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Abbreviation list

ADP=adenosine diphosphate

ATP=adenosine triphosphate

ASL=arterial spin labeling

ASL-sCoV=spatial coefficient of variation of CBF (by ASL)

ATT= arterial transit time

CO_2=carbon dioxide

CVR=cerebrovascular reactivity

GRASE=gradient and spin echo

HR=heart rate

O_2=oxygen

P_i=inorganic phosphates

pCO_2=partial pressure of carbon dioxide

pO_2=partial pressure of oxygen

^{31}P-MRS= ^{31}Phosphate MRS

PCr=phosphocreatine

ROI=region of interest

pCASL=pseudo-continuous ASL

SpO_2=oxygen saturation

INTRODUCTION

Freedivers acquired the ability to voluntarily hold breath over several minutes: One breath-hold can be extended up to the world record of 11.5 minutes[1]. However, animal studies suggest that threshold times for hypoxia-induced neuronal cell death can be as low as 2.5 minutes[2]. The regulation and sufficiency of cerebrovascular reactivity (CVR) to prevent ischemic brain damage during prolonged breath-hold is unclear. Such knowledge may hold clues to explain patterns of brain damage in adverse diving outcomes and provide suggestions for targeted therapies. It can also be useful to deduce CVR in medical conditions of repetitive hypoxemia such as obstructive sleep apnea syndrome[3].

Previous MRI breath-hold experiments involved hypercarbic air composition or the use of relatively short breath-holds[4]. There are, however, crucial differences between experimental set-ups applying altered gas composition and those using voluntary breath-hold. In the latter, the human body can be examined as a closed system of internal metabolic gas exchange, while altered ventilation gas studies are an “open system” approach with ventilation at variable frequency as a complicating factor[5]. Quite different from short breath-holds, prolonged breath-hold over several minutes is furthermore a unique mental challenge demanding an emotional preparation, which freedivers need to train for and which may have an independent effect on CVR[6]. The putative effect of experience from previous prolonged breath-holds on CBF must be mentioned in this context and was not explored before.

Arterial spin labeling (ASL) perfusion MRI allows an *in-vivo* assessment of absolute CBF both regionally and on a vessel-selective level. ASL studies already successfully identified chronic CVR alterations due to obstructive sleep apnea as well as acute alterations during very short breath-hold[5,7]. A corollary ASL measure is the spatial coefficient of variation (ASL-sCoV) of the CBF image itself, which was recently identified as a correlate for the arterial transit time (ATT) and is a useful alternative when direct ATT estimations are not feasible[8].

For these reasons, ASL seems a promising technique to study CVR in prolonged breath-hold.

Blood gas alterations in general trigger a cerebrovascular but also peripheral reaction. During breath-hold, e.g., the CBF increase and hence faster O_2 supply facilitates the maintenance of an aerobic brain metabolism under hypoxemia, i.e. to prevent the depletion of adenosine triphosphate (ATP) and the accumulation of lactate from anaerobic glycolysis. The sufficiency of CVR can be studied indirectly by ^{31}Phosphate (^{31}P)-MRS, which provides estimates of ATP and related metabolites[9], as well as by ^{1}H-MRS, which may show lactate if the metabolite is elevated beyond physiological ranges.

The goal of the present study in experienced freedivers is to explore 1) the acute CVR during a prolonged breath-hold challenge with ASL and 2) the sufficiency of CVR to maintain aerobic cerebral energy metabolism as measured by ^{31}P- and ^{1}H-MRS. In addition, this study investigates whether pre-existing experience with prolonged breath-holds has an influence on the CVR during the breath-hold experiment.

METHODS

Participants

Fifteen experienced male freedivers (median age 36.0, CI 32.0 – 50.0 years) participated (table 1). Inclusion criteria were adult age and ability to hold their breath for longer than four minutes without stress symptoms (i.e. tachycardia, oxygen saturation (SpO_2) below 60%, delirium). Exclusion criteria were: pre-existing cardiac or neurological disorders and current smoking. The participants' age and cumulative number of breath-holds longer than 2.5 minutes during the volunteer's lifetime (estimated by interview report) were registered as potential influential factors.

