic use of pyruvate. The incorporation of pyruvate into neurocritical care has made it increasingly clear that the production and clearance of lactate in any tissue is multifactorial and far more complex than expected.[22,61] In brief, lactate concentration is a highly sensitive indicator of upregulated glycolytic flux but pyruvate levels are necessary in order to differentiate whether the upregulation of the glycolytic flux is anaerobic (i.e., hypoxic) or simply an indicator of an increased use of the glycolysis under aerobic conditions.

3.3. Metabolic patterns based on lactate and LPR

In our first study, we confirmed that some sort of metabolic impairment was present in 57% of the samples analyzed when we used a lactate threshold as a criterion. In the analysis of our data, we found it useful to partition the entire data set in the four patterns described in **Figure 8**. According to our data, only one third of the samples were compatible with a normal metabolism. In an attempt to interpret the patterns we suggested the following working terminology: **Normal metabolism** for **pattern 1**, **Aerobic hyperglycolysis** for **pattern 2**, **Anaerobic metabolism** for **pattern 3** and **Low pyruvate** for **pattern 4**. The frequency of each pattern and the descriptive statistics are summarized in **Table 5**. We believe this interpretation may be controversial and for a better understanding of these patterns there is need to combine MD data with brain O_2 monitoring data to verify or refute patterns 2 (“aerobic hyperglycolysis”) and 3 (“anaerobic metabolism”) and to prove whether our interpretation is reasonable.

3.3.1. Aerobic hyperglycolysis pattern

Hypermetabolism was considered in the past a form of "hypoxia". In a pivotal paper by Siggaard-Andersen et al., "hypermetabolic hypoxia" was defined as a situation in which ATP hydrolysis was not balanced by an increase in oxidative ATP synthesis. As a consequence, glycolysis was activated and lactate increased.[24] However, strictly speaking, we know that this pattern is present when O_2 delivery and utilization is within the normal range; therefore, this pattern has to be differentiated from any other form of tissue hypoxia. The hyperglycolytic pattern was defined in TBI by a pivotal study from Hovda's group[22,28] and emerges only when both lactate and the LPR are used. Bergsneider et al.[28] showed hyperglycolysis in 56% of severe TBI patients studied using PET scanning within one week of injury and in patients with sufficient brain O_2. This pattern has also been reported in the brain of patients with SAH.[50,113,114] In a pivotal paper, Hutchinson et al.[36] in 17 patients with severe TBI used both MD and PET parameters— using the glucose analogue [F^{18}]-fluorodeoxyglucose (FDG) — to correlate the extracellular levels of glucose, lactate and pyruvate with the CMRglc in the same region of interest. The most relevant finding of this study is that there was a linear relationship between CMRglc and the MD levels of lactate and pyruvate but not with the LPR.[36] These data confirm the general view that the increase in CMRglc in these patients is often indicative of hyperglycolysis and not of a shift towards anaerobic metabolism.

The main difference between hyperglycolysis and an anaerobic profile is that the LPR is significantly increased in the latter. It has been postulated that glycolysis upregulation in an injured brain indicates a hypermetabolic state directed toward restoring perturbed ionic homeostasis or the reuptake of high extracellular levels of glutamate. Hypermetabolism following TBI occurs because oxidative phosphorylation normally runs at near maximal capacity and, consequently, an increased energy demand should be supplied by an increased glycolysis.[114] Different authors have shown that an hyperglycolytic pattern is a predictor of good neurological outcome in patients with SAH compared with patients with an anaerobic pattern.[50,114]

3.3.2. Anaerobic metabolism pattern

The anaerobic pattern (high lactate/high LPR) was observed in one third of the analyzed samples. This pattern is well consolidated and it has been the only pattern detected in studies that have used only lactate for profiling energy metabolism. The LPR reflects the equilibrium between product and substrate of the reaction catalyzed by LDH and the LPR is a good surrogate for the cytosolic redox status.[138] In patients with SAH, an anaerobic pattern is related to poor neurological outcomes.[50,114] Cesarini et al.[114] showed that when this pattern is associated with ischemia in a sample of patients with SAH, it is also linked to concurrent low brain glucose concentrations. However, although reduced ECF concentration of glucose is a hallmark of hypoxic ischemia, its concentration may be normal (or even high) in non-ischemic forms of hypoxia (anemia, hypoxemia, high-affinity hypoxia, etc.). This pattern is the most important in clinical settings; when detected, it requires a systematic approach to rule out all the classes of brain hypoxia.

3.3.3. Low pyruvate pattern

The low pyruvate pattern detected in 10.7% of our samples was quite unexpected, given that this profile was already described by Hlatky et al. and confirmed by others.[36,120,136,139] Hillered et al. called this pattern a type 2 elevation of the LPR.[139] This pattern, in which lactate is within the normal range, is misleading and, if not detected, can be easily considered an indicator of anaerobic metabolism and thus indicative of brain hypoxia when it is not. In our series, this pattern was associated with a low brain ECF glucose concentration, as was described by Marcoux et al.[136] Because the brain is an obligate glucose consumer, its fuel depends on the glucose plasma levels. With all the limitations of our study (lack of matched O_2 data for this cohort), a low ECF glucose was associated with the drop in pyruvate concentrations and the increase in the LPR. However, hypoglycemia was infrequent. In all the samples in which we had good quality subcutaneous data, only 16% presented a low subcutaneous concentration of glucose; hypoglycemia might therefore have been the cause. Marcoux et al. suggested that a low pyruvate and a high LPR may be secondary to mitochondrial dysfunction.[136] However, when mitochondrial dysfunction has been

reproduced in experimental models, a high lactate level, together with a high LPR has been consistently found.[135,140] A third alternative to explain this pattern and the concomitant low levels of glucose is the shunting of glucose-6-phosphate toward alternative metabolic pathways, specifically the pentose phosphate pathway. This process has been observed in both experimental models and in clinical studies of TBI.[79,141] It has been described that neurons prioritizes the metabolism of glucose via the PPP to maintain their antioxidant status and viability rather than its metabolism for bioenergetic purposes.[142]

Hutchinson et al. in their combined MD/PET-FDG study suggested that the low levels of glucose in the ECF of head-injured patients are a consequence of an increase in substrate demand rather than inadequate substrate delivery.[36] However, a better understanding of this pattern requires additional studies with simultaneous measurements of plasmatic glucose, lactate, pyruvate, and brain tissue O_2. When mitochondrial dysfunction is present, as hypothesized by some, the tissue O_2 is not used and the amount of dissolved O_2 is significantly increased.[135,140]

3.4. Metabolic impairment and brain hypoxia

Evidence accumulated in the last two decades shows that non-ischemic causes of brain hypoxia are frequent. The most comprehensive classification of tissue hypoxia was developed by Siggaard-Andersen et al. in 1995.[20,24] However, so far, this classification has been rarely used in neurocritical care. The brain is a highly aerobic organ that requires a sufficient supply of $O_2$to the mitochondria to maintain adequate ATP production. The supply of O_2 to the brain is multifactorial and depends on CBF, the ability of the blood to transport O_2, Hb oxygen affinity, Hb characteristics, O_2 diffusive conductance from arterial capillaries to the cells, and the arterial O_2 pressure gradient between the capillaries and the intracellular compartment.[143] It is thus obvious that the potential causes of hypoxia are multiple and not limited to ischemia (i.e., ischemic hypoxia), a term that should be reserved to describe brain hypoxia caused by a reduction in CBF uncoupled to brain metabolism. In a recent brain MD study conducted by Nelson et al.[43] on TBI patients, highly impaired energy metabolism was very prevalent. However, the relationships between MD and either intracranial

pressure and/or cerebral perfusion pressure were very weak and did not explain the observed energetic disturbances. These findings suggest that other factors besides pressure and/or flow may be the main cause of metabolic perturbations in these patients.[43]

The discussion about lactate has been plagued by the same problems as that of hypoxia. For many years, the primary causes of lactate production by any tissue were thought to be either low levels of blood flow (ischemia) or low levels of blood O_2 content (hypoxemia). However, this oversimplification is misleading. In the Siggaard-Andersen classification, nine types of tissue hypoxia were described. If "hypermetabolic hypoxia" is excluded and the uncoupling hypoxia is merged with histotoxic hypoxia (i.e., mitochondrial dysfunction), seven profiles remain that are very useful as a theoretical framework (see **Table 1**).[20,24] In the original Siggaard-Andersen classification, histotoxic hypoxia is a term that is equivalent to mitochondrial dysfunction and reflects the situation in which O_2 delivery is sufficient but the ETC cannot utilize it. This situation stimulates glycolysis but without a decrease in pyruvate. There is increasing experimental and indirect clinical evidence suggesting that severe TBI may be associated with mitochondrial dysfunction. In the presence of adequate oxygenation, the only available BMs that indicate mitochondrial dysfunction are lactate and the LPR. In the last decade, different groups have provided important experimental data that point to mitochondria as a cause of many metabolic disturbances and therefore the potential therapeutic target.[144,145]

3.5. Brain glucose thresholds

Reinstrup et al.[49] established the traditional clinical upper threshold for MD brain glucose at 3.5 mmol/L. This upper limit was similar to the values found in our awake and anesthetized patients at the same perfusion rate. This threshold corresponds to a true ECF glucose of ≈4 mmol/L, similar to the upper reference limit calculated for $[Glu]_{brain}$ in our anesthetized patients (**Table 10**). This cut-off agrees with the upper limit determined in awake epileptic patients (3.1 mmol/L)[110] and with the upper threshold in the CSF determined for adults.[115] Therefore, taking together all these studies, it can be safely assumed that in patients under general anesthesia or heavy

sedation, an upper limit of 3.5 mmol/L is appropriate when using the standard flow rate of 0.3 μL/min. Our lower $[Glu]_{brain}$ was 0.43 mmol/L in the absence of hypoglycemia and with normal or moderately increased glucose plasma levels, a threshold significantly lower than the lower limit reported for the CSF (2.8 mmol/L).[115] Based on our data, $[Glu]_{brain}$< 0.43 mmol/L should be indicative of substrate limitation or ischemia, which reinforces the reference limits recommended by the 2014 cerebral microdialysis consensus statement.[97] Our data also supports the findings of Abi-Saab et al., who showed that $[Glu]_{brain}$ levels are substantially lower than $[Glu]_{plasma}$.[110]

3.6. The anaerobic threshold revisited

The best available estimate for upper brain lactate levels derives from studies of CSF and from a few studies of patients operated on in neurosurgical procedures in whom brain MD were monitored under general anesthesia and using different anesthetic management techniques.[146] These studies and others —conducted in severe TBI and patients with spontaneous SAH— found that certain lactate thresholds were related to poor clinical outcomes, and the upper limit for brain lactate most widely used in neurocritical patients is extremely variable (range: 1.50 to 5.10 mmol/L).[50–52,110,111]

The 2014 cerebral microdialysis consensus statement[97] recommended 4 mmol/L as the upper reference limit based on the Reinstrup et al. study and three additional studies conducted in patients with spontaneous SAH (i.e., comatose or requiring external ventricular drainage) or severe TBI.[50,51,123] Most papers in the last decade that patients with TBI and aneurysmatic SAH used an upper threshold of 4.0 mmol/L, a threshold that Timofeev et al. showed was useful in predicting poor outcomes in TBI patients.[51] Indeed, Oddo et al. used a threshold of 4 mmol/L, based on findings in patients with severe TBI and a poor outcome.[50]

When we were conducting the first study presented in this thesis, we believed that such a high threshold (4 mmol/L) was difficult to justify, especially when much lower thresholds are used in blood or other organs.[147,148] We decided to use an upper threshold of 2.5 mmol/L for lactate corresponding to a real ECF lactate of around 3.7

mmol/L according to lactate's relative recovery of 67% calculated by Hutchinson et al. using the same methodology as our study.[109] For the LPR, we selected the threshold of 25 used in most studies because this ratio is robust to changes in fluid recovery.[22,50–52,114,123]

Posterior data obtained in the RI study suggest that the upper lactate reference limit in anesthetized patients, when used independently of the LPR, should be reduced from the recommended 4 mmol/L to ≈3.5 mmol/L when using a fixed perfusion rate of 0.3 μL/min. This corresponds to a true ECF lactate of ≈5 mmol/L, as shown in our cohort by using the ZFM. However, values above 3.0 mmol/L were unusual (13%) and most patients had an ECF lactate ≤ 3 mmol/L (**Figure 12**). Differences in the depth of anesthesia or anesthetic agents used might account for these differences. In awake patients, the upper lactate level should be raised significantly such that an upper level of 5.5 mmol/L falls within the normal range. This recommendation is consistent with prior findings where a true ECF upper lactate limit of 5.7 mmol/L was observed in the non-epileptic cortex and hippocampus of 38 awake epileptic patients during the interictal period.[112] Also, other studies suggest that lactate increases in the brain during physiological stimulation, due to a transient boost of glycolysis that occurs as a normal response to physiologic stimulation.[149]

A second relevant finding in our study was that the $[Lac]_{brain}$ is usually higher than expected in both the awake and anesthetized patient. Our findings support the 1994 astrocyte–neuron lactate model suggested by Pellegrin and Magistretti,[39] which suggests that lactate is an effective fuel for the brain. Therefore, lactate formed within the brain parenchyma from glycolysis in astrocytes may be used by activated neurons that take lactate from the ECF by MCT-2.[39] Our data support the notion that an increase in lactate by itself does not indicate ischemia if the LPR is below 35 and may only indicate active aerobic metabolism with increased glycolysis.

3.7. Thresholds for pyruvate and the lactate-pyruvate ratio

Increases in lactate can be indicative of either hyperglycolysis or hypoxia/ischemia. In patients with spontaneous SAH, Oddo et al. found that brain lactate elevations (> 4

mmol/L) were more often caused by cerebral hyperglycolysis than by brain hypoxia, and that hypoxic lactate was associated with increased mortality whereas hyperglycolysis was a predictor of good outcomes.[50] LPR is generally thought to be a more reliable indicator of anaerobic metabolism than lactate alone, and furthermore is unaffected by changes in the probes' recovery. When O_2 is present, pyruvate is converted into acetyl-CoA in order to enter the citric acid cycle. At 0.3 μL/min we found a lower pyruvate limit of 39 μmol/L in anesthetized patients, corresponding to a true $[Pyr]_{brain}$ of ≈55 μmol/L. This value was similar to the lower limit obtained by Reinstrup et al.[49]

Our upper limit for the LPR was 34.2 at a perfusion rate of 0.3 μL/min, and it was 39.2 when using the ZFM data (**Table 10**); this corresponds to a LPR that is above 35-40, as suggested by the 2014 microdialysis consensus conference.[97] Therefore, the limit of 25 we used in the study of lactate and LPR in TBI is likely too low to indicate ischemic or non-ischemic brain hypoxia or mitochondrial dysfunction. As additional data are not available, a pragmatic upper limit for the LPR in both awake and anesthetized patients appears to be 35, and this threshold should be used in future classifications of the metabolic disturbances in neurocritical patients.

3.8. Glycerol threshold for prediction of tissue viability

Glycerol is an end product of phospholipids degradation. Some studies have used it as a biomarker for cell membrane deterioration (and therefore cell destruction) in TBI injury and other acute brain injuries.[150,151] In a small cohort of TBI patients, Peerdeman et al. found that values of $[Gly]_{brain}$> 150 μmol/L in the normal-appearing regions of the brain had a positive predictive value of 100% for an unfavorable outcome.[150] In a previous paper where we studied the ionic profile of normal and injured brains, we found a significant increase in $[Gly]_{brain}$ in both the ischemic and traumatic core, but the $[Gly]_{brain}$ levels were always below the upper reference threshold in both the normal-appearing brain and the traumatic penumbra.[99] Our findings indicated that a $[Gly]_{brain}$< 209 μmol/L is a good predictor for tissue viability, with high sensitivity (99.4%) but modest specificity (52.0%). In our study, the upper reference limit for $[Gly]_{brain}$ at a perfusion rate of 0.3 μL/min was 203 μmol/L,

corresponding to a $[Gly]_{brain}$ of 227 µmol/L. In awake patients, two cases had $[Gly]_{brain}$ above 300 µmol/L without any abnormalities in the control CT scan around the tip of the MD probe.

3.9. The effects of anesthesia on brain metabolism

In the RIs study, we obtained energy metabolites values in the same patients under two conditions: while anesthetized and while fully awake. We consistently found that upper reference limits were significantly higher in awake patients for pyruvate, lactate, and the LGR (**Figure 12**), but remained unchanged for glucose, the LPR, and glycerol. Although our data need further verification in larger cohorts, they suggest that anesthesia depresses the glycolytic pathway and that brain lactate levels increase when the patient is awake. However, increased lactate was never accompanied by an increase in the LPR, indicating a normal redox status.

In humans, PET studies have shown that both isoflurane and propofol may reduce whole brain glucose metabolism by ≈50%.[152–154] Propofol has shown neuroprotective effects in experimental models of focal and global ischemia.[154] Volatile anesthetics also reduce CMR and energy consumption, as well as protect the brain against short-term ischemia. Similar neuroprotective effects have been shown for isoflurane, sevoflurane, or desflurane.[155] Similarly to propofol, volatile anesthetics induce a strong concentration-dependent suppression of electroencephalographic (EEG) activity and therefore of the brain's functional metabolism. The previous aspects explain an increase of tissue glucose concentration observed during anesthesia in animals.[156]

Following the lactate-shuttle hypothesis,[39] a drug-induced reduction in synaptic activity during anesthesia may cause a reduction in aerobic glycolysis and therefore in $[Lac]_{brain}$. Our data suggest that reference limits for neurocritical patients–in whom both propofol and midazolam are widely used—should be identical to those obtained in patients under general anesthesia.

Recent experimental models in rats showed that brain lactate was five-fold higher for isoflurane compared with propofol anesthesia and that this increase was independent

of blood lactate levels.[134,157] Horn and Klein concluded that volatile anesthetics like isoflurane, halothane and sevoflurane (but not intravenous anesthetics) caused a specific, dose-dependent rise in extracellular lactate and pyruvate levels in mice brains.[134] There is a lack of consistency between data obtained in human and rat studies and also in studies conducted with the same anesthetic agent in the same species.[152] Alkire et al. described the variability in the cortical and subcortical metabolic effect of propofol and isoflurane. They showed a significantly more depressed relative cortical metabolism during propofol anesthesia than during isoflurane anesthesia but a higher relative subcortical metabolism during propofol anesthesia in comparison with isoflurane anesthesia.[152] We obtained data during anesthesia in 14 patients (9 anesthetized with inhalation anesthesia [desflurane] and 5 with continuous infusion of propofol [TIVA]) (see **Table 9**), and no statistically significant difference in lactate or glucose ECF levels between both groups was observed (data not shown). Our data suggest that the changes in the metabolomic profile shown with volatile anesthetics can be species-specific and must be verified in the human brain before any conclusion can be reached.

3.10. Variability in reference ranges

All metabolites in our RIs study presented wide reference ranges, which demonstrate the variability in metabolite levels between different subjects and under different conditions (under general anesthesia or awake). Our data highlight that no single metabolic value can be interpreted outside the context of the patient. The importance of monitoring systemic parameters in parallel to brain MD must be emphasized. $[Glu]_{brain}$ can be influenced by the local metabolic rate, the local blood flow, and the systemic glucose level.[49] In patients with SAH, Schlenk et al.[158] showed that hyperglycemic episodes were not reflected in the levels of $[Glu]_{brain}$ but episodes of $[Glu]_{brain} < 0.6$ mmol/l induced metabolic derangements in the brain and a significant increase in $[Gly]_{brain}$ and in the LPR. In the case of lactate, we have reported on the misleading interpretation of an isolated increase in $[Lac]_{brain}$ and the low level of agreement between $[Lac]_{brain}$ and the LPR in indicating brain metabolic dysfunction. In monitoring brain metabolism, integrating all data is essential and, in the case of

ischemia, the use of multiparametric monitoring may help. The use of a regional method for monitoring CBF or $PtiO_2$ close to the MD probe may aid interpretation of the absolute and/or the observed values. This is crucial for attributing any metabolic abnormality to either ischemic or non-ischemic episodes by considering both the $PtiO_2$ values and brain glucose levels. A reduced $PtiO_2$ and/or reduced $[Glu]_{brain}$, the latter in the absence of hypoglycemia, is a clear indication of ischemia.