Study design

Preparatory evaluations involved questionnaires regarding claustrophobia, noise tolerance or issues with restraints. All participants were instructed to refrain from meals and caffeine at least 2 hours prior to MRI to reduce perfusion confounders[10]. Preparation (e.g. meditation, test placement on MRI table) was allowed. MRI-ECG and finger oximetry guaranteed continuous monitoring of SpO_2 and HR. Arterial CO_2 measurements were not permitted by the hospital ethics committee, but venous blood gas was repetitively analyzed. Continuity of breath-hold and consciousness were visually monitored (L.E.) to guarantee safety and correct measurements.

The MRI protocol consisted of a 3D T1W acquisition followed by five ASL scans. Participants were instructed to breathe with normal frequency to obtain the baseline CBF (baseline phase). Hyperventilation to reduce blood CO_2 or increase O_2 concentrations was a forbidden confounder. The participants gave an acoustic signal when starting the five minute breath-hold, on which two consecutive ASL scans were acquired (early and late breath-hold phases). After the second breath-hold scan the participant was instructed to breathe again at normal frequency (ca. 16 /min.). Without delay two normal breathing ASL scans (early and late recovery phases) were performed. A subset of participants was available for ^{31}P- and ^{1}H-MRS (n=11 and 8 participants respectively) during separate sessions to determine relative

brain metabolites and again venous blood gas. The study was approved by the university ethics committee. All participants provided written informed consent.

MRI sequences

All imaging was performed on a 3T Ingenia MRI (Philips Healthcare, Best, Netherlands). The T1W MPRAGE sequence was 1x1x1 mm^3. The five identical pseudo-continuous ASL (pCASL) sequences were acquired with a 3D Gradient and Spin Echo (GRASE) readout (5 segments, acquisition voxel size 3.75 x 3.75 x 6 mm^3; FOV 240 x 240 x 96 mm^3, TE/TR 8 ms/ 4280 ms, labeling duration 1800 ms, RF labeling pulse duration/interval 0.7/1.41 ms, post-labeling delay 2000 ms, 4 background suppression pulses, scan time 2.5 minutes per scan phase). Each sequence consisted of two M0 images and two control-label pairs. A labeling distance of 13 cm (middle slice of the ASL stack to labeling plane) was chosen.

Image processing

Image processing was performed with "*ExploreASL"*, a toolbox based on SPM12 routines (Statistical Parametric Mapping 12, Wellcome Trust Centre for Neuroimaging, University College London, UK) that was initiated through the EU-funded COST action "ASL In Dementia", aiming at harmonizing ASL image processing for single- and multi-center ASL studies[11]. Image processing steps were: automated segmentation of 3D T1W images using CAT12 toolbox, rigid-body registration of CBF to the gray matter (GM) partial volume map and spatial normalization into common space using Diffeomorphic Anatomical Registration analysis using Exponentiated Lie algebra (DARTEL)[12]. M0 images were masked, iteratively smoothed and extrapolated also outside the mask, and CBF was quantified using a single compartment quantification model[13]. The regions of interest (ROI) analyzed were the total
(cortical) GM and the total white matter (WM). The GM was subsegmented into vascular territories of the anterior, middle and posterior cerebral arteries (ACA, MCA and PCA). The vascular territories were delineated in the common space on the MNI atlas according to Tatu et al.[14].

The ASL-sCoV was defined as the standard deviation of CBF divided by the mean CBF, within the total cortical GM ($p_{GM} > 0.7$)[8]:

$$ASL\text{-}sCoV_{ROI} = \frac{\sigma(CBF_{GM})}{\mu(CBF_{GM})} * 100\% \quad [1]$$

Partial volume effects within the GM and WM ROIs were corrected using the Asllani method[15].

MR spectroscopy

Proton-decoupled ^{31}P spectra were acquired with a dual-tunable $^{31}P/^{1}H$ birdcage transmit/receive head coil (1024 data points, 3 kHz sampling, repetition time 4s, 4 signal averages) as a continuous time series with 16 s duration for each spectrum and comprising a total of 40 scans before, during, and directly after breath-hold in a 25 mm thick axial slice on the level of the basal ganglia. ^{31}P signals were processed by the AMARES algorithm of the JAVA-MRUI software quantifying 15 peaks in each spectrum arising from phosphocreatine
(PCr), inorganic phosphates (P_i), ATP, and from phosphomono- and -diesters (PE, PC, GPE, GPC)[16]. The cerebral pH was determined from the frequency separation between P_i and PCr. Five consecutive spectra sets within the ^{31}P time series were averaged over 2.5 minutes, which represent the metabolic baseline, first and second halves of breath-hold as well as early and late metabolic recovery phases. This was also done to improve the signal-to-noise ratio. In this way, the time course of pH and of the ratios P_i/PCr, P_i/β -ATP, PCr/β-ATP as well as the ratios of all ^{31}P metabolites relative to (ATP+ADP), calculated from the mean of the γ– and α-ATP peaks, were obtained in the same 2.5 minute intervals as the ASL sequences. γ– and α-ATP peaks include the signals from nucleotide diphosphates and thus are expected as near constant.