4. LIMITATIONS OF THE STUDIES AND FUTURE DIRECTIONS

4.1. Reference interval estimation

RI estimation methodology has significantly evolved over the past few decades. Once RIs are established, the immediate clinical consequence is that any value outside this range is flagged.[159] Although our cohort was accurately selected, our samples were not representative of healthy individuals, which limits the generalizability of our findings. In addition, NCCLS guidelines recommend the use of nonparametric RIs and that sample sizes consist of at least 120 values.[159] For obvious reasons, to include 120 patients in a single-center study is difficult and therefore a multicenter study is needed.

The ZFM described by Jacobson in 1985 relies on the fact that the brain concentration stays constant during the study.[102] This assumption could be challenged in our study, as the median experiment time in our cohort was 6.5 hrs. However, we believe that, under general anesthesia and when surgery was uneventful and with no intraoperative adverse events, the margin of error introduced by the length of the experiment is acceptable and that the randomization of the perfusion rates that we performed reduced the risk of bias.

The differences in the depth of anesthesia or the different anesthetic agents used might account for some of the differences among studies. The reliability of future studies could be significantly improved if a measure of brain activity suppression (EEG or bispectral index) was included in routine monitoring.

4.2. *In vitro* experiments conditions

In our *in vitro* experiments, we did not use stirring because it produced significant and unpredictable changes in volume recovery, as explained in the *in vitro* experiments section. However, in non-agitated solutions, changes in solute concentration can occur at the surface of any porous semipermeable membrane, as long as the membrane shows different permeability for the various components of the solution. This phenomenon is known as polarization by concentration and its immediate consequence

is that concentrations at the membrane surface are not the same as in the bulk fluid (matrix), and can produce overestimates or underestimates of the true concentration.[160] Due to the polarization by concentration phenomenon, when a semipermeable membrane is put in a matrix containing a solute with low permeability (i.e., albumin), solutes are transferred from the concentrated solution to the membrane but do not cross the membrane. However, the solvent crosses the membrane and the final effect is increased solute concentration in the boundary layer of the membrane wall.[161] The effect of this layer reduces the mass transfer process, since the concentration in this layer is always greater than in the matrix.[160,161] The reverse situation occurs when the membrane is completely permeable to the solute of interest; the solute concentration around the boundary layer has a lower concentration than in the bulk of the solution. In our *in vitro* experiments, we cannot disregard the possibility that this phenomenon was the cause of underestimating the true concentrations in the matrices. Therefore, we believe this may affect all concentrations and the true *in vitro* concentrations calculated by the ZFM. However, the main goal of these experiments was to replicate Jacobson's finding and to show that this method can be applied *in vivo*. This phenomenon could also justify the approximately 5% difference in RR found for all metabolites in our *in vitro* studies and the statistically non-significant underestimation shown in **Table 8** between the estimated *in vitro* concentrations and the real concentrations in the matrices (except for glycerol).

4.3. Tissue response to probe implantation

An additional limitation of the Jacobson method, when used *in vivo*, is that it inherently assumes that the unperturbed tissue next to the probe is representative of tissue far from the probe. However, it has been shown that the physical insertion of any probe into the brain causes a local injury that initiates a progressive inflammatory tissue response that alters the microenvironment and the function of the tissue from which metabolites are sampled.[100,162] Kozai et al. published a comprehensive review of the acute and more chronic changes induced by inserting any probe into the brain. In the acute phase, blood-brain barrier disruption occurred through unavoidable damage to the capillaries. An immediate response is microglial activation in the 130 μm around

the probe, which produces a thin cellular sheath of microglial cells surrounds the probe and therefore limits solute exchange.[162] This response is the rationale for the long-term phenomenon of biofouling that limits solute exchange after a few days. Although data obtained from any MD probe needs to take this into consideration, this approach does bring us closer to exploring the healthy brain *in vivo* and to improving our understanding of the pathophysiology of acute brain injuries.

In addition, our studies during surgeries of posterior fossa lesions were limited in time and therefore only the more acute changes induced by inserting a probe need to be taken into consideration. We excluded all patients with any CT scan abnormality after insertion and therefore we minimized the odds of obvious bleeding or edema around the probe.

4.4. Microdialysis temporal resolution

MD has—with its present methodology— some intrinsic and unavoidable limitations. Probably the most important one is that it only provides an estimate of the time-averaged metabolic profile of the time—30–60 minutes— before the microvials are collected. Therefore, MD is quite insensitive to rapid changes in metabolite levels. However, trends are still very useful as a method for identifying brain tissue at risk for patients in neurocritical care, detecting changes in metabolic profiles, and detecting ischemia. The future of the technique is real-time online techniques already introduced into clinical research. Rogers et al. reported on the use of continuous online MD by using microfluidic sensors to study glucose, lactate and potassium in cortical spreading depolarization.[163,164] Ideally, when commercially available and approved for human use, continuous MD might improve its temporal resolution and therefore will be able to detect metabolic changes that are short in duration and thus missed with conventional MD.

4.5. Microdialysis data processing and interpretation

Because the main goal of our first study was to evaluate the frequency and agreement between lactate and the LPR, hourly data were pooled from the entire cohort of patients. Therefore, we cannot analyze the influence of the metabolic profile in the clinical evolution and short- or long-term patient's outcome and caution needs to be exercised in the interpretation of our findings. However, in the study of Sanchez et al.[61] conducted in 12 patients with severe TBI, an increase in the ECF levels of lactate above a threshold of 4.0 mmol/L and a LPR above 25 was related to poor neurological outcomes.[61] This relationship has also been shown previously by others.[165] Belli et al. showed that high LPR (> 25) in TBI patients could predict the development of high ICP within the next 3 hours in 89% of cases.[166] Unfortunately, only the LPR (not lactate levels) was reported in this study.

TBI patients may show different metabolic patterns at different time points after injury. This fact was not explored in our study, nor was the coexistence of different metabolic patterns in the same patient. This would have made the analysis extremely complex and flawed because we had not anticipated conducting a multivariant analysis. An additional limitation is that our study did not consider the potential causes of specific profiles, nor we did correlate them with the more common variables that are routinely monitored in TBI patients (ICP, CPP, brain oxygenation, etc.).

In addition, our study in TBI patients focused on the metabolic disturbances found in brain tissue that appeared normal. Therefore, although this sample is probably representative of most of the non-injured brain, further studies need to be conducted to clarify whether brain metabolism in such areas is representative of the energetic metabolism in the whole brain or at least of the hemisphere where the probe is implanted.

We believe one of the next steps in neurocritical care is to establish a clear classification of the metabolic disturbances found in patients who suffer acute brain damage and to clarify its pathophysiology. For an in-depth analysis of the different metabolic patterns discussed in the present thesis, ICP, CPP, ctHb, brain $PtiO_2$ and

other hemodynamic variables should be included. This is especially relevant for differentiating hypoxic-induced metabolic crisis from aerobic hyperglycolysis or mitochondrial dysfunction, in which O_2 limitation is not the cause. A better characterization of the metabolic response of the brain to injury and its pathophysiology is an essential step for implementing better and more targeted therapeutic strategies for managing patients with acute brain damage.

CONCLUSIONS

1. Metabolic abnormalities, defined by elevated lactate and lactate-to-pyruvate ratio (LPR), are frequent in the macroscopically normal brain of patients in the acute phase of TBI. These disorders by their nature are clinically silent until their late stages, at which point patients may suffer significant irreversible brain damage; therefore, early detection is important.

2. A very poor agreement between lactate and the LPR is found when classifying normal or abnormal metabolism at different thresholds used in bedside neurocritical care. The concentration of lactate in MD should always be interpreted while taking into consideration the LPR to distinguish between anaerobic metabolism and aerobic hyperglycolysis. A revision of the traditional concepts regarding conventional anaerobic thresholds should be reconsidered.

3. Both lactate and the LPR can be used at the bedside to classify the metabolic profiles in four patterns that can be useful for further exploring their characteristics, causes and prognostic values. Its definition and better characterization also provides an opportunity to modulate the causes and potentially influence the outcome of patients with acute brain injuries.

4. *In vitro* use of the extrapolation to zero-flow rate method (ZFM) allows to estimate the actual metabolite concentrations in a known matrix by a nonlinear regression model. Therefore, this methodology can be applied *in vivo* to determine the real ECF concentrations in the normal brain.

5. *In vivo* relative recovery (RR) of metabolites can be estimated with the values extrapolated with the ZFM. The RR values obtained are consistent with those described previously in the literature.

6. Our findings review the normal thresholds routinely used at the bedside and define reference levels to detect pathological brain metabolite levels in the neurocritical patient. Accurate reference intervals (RIs) can be used to guide clinical interventions based on cerebral microdialysis.

7. The RIs for brain metabolites reported present wide reference ranges that demonstrate the variability in metabolite levels between different subjects and under different conditions (under general anesthesia or awake). For this reason, no single metabolic value can be interpreted outside the context of the patient. The RIs reported raises the need to reconsider traditional definitions of brain metabolic disturbances.

8. Although our data need further verification in larger cohorts, the statistically significant differences in lactate and pyruvate levels between the anesthetized and the awake patient suggest that anesthesia depresses the glycolytic pathway. This finding emphasizes the importance of using different thresholds for awake patients and patients under anesthesia or deep sedation.

9. The use of other monitoring variables such as ICP, CPP, brain $PtiO_2$ and other hemodynamic variables can be helpful to establish a clear classification of the metabolic disturbances found in patients who suffer acute brain damage and to clarify its pathophysiology. It is an essential step for implementing better and more targeted therapeutic strategies for managing patients with acute brain damage.

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ANNEX

In this section, the results of an extension of the "Normal range for brain metabolites" study are presented. We included more patients with the aim to obtain more robust RIs for brain metabolites. Also, we wanted to study the differences in metabolite levels between anesthetized and awake patients and the influence of the type of anesthesia in these differences.

On the other hand, we conducted a study to describe the effects on energy metabolites after 4h of normobaric hyperoxia (NBO) in 34 patients with TBI. The metabolic results obtained have been included as a part of a more complete study that has been submitted to an international journal.

1. NORMAL RANGE FOR BRAIN METABOLITES STUDY EXTENSION

Additional patients were included in the study of RIs after the article was submitted for publication; information regarding these patients were included and discussed in the present doctoral thesis. The aim of extending the inclusion period was to increase the number of patients to obtain more robust results. Moreover, we wanted to include more patients with continuous infusion of propofol (TIVA anesthesia) in order to study the variability in metabolism related with the type of anesthesia applied, as it has been described in the literature.[1–3] In the published study, we obtained data during anesthesia in 14 patients (9 anesthetized with inhalation anesthesia and 5 with TIVA), and no statistically significant difference in metabolites between both groups was observed. The goal of the present extension was to include more patients with TIVA anesthesia to obtain a balanced number of patients in both groups of anesthesia and to study the metabolite levels differences between them.

1.1. Study group

Eight new patients were included in the study, and 3 of these patients were excluded from the analysis. In one case, the MD probe was implanted next to a hematoma that resulted from the surgical procedure. In the second case, the MD probe was implanted in a brain groove, and the volumetric recovery was not correct.

Table 1. Demographic and clinical characteristics of the patients included in the complete study

Case No.	Age (yrs)/ Sex	Diagnosis of pathology	Evans index	Duration of microdialysis on surgery (h)	Duration of microdialysis on awakeness (h)	Anesthesia
1	38/F	Cerebellar tonsil lesion	0.28	4.67	7.42	Inhalation
2	32/F	Ependymoma	0.28	8.00	11.90	TIVA
3	61/F	Atypical fibrous meningioma	0.33	6.83	NA	Inhalation
4	21/F	Hemangioblastoma	0.29	3.30	5.17	Inhalation
5	43/F	Chiari malformation	0.27	4.93	13.17	Inhalation
6	22/M	Classic medulloblastoma	0.27	7.72	7.42	Inhalation
7	34/F	Cerebellar metastasis	0.24	10.83	5.08	Inhalation
8	60/F	Vestibular Schwannoma	0.30	6.53	15.83	Inhalation
9	65/M	Cerebellar metastasis	0.31	5.00	5.33	Inhalation
10	65/F	Hemangioblastoma	0.28	5.00	9.33	Inhalation
11	66/M	Cerebellar metastasis	0.28	8.00	NA	Inhalation
12	55/F	Anaplasic glioma	0.30	5.83	7.67	Inhalation
13	42/M	Transitional meningioma	0.24	6.83	39.00	Inhalation
14	31/M	Vestibular schwannoma	0.21	6.50	14.00	Inhalation
15	69/F	Mixed meningioma	0.27	6.25	10.00	TIVA
16	32/F	Central neurocytoma	0.30	5.08	13.42	TIVA
17	65/F	Low grade glioma	0.20	7.10	14.00	TIVA
18	66/M	Cerebellar metastasis	0.29	7.17	13.17	TIVA
19	44/M	Epidermoid cyst	0.30	7.17	12.67	TIVA
20*	40/F	Tentorial meningioma	0.26	8.75	14.92	TIVA
21*	50/F	Vestibular Schwannoma	0.29	6.75	13.72	TIVA
22*	48/F	Tentorial meningioma	0.23	7.25	10.67	Inhalation
23*	26/F	Chiari malformation	0.31	5.50	14.50	TIVA
24*	28/M	Epidermoid cyst	0.25	5.33	13.80	TIVA
Median	45/F		0.28	6.64	12.92	

M: male; F: female; TIVA: total intravenous anesthesia; NA: not applicable.
* New patients included in the study extension.

Finally, the third patient had had an endoscopic ventriculostomy in the months prior to the surgery, and the MD probe was implanted in the area manipulated during this procedure, so we could not consider it as normal brain tissue.

Together with the patients of the published study, a total of 24 patients including 8 males and 16 females with a median age 45 (range: 21-69 years) and a median Evans index of 0.28 (min: 0.20, max: 0.33) were included in the new analysis. **Table 1** shows a summary of the demographic and clinical data of all of the patients.

1.2. Results

We obtained valid intraoperative data to apply the extrapolation to zero-flow rate method in 21 patients. We recalculated the fixed *k* value for each metabolite by merging the data for all patients. With the new *k* values, we obtained the value extrapolated to zero flow for each metabolite and each patient. Metabolite concentrations at 0.3 μL/min in the anesthetized patient were calculated using samples recovered at the mentioned flow rate during the ZFM *in vivo*. Of the 24 patients included, data from 20 patients were included in the analysis. Metabolite concentrations at 0.3 μL/min from awake patients were obtained for a total of 22 patients. Data for the analyzed metabolites calculated by the ZFM and those measured at a flow rate of 0.3 μL/min in anesthetized and awake patients are summarized in **Table 2**.

Levels at a perfusion flow rate of 0.3 μL/min were significantly higher for lactate, pyruvate, glycerol and LGR when the patient was fully awake as compared to the values obtained when the patient was under anesthesia. We did not find statistically significant differences between glucose and LPR levels of awake versus anesthetized patients (**Table 2**).

From the 24 patients included, we obtained data in the same patient during anesthesia and when the patient was fully awake in 19 cases (10 anesthetized with inhalation anesthesia and 9 with TIVA). **Table 3** summarizes values obtained for each metabolite depending on the type of sedation. Glycerol levels obtained in patients with inhalation

anesthesia were significantly lower than those in patients anesthetized with TIVA (Wilcoxon test, p=0.02) (**Figure 1 and Table 3**). The rest of metabolites did not show statistically significant differences between both types of anesthesia (**Table 3**).

Table 3. Brain tissue concentrations of metabolites depending on the type of sedation

Metabolites	**TIVA** (n = 9)	**Inhalation** (n = 10)	**p-value***
Glucose (mmol/L)	**1.15** (0.51-1.98)	**1.22** (0.74-3.53)	0.41
Lactate (mmol/L)	**1.51** (0.62-3.84)	**1.36** (1.10-3.23)	0.74
Pyruvate (µmol/L)	**62.8** (33.6-114)	**69.5** (36.6-150)	0.84
Glycerol (µmol/L)	**84.8** (41.2-205)	**41.5** (24.4-199)	0.02
LPR	**26.6** (9.83-33.8)	**22.6** (12.8-35.2)	0.97
LGR	**1.42** (0.36-3.11)	**1.32** (0.50-1.55)	0.45

LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio; TIVA: total intravenous anesthesia.
Results are expressed as median (min, max).
*Comparisons between TIVA and Inhalation variables were made using the Wilcoxon Signed Rank Test.

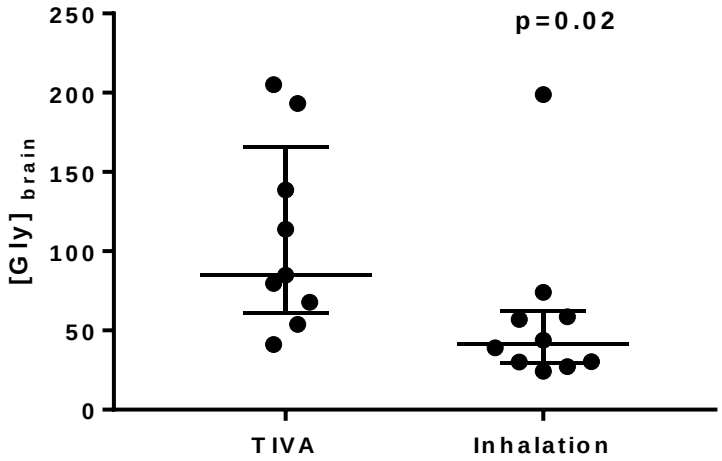

Figure 1. Box plots showing the concentrations of glycerol ([Gly]brain) obtained in patients anesthetized with TIVA and with inhalation anesthesia. A summary of the data is shown in **Table 3**. TIVA values were compared with Inhalation values by the Wilcoxon signed-rank test. Glycerol values were significantly lower in patients under inhalation anesthesia (p=0.02).TIVA: total intravenous anesthesia.

Table 2. Brain tissue concentrations of metabolites

Condition	Glucose (mmol/L)		Lactate (mmol/L)		Pyruvate (µmol/L)		Glycerol (µmol/L)		LPR		LGR	
	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*	Median (min-max)	RI*
ZFM (n = 21)	**1.96** (0.85-5.72)	1.10-4.78	**2.08** (0.79-5.92)	0.98-5.78	**94.7** (47.9-306.8)	55.3-258.1	**61.6** (22.2-234.7)	24.7-232.9	**23.1** (9.0-33.6)	10.9-33.3	**1.03** (0.36-3.13)	0.39-2.98
Anesthetized (0.3µL/min) (n = 20)	**1.22** (0.51-3.53)	0.57-2.96	**1.42** (0.62-3.84)	0.75-3.55	**64.0** (33.2-149.7)	35.0-132.6	**57.8** (24.4-205.1)	25.7-202.2	**23.9** (9.8-35.2)	11.3-34.9	**1.31** (0.36-3.11)	0.43-2.95
Awake (0.3µL/min) (n = 22)	**1.62** (0.29-3.01)	0.48-2.92	**3.18** (1.32-5.62)	1.45-5.46	**135.5** (55.3-198.1)	70.9-198.1	**89.6** (29.3-346.4)	32.5-336.2	**23.6** (16.9-35.1)	17.2-33.0	**2.14** (0.62-15.0)	0.66-10.2
p-value*	0.25		<0.0001		<0.00001		0.04		0.83		0.005	

ZFM: zero-flow method; RI: reference interval; LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio. *2.5% and 97.5% percentile values, as lower and upper reference interval limits, respectively.
*Comparisons between Anesthetized and Awake variables were made using the Wilcoxon Signed Rank Test.