In a consecutive single-volume ^{1}H-MRS breath-hold experiment, ratios of the ^{1}H-MR signals of N-acetyl aspartate, total creatine, choline and lactate were determined from an 8 mL

volume in the left basal ganglia (PRESS-localized spectra, TR 2000 ms, TE 140 ms, 128 signal averages). Recording periods were split to match with ASL-MRI and ^{31}P-MRS phases of the breath-hold experiment.

Blood gas analysis

10 mL of venous blood were drawn before breath-hold, after 2.5 minutes of breath-hold, at first breath after breath-hold and after 2.5 minutes of recovery. Immediate analysis (Rapilab 1265, Siemens Healthcare, Erlangen/Germany) involved partial pressure of carbon dioxide and oxygen (pCO_2, pO_2), glucose and lactate levels. With exception of pCO_2 these parameters were shown to correlate well with arterial values[17].

Statistics

Overall CBF variability was analyzed by a mixed linear model with the participant as random factor, while differences between single time points were analyzed by a paired t-test, which considers CBF differences compared to baseline (Δ CBF/CBF_0) as the expression of CVR without division by a fixed rate of stimulus such as per unit CO_2[18]. The same tests were applied for the partial volume corrected ASL-sCoV. MRS parameters were analyzed in a mixed linear model and with Pearson's R correlation. The GM CBF in the vascular territories was analyzed separately by a paired t-test to identify differences between the anterior and posterior circulation. The influence of age and experience with prolonged breath-holds estimated from cumulative breath-hold events >2.5 minutes were tested for correlation with CBF and ASL-sCoV using a mixed linear model with the participant as random factor. Similarly, the relationships of the ASL parameters with SpO_2 and HR were analyzed. Parametric testing occurred after testing for normal distribution. Generally, absolute CBF differences (Δ in mL/100 g/min.) were used for descriptive statistics and statistical analyses, while ratios (in %) are mentioned for illustrative purposes.

RESULTS

Cerebrovascular reactivity

All participants showed a significant CVR by an increase in CBF until five minutes of breath-hold with subsequent decline at recovery (*P*<.0001 for all vessel territories, GM and WM; table 2, fig. 1 and fig. 2 a, b). While CBF increase from baseline was substantial after five minutes (late breath-hold scan phase; for the total GM ROI: mean Δ CBF±standard deviation (SD) 18.3 ±14.4 mL/100g/min. (+51.8 %); *P*<.0001), it was only subtle within the first 2.5 minutes of breath-hold (total GM ROI: mean ΔCBF±SD 6.3±11.1 mL/100 g/min. (+17.8 %); *P*=.04). In four volunteers, early breath-hold phase CBF was indeed lower than baseline CBF (mean -22.4 %; *P*=.01). Baseline to late recovery phase CBF differences were not significant (*P*=.55 for the total GM ROI). Return to baseline CBF or below occurred during the early recovery phase with a mild secondary CBF increase during the second 2.5 minutes of recovery (*P*=.03 in total GM ROI).

The absolute CBF and CVR of the anterior circulation (ACA, MCA) were at all times higher than in the posterior circulation (*P*=.001; table 2). The mean difference between CBF of the MCA and the PCA increased steadily during breath-hold from 8.8±6.6 mL/100 g/min. at baseline to 14.6 ±6.1 mL/100 g/min. at late breath-hold (*P*=.001).

During all scan phases between-participant CBF variability showed a more narrow range in the GM than in the WM as well as a lower overall CVR (CVR variability in GM: 83.8 – 152.8% from baseline, CVR in WM: 74.2 – 231.5% increase from baseline CBF; *P*=.0001; table 2).