1.3. Discussion

The RIs obtained were similar to those published in the previously presented "Normal range for brain metabolites" study. Therefore, the thresholds defined in the present doctoral thesis are valid and consistent with the new results.

The main difference is that glycerol levels were significantly higher in awake patients compared with values in anesthetized patients. Glycerol levels obtained in anesthetized patients with TIVA were similar to the levels in the awake patients. Moreover, glycerol levels in patients with inhalation anesthesia were significantly lower than levels when the patients were awake. For this reason, we could hypothesize that inhalation anesthesia decrease glycerol levels.

Reinstrup et al.[4] found similar results, although the number of patients included was less and the number of patients anesthetized with TIVA and inhalation anesthesia was not balanced (3 and 6, respectively). In this study, glycerol levels tended to be reduced during general anesthesia compared with levels measured before and after surgery.[4]

Catecholamine-induced lipolysis of triglycerides can increase glycerol systemic levels after surgery, producing a concomitant increase in brain levels.[4] In our study, this factor could be ruled out because glycerol levels in patients anesthetized with TIVA were similar to those in awake patients. Moreover, adipose tissue levels of glycerol during surgery were not statistically different between both types of anesthesia (data not shown).

Besides its role as a biomarker for cell membrane deterioration (end product of phospholipids degradation), glycerol can also be derived from glycolysis. In our initial study, we concluded that anesthesia depresses the glycolytic pathway. This could produce a decrease of glycerol levels during anesthesia, although this factor would not explain the difference observed between both types of anesthesia.

Further studies in humans are needed to verify the changes in the metabolomic profile depending on the type of anesthetics (volatile or intravenous) used.

2. METABOLIC RESPONSE TO NORMOBARIC HYPEROXIA IN TRAUMATIC BRAIN INJURY

2.1. Introduction

Normobaric hyperoxia (NBO) is a common therapeutic maneuver in patients with TBI, but its benefit has not been irrefutably demonstrated. TBI pathology is characterized by secondary injuries caused by ischemia or non-ischemic tissue hypoxia lesions, leading to a metabolic dysfunction in brain.[5] The therapeutic management of these patients is directed to maintain sufficient O_2 delivery during the first hours after injury.

NBO using 100% FiO_2 at 1 ATA is considered an attractive treatment because it can be applied at the bedside of the patient, and it is an easy maneuver. This therapy could be used as a potential treatment to improve brain oxygenation and consequently the metabolic disorders resulting from TBI. Several clinical studies have been completed since the pioneering study published by Menzel et al.[6] in 1999. However, metabolic contradictory results have raised significant controversy regarding this treatment.[6–12]

In a previous study, our group showed that NBO significantly decreased the LPR in TBI patients who had increased brain lactate levels (>3 mmol/L) at baseline, suggesting that the response to NBO could be influenced by the metabolic state at baseline.[11]

Other clinical studies have demonstrated deleterious effects (increased production of ROS and cell death, reduction of antioxidant defenses) and a lack of effectivity of NBO in neurocritical care patients.[13–15] The use of supranormal levels of O_2 may be potentially toxic and worsen outcome in TBI patients. Talley et al.[16] described an increase in the lesion volume after hyperoxia treatment in an animal model of moderate TBI pathology. Increase in oxygenation can lead to cerebral vasoconstriction and the subsequent reduction in cerebral perfusion pressure.

The aim of this study was to demonstrate whether NBO (FiO2 100%, 1 ATA) for a period of 4 hours is an effective therapeutic maneuver in patients with severe TBI to improve brain energetic metabolism.

2.2. Material and methods

2.2.1. Study group

A prospective study was conducted from a cohort of 35 adult patients (aged ≥18 years) who had sustained a moderate or severe T I (GCS≤ 13), had an abnormal CT scan upon admission, and required ICP monitoring. This study was approved by the institutional ethics committee of Vall d'Hebron University Hospital (arcelona, Spain; protocol PR-AG-140/2011) and written informed consent was obtained from the patients' next of kin. Outcome was assessed by an independent evaluator at 6 months after injury using the Glasgow Outcome Scale Extended (GOSE). The scores obtained were dichotomized into two categories: bad outcome (GOSE: 1–4) and good outcome (GOSE: 5–8).

2.2.2. Multimodal monitoring

Continuous ICP monitoring was performed for all patients using a Camino 110-4B intraparenchymatous ICP sensor (Integra Neurosciences, Plainsboro, NJ, USA). The ICP sensor was always implanted in the pre-coronal region in the "worst" hemisphere, which was defined as the hemisphere with the most evident lesion in the initial CT scan. All patients received standard treatment based on the BTF guidelines, which aim to maintain the following therapeutic targets: a CPP above 60 mmHg and an ICP below 20 mmHg.[17]

Regarding brain tissue O_2 monitoring, all included patients were monitored with a Licox® CMP system (Integra Neurocare, Plainsboro, NJ). A CC1.SB sensor (Integra Neurocare) was used in 30 patients, and a CC1.P1 sensor (Integra Neurocare) was used in 4 patients. The brain tissue O_2 probe was implanted close to the MD catheter. Probes were allowed to stabilize for at least 3 hours after insertion before any oxygen challenge was performed. End-hour ICP and $PtiO_2$ readings were recorded either manually by the patients' nurses or by a custom-built system, PowerLab (ADInstruments Ltd, Oxford, United Kingdom), running LabChart software v7.0.3 (ADInstruments Ltd.). Data were exported to a flat file for statistical analysis.

A CMA-71 microdialysis probe (M Dialysis AB) was implanted close to the $PtiO_2$ probe in all patients. Cerebral catheters were perfused with a sterile isotonic CNS fluid containing 147 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 2.7 mmol/L KCl, and 0.85 mmol/L $MgCl_2$ (M Dialysis AB) at a fixed flow rate of 0.3 μL/min using a CMA-106 pump (M Dialysis AB). The dialysate were collected hourly by the nurse in charge of the patient via capped microvials. Lactate, pyruvate, glucose and glycerol were routinely monitored at the bedside using an ISCUS Flex microdialysis analyzer (M Dialysis AB). No clinical decisions were based on MD data except for decisions to screen for potential secondary intra- or extra-cranial insults. The CT scan closest to the time of the hyperoxic challenge was used to determine the position of the MD and $PtiO_2$ probes. The region of the brain sampled by the probes was assigned to one of the following previously described categories (under the "Introduction" section of the present doctoral thesis): apparently normal injured brain (NB), traumatic penumbra (TP) and traumatic core (TC).

2.2.3. Normobaric hyperoxia protocol

Hyperoxic challenge was performed as soon as possible after implanting the MD and $PtiO_2$ probes. All patients received continuous sedation and analgesia by continuous infusion of midazolam and morphine. Patients had to be hemodynamically stable and correctly sedated at the beginning of the hyperoxic test, as previously described.[11] Care was taken to maintain stable CPP, temperature, and partial pressure of arterial carbon dioxide ($PaCO_2$) to avoid confounding factors that could influence the cerebral metabolism. As a first step, we extracted a baseline arterial blood sample to determine the oxygenation status of the patient. If the partial pressure of arterial oxygen (PaO_2) was > 170 mmHg, the ventilator or FiO_2 settings were modified to obtain a basal PaO_2 between 100 and 150 mmHg and a $PaCO_2$ between 35 and 40 mmHg. After these changes, at least 1 hour was allowed to pass before conducting the hyperoxic challenge. Before hyperoxia was induced, systemic variables (systolic and diastolic blood pressure, heart rate), ICP, CPP, brain microdialysates and brain $PtiO_2$ values were recorded. Baseline blood samples were taken, and the ctHb, PaO_2, and $PaCO_2$ were recorded. As a second step, FiO_2 was increased to 100% and maintained at this

level for 4 hours. Arterial blood samples were extracted again 2 hours after starting hyperoxia and at the end of the test, and the same physiological variables mentioned above were recorded. Immediately after completing the NBO challenge, FiO_2 was modified again to maintain a PaO_2 of ≈100 mmHg. In patients with a catheter in the jugular bulb, a jugular venous blood sample was extracted at the same time as the arterial blood sample. The baseline and post-hyperoxia values recorded for both systemic and cerebral parameters correspond to the mean of the 3 hours prior to and after the hyperoxic challenge. The parameters recorded during the hyperoxic challenge are the mean values obtained during the 4 hours of hyperoxia.

2.2.4. Monitoring brain oxygen and hypoxic thresholds

A $PtiO_2$ threshold for hypoxia-ischemia of 15 mmHg is commonly used because Jödicke et al.[18] showed that this value is the most sensitive threshold and has the highest positive predictive value for detecting intraoperative ischemia that causesirreversible brain damage.[18] In a recent work from our group, a $PtiO_2/PaO_2$ ratio less than or equal to 0.10 was defined as athreshold for detecting intraoperative hypoxia and avoiding artifactually high $PtiO_2$ in the presence of high PaO_2 in mechanically ventilated patients.[19] Thus, we considered a hypoxic pattern when $PtiO_2$ was < 15 mmHg or when the $PtiO_2/PaO_2$ ratio was less than 0.10. In the absence of hypoxemia (low PaO_2) and at a constant cerebral metabolic rate of oxygen ($CMRO_2$), ratios below 0.10 indicate covert hypoxia and a deficient delivery of $O_2$to the brain. A $PtiO_2/PaO_2$ ratio above 0.35 indicates a relative hyperoxic status.[19]

2.2.5. Statistical analysis

Descriptive statistics were obtained for each variable. For exploratory data analysis, variables were presented graphically using box-and-whisker plots. For continuous variables, the mean, median, range, and standard deviation were used for normally distributed data, and the median, minimum, and maximum values were used for non-Gaussian distributions. The Shapiro-Wilk test and the inverse probability plot were

used to test whether the data followed a normal distribution. For the sake of clarity, in those cases in which both normal and non-normal data were present in the same table, we used the non-parametric approach to summarize the variables. The percentages and sample sizes were used to summarize the categorical variables. The two-tailed paired t-test was used to compare pairs of variables that followed a normal distribution and the Wilcoxon Signed Rank Test was used for variables that did not follow a normal distribution. Statistical analyses were carried out with R v3.2.0 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) and the integrated development environment R Studio v0.99.902 (RStudio, Inc., Boston, MA, USA; http://www.rstudio.com). Statistical significance was set at $p \leq 0.05$.

To define whether or not NBO changed the metabolic profile, all patients were first analyzed in function of the region monitored and afterwards they were divided into two groups according to baseline brain lactate levels: patients with baseline brain lactate concentrations below or equal to 3.5 mmol/L and patients with brain lactate concentrations above 3.5 mmol/L (protocol described previously) (**Figure 2**).[11] The lactate threshold used in our present study was selected based on the reference range of normality published in the study presented in this thesis.

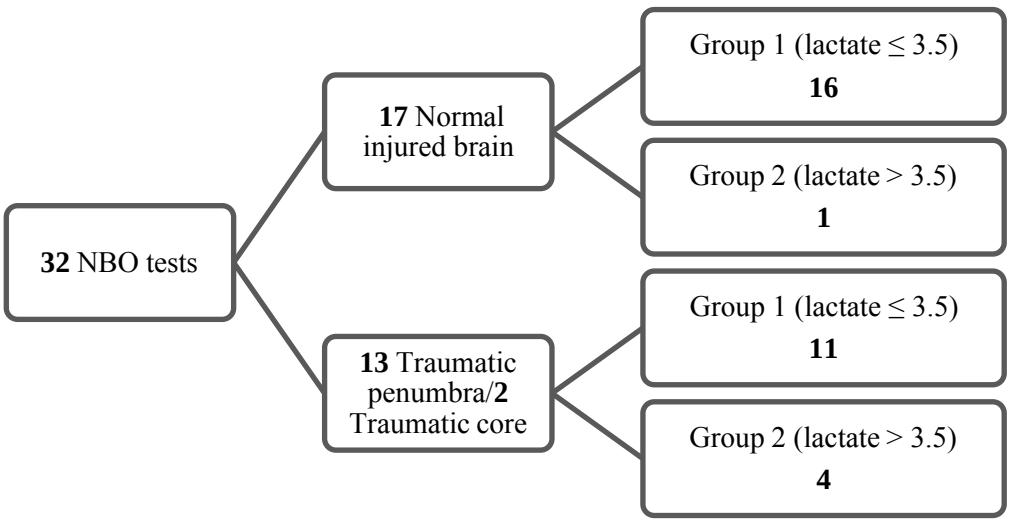

Figure 2. Schematic diagram of group classification. To define whether or not NBO changed the metabolic profile, all patients were analyzed in function of the region monitored and afterwards they were divided into two groups according to baseline brain lactate levels: patients with baseline brain lactate ≤ 3.5 mmol/L and patients with brain lactate > 3.5 mmol/L.

2.3. Results

Thirty-four patients were included in the study and a total of 35 hyperoxic tests were performed. In one patient the test was repeated twice with an interval period of 3 days. Three tests were excluded from analyses: two were excluded because a significant increase in PaO_2 (270.6 and 309.1 mmHg) did not induce any increase in $PtiO_2$, and an additional case was excluded because PaO_2 levels did not increase after the hyperoxic challenge. The remaining 31 patients (32 tests) were included in the analysis. The median time from injury until hyperoxic challenge was 57 hrs (min: 22, max: 96). **Table 4** shows a summary of the demographic and clinical data of the included patients. According to the described criteria, 17 patients had the cerebral MD probe inserted in the NB, 12 patients in the TP, and in 2 patients the probe was inserted in the TC. Patients where the probe was inserted in the TP and TC were grouped together for the analysis (TP/Core group).

2.3.1. Systemic and intracranial changes after the hyperoxic challenge

Summary measures of the systemic and cerebral parameters at baseline and during the hyperoxic challenge are summarized in **Table 5**. Jugular blood gas variables were only available in 15 tests (9 in the NB and 6 in the TP/Core). Five patients (2 in the NB and 3 in the TP/Core) presented with brain hypoxia at baseline ($PtiO_2$< 15 mmHg and/or a $PtiO_2/PaO_2$ ratio < 0.10). As expected and compared to baseline, most O_2-related arterial and venous parameters ($PtiO_2$, PaO_2, SaO_2 and CaO_2) changed significantly in both groups during hyperoxia, as shown in **Table 5**. Some of the increases in $SjvO_2$ and $CjvO_2$ that were observed on both groups were not statistically significant because of the small sample size. Statistically significant—but clinically irrelevant— changes were found in arterial pH in the NB group.

At baseline, 7 patients (23%) presented with poor pulmonary function, defined as PFR ratio ≤ 250. The median PFR at baseline was 317 (min: 165, max: 470) and did not change significantly at the end of NBO (median 320, min: 124, max: 483), indicating that pulmonary function was not affected by 4 hrs of NBO. Median PaO_2 after hyperoxia increased from 115 mmHg to 445 mmHg in the NB group and from 127 mmHg to 435 mmHg in the TP/Core group. This produced a modest but statistically

significant increase in CaO_2 in both groups. Hyperoxia induced a median $\Delta PtiO_2$ of 54.4 mmHg (min: 11.9, max: 180 mmHg) in the NB group and a similar median $\Delta PtiO_2$ of 56.2 mmHg (min: 16.2, max: 179 mmHg) in the TP/Core group (**Figure 3**). Non-statistically significant differences were observed in ICP levels in both groups, and a statistically significant but clinically irrelevant increase in mean arterial blood pressure (MABP) was also observed (**Table 5**).

Table 4. Demographic and clinical characteristics of the patients

Sex	
Man	24 (77%)
Woman	7 (23%)
Age	36 (18-63)
Initial GCS	4 (3-11)
Initial CT scan classification	
Diffuse injury II	10 (32%)
Diffuse injury III	4 (13%)
Diffuse injury IV	0 (0%)
Evacuated mass lesion	10 (32%)
Non-evacuated mass lesion	7 (23%)
Neurological outcome (GOSE)	
Good outcome	8 (26%)
Bad outcome	21 (68%)
Dead at discharge	4 (13%)
Lost to 6 month follow up	2 (6%)
Probe location	
Normal injured brain	17 (55%)
Traumatic penumbra	12 (39%)
Traumatic core	2 (6%)

Sex, initial CT classification, GOSE and probe location are expressed as number of cases and percentage. Age and Initial GCS are expressed as median (min-max). GCS: Glasgow Coma Scale; GOSE: Glasgow Outcome Scale Extended.

Table 5. Intracranial and laboratory parameters before and after the hyperoxic challenge

Variable	Normal Injured Brain (n=17)			Traumatic Penumbra-Core (n=15)		
	Baseline	Hyperoxia	p*	Baseline	Hyperoxia	p*
FiO_2	0.35 (0.24, 0.67)	1 (0.95, 1.00)	0.0003	0.40 (0.30, 0.60)	1.00 (0.90, 1.00)	0.0007
$PtiO_2$ (mmHg)	25.6 (7.5, 40.7)	74.9 (35.7, 218)	<0.0001	24.8 (4.90, 44.3)	79.3 (21.1, 204)	<0.0001
Brain hypoxia (n,%)	2 (12%)	2 (12%)	NS	3 (20%)	2 (13%)	NS
PFR ≤ 250 (n,%)	5 (29%)	5 (29%)	NS	2 (13%)	3 (20%)	NS
HR (mmHg)	81 (49, 109)	77 (51, 102)	NS	71 (47, 121)	69 (46, 109)	0.008
MABP (mmHg)	76 (68, 95)	78 (67, 98)	0.035	80 (69, 103)	84 (67, 105)	0.008
ICP (mmHg)	10 (2, 19)	11 (3, 21)	NS	11 (6, 25)	13 (2, 27)	NS
CPP (mmHg)	68 (58, 83)	69 (53, 92)	NS	69 (57, 72)	71 (57, 91)	0.027
Arterial blood gases						
ctHb (g/dl)	10.1 (7.8, 14.9)	10.1 (7.0, 15.6)	NS	10.2 (8.6, 12)	10.0 (8.6, 11.5)	NS
pH	7.45 (7.34, 7.48)	7.42 (7.31, 7.46)	0.033	7.45 (7.38, 7.50)	7.45 (7.28, 7.49)	NS
$PaCO_2$ (mmHg)	37.2 (31.4, 48.9)	38.5 (33.5, 49.1)	0.036	37.5 (32.1, 42.2)	37.5 (30.5, 56.5)	NS
PaO_2 (mmHg)	115 (81.0, 171)	445 (253, 615)	<0.0001	127 (89.0, 204)	435 (266, 621)	<0.0001
SaO_2 (%)	98.5 (96.2, 99.2)	99.8 (99.1, 99.9)	0.0003	98.9 (97.3, 99.5)	99.7 (99.4, 100.1)	0.001
CaO_2 (mL/100mL)	14 (11, 20)	15 (11, 22)	0.0007	14 (12, 16)	15 (13, 17)	0.002
Jugular blood gases						
$PjvCO_2$ (mmHg)	42.5 (38.1, 46.0)	45.5 (41.5, 50.1)	0.004	43.9 (38.4, 42.4)	45.5 (39.2, 60.0)	NS
$PjvO_2$ (mmHg)	42.4 (34.2, 52.6)	56.0 (40.0, 103.6)	0.004	44.1 (38.8, 56.4)	50.9 (48.2, 84.5)	NS
$SjvO_2$ (%)	80.0 (65.5, 95.8)	90.9 (73.10, 97.6)	0.008	81.0 (76.2, 88.6)	86.9 (81.9, 97.9)	NS
$CjvO_2$ (mL/100mL)	11.5 (8.5, 17.3)	13.2 (9.2, 19.2)	NS	11.3 (10.5, 14.2)	12.7 (10.8, 13.92)	NS
$AVDO_2$ (mL/100mL)	3.17 (0.50, 4.54)	2.74 (1.49, 4.73)	NS	2.37 (1.82, 3.61)	2.92 (1.16, 3.40)	NS

Jugular blood gases variables were determined in 15 tests (n=9, normal injured brain; n=6, traumatic penumbra-traumatic core). Results are expressed as median (min, max). * Comparisons between variables were made using the paired t-test or the Wilcoxon Signed Rank Test depending of the normal or non-normal distribution of the variables. HR: heart rate; MABP: mean arterial blood pressure; ICP: intracranial pressure; CPP: cerebral perfusion pressure; NS: not-significant ($p < 0.05$).