The spatial CoV

The ASL-sCoV varied over the course of the experiment (*P*<.0001). In the majority of cases (n=12/15) there was an ASL-sCoV decrease between baseline and the early breath-hold phase (mean decrease: -30.0±21.6 %; *P*=.002; fig. 2 c). The ASL-sCoV remained reduced during the five-minute breath-hold (*P*=.81 for the difference between early and late breath-

hold), and rose again during recovery (*P*=.01). There was no difference between baseline and recovery phases (*P*=.29).

The range of GM ASL-sCoV between participants was smaller during breath-hold, than during normal breathing: mean±SD 2.8±1.0%; range 1.9–5.3% vs. mean±SD 1.8±0.4%; range 1.5–3.0%, for baseline vs. early breath-hold phases respectively. This equates into a 35.7% decrease of ASL-sCoV variability between participants (*P*=.02).

Physiological correlations

The dynamics of SpO_2 and HR are presented in table 2. SpO_2 correlated with CVR, with an estimated increase of CBF of 0.82 mL/100 g/min. with each 1% SpO_2 drop (CI 0.6–1.1 mL/100 g/min.; *P*=.0001; fig. 3 a and b). HR was not correlated with CVR (*P*=.36; fig. 3 c and d). Similarly, age was not identified as an influential co-factor on CVR (*P*=.32).

Taking the entire group of 15 freedivers into account, previous prolonged breath-hold events were not correlated with absolute CBF values (*P*=.56). However, there were two outlier participants (diver 11 and 13) with exceptionally large experience and comparatively high CBF. After their exclusion a relationship between previous breath-hold experience and absolute CBF was found with 1,000 previous prolonged breath-holds reducing CBF in GM by 2.2 mL/100 g/min. (CI 0.7–3.7 mL/100 g/min.; *P*=.01; fig. 4) for the remaining thirteen cases. This finding was similar for CBF in WM: 1000 previous breath-hold reduced WM CBF by 0.6 mL/100 g/min.; CI 0.15–1.1 mL/100 g/min. (*P*=.01). Mean CVR, however, as defined by the Δ CBF/CBF_0 was not correlated with previous experience with prolonged breath-holds (*P*=.23). Similarly, ASL-sCoV was not correlated with SpO_2, HR, age or diver experience with prolonged breath-holds (P=.45, .53, .90 and .69 respectively).

MR spectroscopy and blood analyses

^{31}P-MRS (fig. 5 a) revealed minor fluctuations in pH (fig. 5 b) and ATP metabolites within physiological ranges during the course of breath-hold (P=.07 for pH; *P*>>.05 for dynamics in relative PCr, P_i, ATP-α, -β, -γ, and phosphomono- and -diester levels). There was a small but

significant decrease of the PCr/(ATP+ADP) ratio between baseline and late phase breath-hold in the matched pairs analysis of the individuals (-6.4%; *P*=.02; fig. 5 c). PCr and ß-ATP differences relative to their baseline values (∆PCr and ∆ß-ATP) were correlated with the differences in pH from baseline (R=.53; *P*<.001 for ∆PCr, and R=.45; *P*=.003 for ∆ß-ATP respectively; fig. 5 d).

^{1}H-MRS never showed the CH_3 doublet of lactate at 1.34 ppm in any of the participants. No significant changes in the levels of N-acetyl aspartate, total creatine or choline occurred (*P*>>.05 for all).

Venous blood samples showed a development of hypoxemia and hypercapnia during breath-hold (table 2; fig. 5 e). A significant pCO_2 increase was noted only at late breath-hold (*P*=.02), while pO_2 significantly dropped already after the early breath-hold phase (*P*=.002). Both parameters returned to baseline after breath-hold, while venous lactate and glucose levels increased until the end of the experiment during the recovery phase (*P*=.001 and .01 respectively; fig. 5 e).

DISCUSSION

This study provides three key findings. First, despite individual variability freedivers show a relatively consistent and vessel territory-specific CVR during a breath-hold challenge, which is measurable with ASL. Second, CBF and the ATT correlate ASL-sCoV deliver independent aspects of cerebrovascular response to breath-hold. Finally, this study identified indicators for an influence of earlier experience with prolonged breath-hold on absolute CBF during the breath-hold challenge but not on CVR itself. Physiological responses can apparently withstand the extreme biochemical challenge induced by a prolonged breath-hold of five minutes, which can be detected by ASL and MRS.