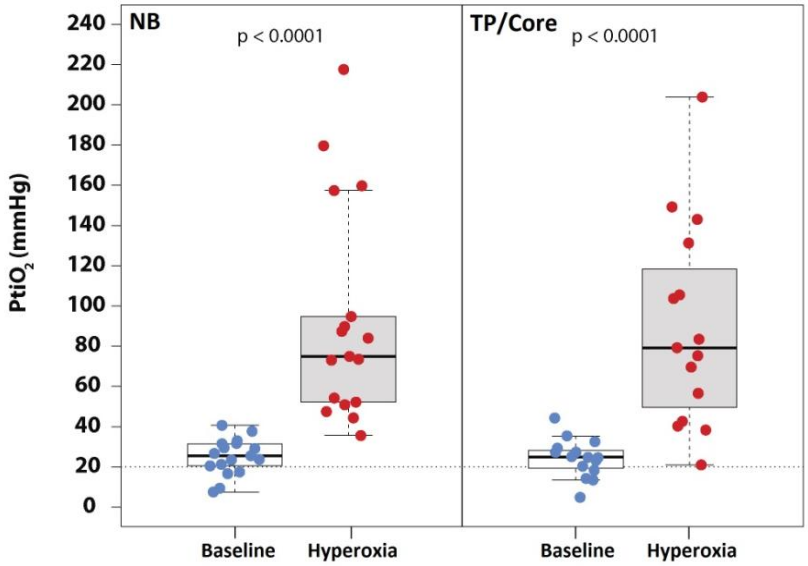

Figure 3. Box-plots showing changes in brain O_2levels at baseline and after hyperoxic challenge in both groups. Median PaO_2 after hyperoxia increased from 115 mmHg to 445 mmHg in the NB group and from 117 mmHg to 435 mmHg in the TP/Core group (see text). This increase in PaO_2 produced a significant increase in $PtiO_2$ in both brain regions classified as NB and TP/Core. The dashed line marks the 20 mmHg threshold.

2.3.2. Metabolic response to hyperoxia in the normal and injured brain

Baseline and hyperoxia values for both groups (NB and TP/Core) and for each metabolite are shown in **Table 6**. In the NB group, NBO induced a statistically significant moderate decrease in $[Lac]_{brain}$ from 1.98 mmol/L (min: 1.06, max: 7.42 mmol/L) at baseline to 1.79 mmol/L (min: 0.90, max: 5.72 mmol/L) at hyperoxia nadir (Wilcoxon test, p=0.0351). A modest reduction in $[Pyr]_{brain}$ was also observed, from 77.8 µmol/L at baseline (min: 55.5, max: 387 µmol/L) to 77.3 µmol/L (min: 45.3, max: 295 µmol/L) during N O treatment (Wilcoxon test, p=0.0305). A statistically non-significant reduction in the LPR was found in the entire cohort. In the TP/Core group, non-statistically significant differences were observed in brain tissue metabolites during hyperoxia. Only five patients in the entire cohort (15.6%) presented with a $[Lac]_{brain}$>3.5 mmol/L before the hyperoxic challenge (group 2, **Figure 2**). In this group, the median baseline $[Lac]_{brain}$ was 6.4 mmol/L (min: 6.1, max: 7.4 mmol/L), with a median LPR of 25.9 (min: 19.2, max: 217). NBO induced a non-statistically significant reduction in both $[Lac]_{brain}$ (median 5.7 mmol/L, min: 4.8, max: 6.4; Wilcoxon test, p= 0.063) and LPR (median 22.7, min: 19.3, max: 243; Wilcoxon test,

p= 0.81). **Figure 4** shows a graphical example of the metabolite and ICP levels changes during and after normobaric hyperoxia in a patient of the group 2.

Table 6. Brain and subcutaneous microdialysis data before and after the hyperoxic challenge

Metabolites	Normal Injured Brain (n=17)			Traumatic Penumbra-Core (n=15)		
	Baseline	Hyperoxia	p*	Baseline	Hyperoxia	p*
Brain tissue						
Glucose (mmol/L)	1.33 (0.29, 3.55)	1.38 (0.27, 3.75)	NS	1.40 (0.11, 4.58)	1.44 (0.16, 5.30)	NS
Lactate (mmol/L)	1.98 (1.06, 7.42)	1.79 (0.90, 5.72)	0.035	2.30 (0.84, 6.64)	2.43 (0.79, 6.37)	NS
Pyruvate (µmol/L)	77.8 (55.5, 387)	77.3 (45.3, 295)	0.031	96.7 (29.9, 273)	82.6 (26.4, 221)	NS
Glycerol (µmol/L)	69.7 (24.2, 378)	63.5 (31.2, 421)	NS	69.1 (18.8, 585)	63.0 (17.7, 549)	NS
LPR	23.5 (9.93, 38.0)	22.3 (10.3, 39.2)	NS	22.4 (11.4, 217)	21.6 (12.8, 243)	NS
LGR	1.43 (0.40, 8.86)	1.44 (0.36, 9.09)	0.031	1.33 (0.50, 58.6)	1.20 (0.54, 49.3)	NS
Subcutaneous tissue						
Glucose (mmol/L)	3.18 (0.94, 9.55)	3.56 (0.50, 10.3)	NS	3.65 (1.07, 7.72)	3.73 (0.90, 7.42)	NS
Lactate (mmol/L)	0.92 (0.27, 2.76)	0.84 (0.25, 2.73)	NS	0.89 (0.21, 3.65)	0.72 (0.24, 3.10)	0.018
Pyruvate (µmol/L)	64.6 (7.98, 231)	60.2 (9.55, 211)	NS	62.1 (11.5, 232)	66.8 (8.43, 162)	NS
Glycerol (µmol/L)	274 (85.0, 1364)	240 (67.5, 1341)	NS	199 (76.1, 535)	231 (88.2, 1184)	NS
LPR	13.4 (9.63, 38.7)	14.8 (9.81, 26.7)	NS	12.0 (9.99, 37.3)	13.0 (8.77, 41.7)	NS
LGR	0.29 (0.16, 0.54)	0.28 (0.15, 0.60)	NS	0.28 (0.13, 0.82)	0.27 (0.14, 0.57)	NS

Results are expressed as median (min, max).* Comparisons between variables were made using the paired t-test or the Wilcoxon Signed Rank Test depending of the normal or non-normal distribution of the variables. LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio. NS: not-significant ($p < 0.05$).

2.4. Discussion

Ischemic and non-ischemic brain hypoxia plays a relevant role in the pathophysiology of the injured brain.[20] Therefore NBO is considered a potential therapy that could be beneficial for TBI patients. In addition, many experimental and clinical studies indicate that metabolic disorders are common in acute brain injuries and that impaired O_2 delivery or O_2 utilization in the mitochondria (i.e., mitochondrial dysfunction) may play a significant role in the acute phase of TBI, ischemic, and hemorrhagic stroke.[21–23] Oxygen diffusion anomalies can alter the O_2 diffusion from the capillaries to the mitochondria.[23,24] Because increased diffusion barriers to O_2 delivery due to brain edema can develop and persist after injury,[23,25] some patients may benefit from interventions that increase O_2 pressure and consequently the pressure drive that moves

O_2 into the brain. Furthermore, a growing body of evidence suggest that mitochondrial dysfunction may play a very important role in the pathophysiology of TBI,[26] and might explain the significant reduction in the $CMRO_2$ found after TBI.[27] Hypothetically, if mitochondrial function is impaired after TBI, then the steeper capillary-cell O_2 gradient induced by hyperoxia may also improve O_2 utilization.

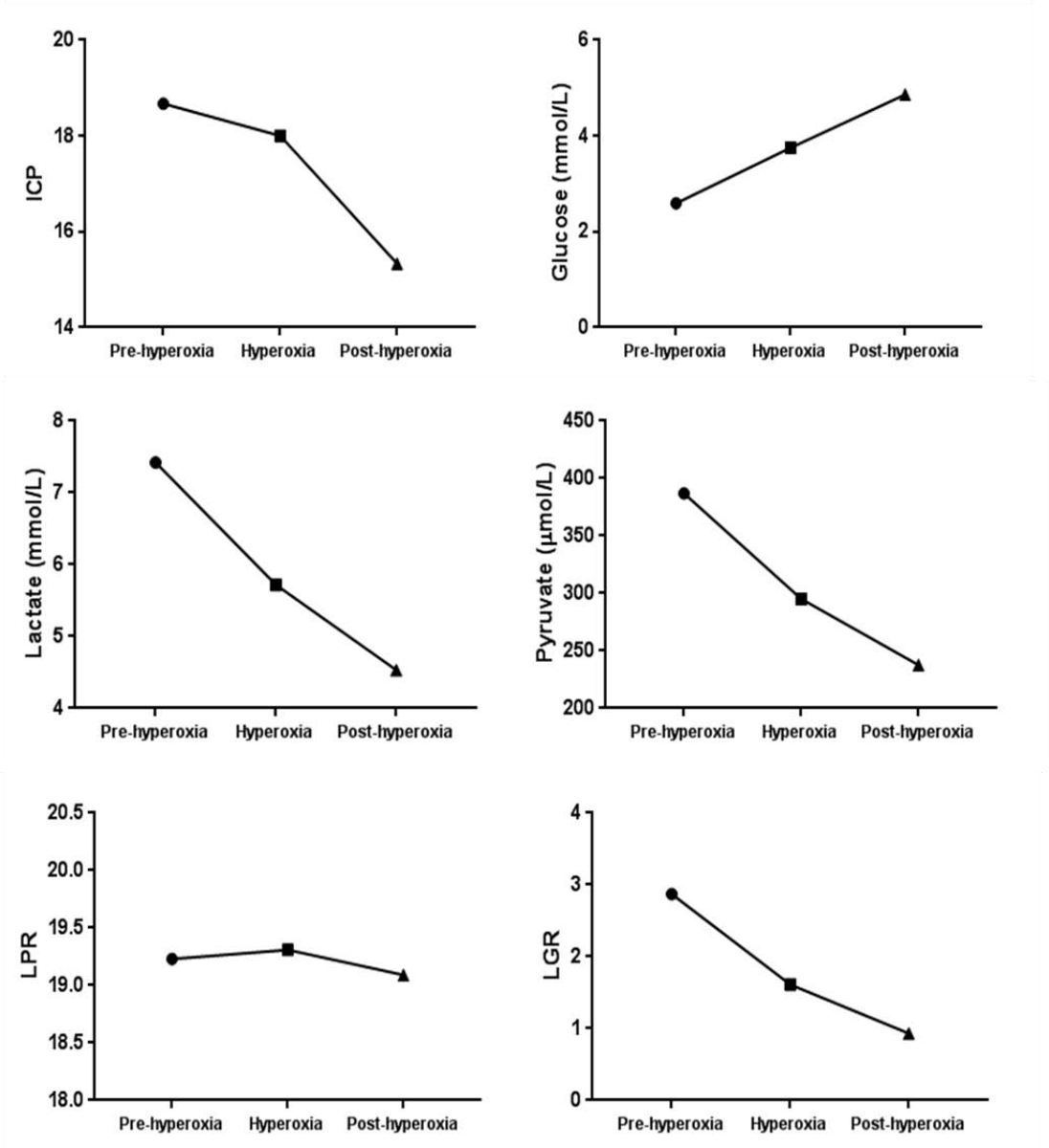

Figure 4. Graphical representation of the metabolic and ICP levels response to normobaric hyperoxia. Data correspond to patient #6, who had the probe implanted in normal injured brain and presented baseline lactate levels >3.5 mmol/L (group 2). ICP: intracranial pressure; LPR: lactate-to-pyruvate ratio; LGR: lactate-to-glucose ratio.

The use of therapeutic O_2 in TBI has focused primarily on hyperbaric hyperoxia (HBO), which it has been demonstrated that improves cellular energy metabolism and outcome and that can be neuroprotective in animal models.[28,29] A few clinical trials have also shown that HBO has short-term neuroprotective effects in both mild and severe TBI[30] and a significant reduction in the risk of death in TBI patients.[31] A randomized phase II study conducted by Rockswold et al. in a single-center study compared combined HBO/NBO treatment (i.e., FiO_2 1.0 delivered for 60 minutes at 1.5 ATA followed by 3 hours of NBO) with standard of care in severe TBI and showed a significant 36% increase in the absolute risk of a favourable outcome in the HBO/NBO arm.[32] Despite the potential advantages of HBO or combined HBO/NBO treatments, the specific and expensive facilities needed to apply HBO make this therapeutic strategy difficult to implement. In addition, HBO can be only provided for a short period of time and can significantly disturb conventional critical care management. In this clinical scenario, the simplicity with which NBO can be applied in any institution makes this low-cost option attractive and easy to implement with no modification to standard of care.

2.4.1. NBO-induced changes in brain oxygenation and metabolism

The NBO-induced metabolic effects and changes in brain hemodynamic variables are heterogeneous and sometimes contradictory. Diringer et al. used PET to show that using NBO in patients with severe TBI for 1-hr induced no significant change in the $CMRO_2$.[33] However, Nortje et al. showed that NBO improved cerebral metabolism in brain tissue (defined in our study as traumatic penumbra), with a significant reduction in the LPR but with no significant global changes in the brain.[34] The same inconsistent results and contradictory findings have been reported for the effects of NBO in brain energy metabolism.[6,9–12] In the present study, NBO induced a significant increase in PaO_2 and a statistically significant but clinically irrelevant increase in CaO_2 of ≈1mL/100 mL (**Table 5**). NBO induced a median increase in $PtiO_2$ of ≈50 mmHg in both the normal brain and regions defined as TP/Core (**Figure 3**). On average, this represented ≈215% increase from $PtiO_2$ baseline levels, significantly lower than the increase (≈600%) achieved by Rockswold et al. in the non-injured brain when using

HBO.[32] An interesting finding we observed was that the increase in $PtiO_2$ was similar for both the normal and the injured brain, suggesting that when the gradient is high enough, O_2 can even diffuse across injured and edematous tissue and can reach the target—the mitochondria—even in the damaged and edematous brain.

A few studies have found that brain metabolism improves after hyperoxic challenge, but with heterogeneous results. The initial studies showed a decrease of $[Lac]_{brain}$ in TBI patients treated with short-lasting hyperoxia.[6,9,13] However, two subsequent studies did not show a significant decrease in the LPR ratio,[8,10] and our group previous study only observed a decrease in the LPR for patients with an increased baseline $[Lac]_{brain}$.[11] Vilalta et al. showed that NBO did not induce any significant change in brain energy metabolism in patients with normal $[Lac]_{brain}$ but that it significantly decreased the LPR in patients with elevated baseline $[Lac]_{brain}$.[11] Tisdall et al. studied the changes in oxidized cytochrome C oxidase (CCO) concentration by near-infrared spectroscopy (NIRS), and observed a decrease in lactate and LPR levels and an increase in CCO oxidized concentration during NBO therapy. These variations correlated with changes in $PtiO_2$ and indicated an increased aerobic metabolism.[35] Similar results have been reported recently by Ghosh et al..[7]

Our study replicates our previous findings that NBO does not change energy metabolism in the whole group of patients. Statistically significant—but clinically irrelevant—changes were observed in $[Lac]_{brain}$ and $[Pyr]_{brain}$ in the macroscopically normal brain, but no significant changes in the LPR were observed (**Table 6**). However, in the five patients (15.6%) that presented $[Lac]_{brain}$>3.5 mmol/L at baseline, NBO induced a non-statistically significant reduction both in the $[Lac]_{brain}$ and in the LPR. The LPR changed from a median of 25.9 at baseline to a median of 22.7 (Wilcoxon test, p= 0.81). Although these differences were not statistically significant due to the limited number of cases, this trend was similar to what we have reported in our previous study and suggests that NBO can improve metabolism in the subgroup of patients with objective findings of disturbed brain metabolism. **Figure 4** shows an example of a patient included in the study that presented a marked metabolic response to NBO. The baseline lactate levels were >3.5 mmol/L and decreased during the hyperoxic challenge. The LPR maintained without variations because pyruvate levels also decreased. This patient did not presented hypoxia at baseline ($PtiO_2$< 15 mmHg

and/or a $PtiO_2/PaO_2$ ratio < 0.10). In this punctual case, the effects of NBO therapy could be clearly observed in the intracranial parameters monitored.

2.4.2. Potential NBO-induced adverse events

The main concern raised by NBO in TBI, stroke, or hypoxic-ischemic encephalopathy is the potential toxicity of using supra-normal levels of PaO_2. The mechanisms by which supra-normal O_2 may worsen outcome in patients with acute brain injuries is not yet clear, but high FiO_2 could induce vasoconstriction, exacerbate oxidative stress (OxS), increase neuroinflammation or induce excitotoxicity.[15] Furthermore, some studies on patients with TBI and cardiac arrest have suggested that NBO is associated with worse outcomes;[36–38] and hyperoxia in patients with aneurysmal SAH was associated with a higher incidence of delayed ischemia and poor neurological outcome.[39,40] The vasoconstriction effect the dissolved O_2 has on the brain and the reduction in CBF it induces constitutes a potential deleterious effect of NBO. Studies in humans have demonstrated that NBO produces a consistent and variable reduction of CBF, nearly to 27% in some cases.[41,42] Experimental and clinical studies have suggested that excitotoxicity may be an alternative or a synergic factor together with OxS for oxygen-induced tissue damage.[15,43] Thus, despite the potential benefits in brain metabolism, NBO is still controversial and raises many concerns that need to be clarified before entering clinical trials or being used routinely in some patients with brain injuries.

2.4.3. Conclusions and future directions

Our study show that NBO increased $PtiO_2$ in both macroscopically normal injured brains and in traumatic regions at risk, suggesting that pressurized O_2 may be delivered in regions with brain edema and impaired O_2 diffusion. It can be hypothesized that TBI patients would benefit from receiving NBO when they show indications of brain hypoxia, objective indicators of metabolic stress, or evidence of increased anaerobic metabolism. New clinical trials should focus on these subgroup of patients to see

whether or not NBO may be a useful tool in patients in whom O_2 delivery, metabolic crisis or mitochondrial dysfunction (either in isolation or in combination) play a role in the pathophysiology of brain damage. An important step in defining potential subgroups of patients who can benefit from NBO will be to use both MD and $PtiO_2$ monitoring to detect such patients. Patients with either low $PtiO_2$—once other causes of brain hypoxia has been ruled out—or in whom $[Lac]_{brain}$ and the LPR are increased—suggesting mitochondrial dysfunction— may be potential targets for NBO. Future studies should investigate this question more closely, identifying the metabolic needs of each patient and defining the potential effect of this therapy to improve energetic metabolism individually.

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