Multiple methods exist to assess CBF including ASL, phase contrast MRI, PET and Doppler ultrasound. Maximum breath-hold studies are rare and CBF evaluations were until now exclusively performed with Doppler ultrasound, which revealed a continuous elevation of flow velocity in the MCA around 100%[19,20]. The mean maximum CBF increase after five minutes of breath-hold observed in this ASL study was 51.8%, which is very close to values observed in ASL studies using hypercarbic gas inhalation or short breath-hold, but indeed in some cases lower than values measured with ASL in a recent maximum breath-hold study (+107%)[5,21,22]. Previous studies comparing CBF assessments with different methods similarly revealed substantial inter-method differences in absolute CBF, but otherwise confirmed a high correlation between methods. PET-estimated absolute CBF was, e.g., consistently lower than phase contrast MRI, while Doppler and ASL differed substantially in relative CBF change in a drug stimulation trial[23,24]. These differences are not surprising and can be explained by different influential factors acting on the respective flow parameters. While ASL can measure absolute CBF, Doppler provides flow velocity in a local vessel segment as a surrogate parameter for CBF. CO_2 is a strong vasodilating agent in cerebral tissue causing an increase in CBF due to increased blood volume (CBV) based on the equation *CBF=CBV/MTT (mean transit time).* The blood flow velocity rises despite vessel dilatation also due to reduced ATT. However, earlier Doppler studies revealed a strong

neuromuscular response in proximal vessel segments of the anterior circulation, while more distal segments and the posterior circulation seemed less responsive[25,26]. This may explain why Doppler-assessed breath-hold experiments identify a higher absolute and relative CVR mostly in the M1 segments of the MCA, than most of those applying ASL, which captures perfusion in most distal and non-muscular vascular segments.

A heterogeneous CVR between vessels was also observed in the present ASL study with lower CBF and CVR in the PCA territories. This finding can be explained by differences in labeling efficiency between vascular territories as well as the longer ATT in the posterior vascular territory. Microanatomical differences leading to a diverse autoregulation capacity between the anterior and posterior circulation as an underlying reason for a diverse CVR are on the other hand more controversially discussed even beyond the field of perfusion studies under extreme conditions[27,28]. A recent Doppler-monitored breath-hold study yet supports our findings of a lower CVR in the PCA and there suggests a different sympathetic activation between the two territories as an additional explanation beyond the labeling aspect, which needs to be considered in ASL[29]. An awareness of a vessel-selective CVR is however crucial when interpreting ASL measurements in focal ischemic lesions after prolonged clinical conditions of apnea.

Another finding of this study is that CBF decreased during the first 2.5 minutes of breath-hold in four out of fifteen participants. The counter-suggestive relatively higher CBF at baseline compared to later time points might be the effect of anticipation anxiety towards the upcoming breath-hold challenge[30]. This also among freedivers well-known mental phenomenon is currently not sufficiently addressed in sports physiological research. Indeed, our own results may only suggest that some freedivers experienced an early sympathetic activation prior to the breath-hold challenge. Due to the limited temporal resolution of the ASL sequence, which delivers a mean CBF over each of the 2.5 minute phases, we cannot readily assess how long the reduction of CBF persists and when exactly an elevation of CBF above took place during the early breath-hold phase in these four volunteers.

We further confirmed a difference between the cortical GM and the deep WM CVR, with the total GM CBF known to be at least two times higher than the WM CBF[31]. Low WM signal is an obstacle for WM CBF assessment in ASL despite the availability of background suppression[31]. However, CVR differences between GM and WM tissue can be interesting as they may help to better understand morphologic findings in brain diseases such as obstructive sleep apnea syndrome[32]. We noted a larger CVR variability and relative increase in WM than in GM, which could be explained by the later arrival of blood in the relatively more distal WM vessels. WM CBF is difficult to measure at baseline due to longer ATT. Direct ATT assessment was technically not possible as part of our experimental setting due to temporal restrictions and a resulting mono-PLD ASL sequence. However, our ATT approximation based on ASL-sCoV confirmed a breath-hold induced decrease in ATT, which might increase SNR of WM CBF, which inflates the measured ΔCBF to a certain extent.

For the interpretation of ASL-sCoV, it is viable to consider the methodological peculiarities of ASL. An ATT increase will cause ASL signal to appear in bigger vessel resulting in vascular artefacts. ASL signal can at the same time decrease in areas with higher baseline ATT as for example the perfusion watershed. These two effects both increase ASL-sCoV[8]. However, ASL-sCoV has a theoretical lower limit attained when all labeled blood has arrived in the tissue and all vascular artifacts have already disappeared. Further ATT decrease beyond this limit will have only minimal effect on the ASL-sCoV. Reaching this lower limit of ASL-sCoV during the early breath-hold could explain the low ASL-sCoV variability in some participants. The ASL-sCoV decrease however appears to occur earlier than CBF increase and levels off during the second breath-hold phase, while CBF further increases. An earlier response to breath-hold leading to decreasing ATT prior to CBF increase in the late breath-hold phase can be the explanation, denoting that the effect is mainly vascular and perfusion changes as detected by ASL follow later. ASL-sCoV may be an earlier CVR marker of hypoxemia than absolute CBF.

CVR is predominantly triggered by changes in blood CO_2, while e.g. hypoxemia detected by peripheral chemoreceptors is considered to play an independent but inferior role in cerebral vasodilation[33]. While Willie et al. already reported that O_2 metabolism has a crucial influence on the breath-hold capability of freedivers, stressing the role of O_2 for breath-hold tolerance and CVR, Cross et al. could not confirm an influence of O_2 on cerebral autoregulation in their prolonged breath-hold study[19,20]. We identified falling O_2 as an influential factor of CVR in this study, which can usually not be observed in CBF studies applying hypercarbic-normoxic gas despite otherwise comparable CVR between breath-hold and hypercarbic-normoxic gas studies[5,26]. Due to the design of this study, which could not rely on arterial CO_2 measurements, it remains however impossible to discern the relative contribution of hypercarbia and hypoxia to CBF increase. CVR increased faster in the second half of the breath-hold experiment in correlation with the secondarily faster SpO_2 decline, which can be explained by pulmonary O_2 stores that allow for normal hemodynamic conditions during the first minutes of breath-hold. Cerebral near-infrared measurements in elite freedivers showed that cerebral O_2 desaturation tends to occur within 175±50 s (mean±SD) but not before, which supports our finding[34].

In this study, CBF itself was not strongly correlated to HR. This finding does yet not allow concluding that CBF in breath-hold is not modulated by cardiac causes. It is however beyond the scope of this study to evaluate cardiac co-factors to CVR such as heart stroke volume or the diving reflex in detail. The factor age (an indirect measure of the cardiac and vascular influence on CVR) was assessed, but was not associated with any of the flow parameters, which is not surprising considering that most volunteers in this study were younger than 40. Also a selection bias of outstandingly healthy and well-trained freediver volunteers must be considered a further contributing factor.

A physiological adaptation to breath-hold was another hypothesis to be tested in this study. A diminished CVR during hypercarbia/hypoxemia was reported for patients with chronic obstructive pulmonary disease (COPD) as well as sleep apnea[35]. In an attempt to investigate

a similar association between experience with hypercarbic/hypoxemic states and CVR in freedivers, we estimated the total amount of previous prolonged breath-holds of the participants before participation in the current breath-hold experiment (defined as cumulative breath-holds >2.5 minutes in a lifetime). Our findings do not unconditionally corroborate that breath-hold experience accounts for an adaptation effect on CBF. First, CVR did not differ between more breath-hold experienced freedivers and their less experienced counterparts. Second, the by far two most prolonged breath-hold experienced freedivers, who additionally stated a high frequency of longer breath-holds per training session, showed relatively high CBF values. However, for the remaining cohort an association between experience and lower absolute CBF in all phases of the experiment could be observed, which may indicate that repetitive previous hypercarbia/hypoxemia has an acute cerebrovascular effect during a breath-hold challenge. It is intriguingly challenging findings in COPD patients which identified a chronically elevated CBF for that group[35]. Due to the limited cohort size, which also included freedivers normally specialized in shorter breath-holds, this interesting and also clinically relevant observation will need to undergo further critical evaluation in the future, favorably in a more homogeneous group concerning age and freediving specialization.

In prolonged breath-hold, the efficacy of the cerebrovascular but also cardiac response to maintain a stable O_2 supply to the brain despite decreasing availability is a crucial health aspect and can be assessed spectroscopically regarding energy metabolism. Direct non-invasive in-vivo measurements of brain energy metabolism during prolonged breath-hold are extremely rare[22,36]. Cerebral lactate accumulation or acidosis were observed in none of our participants suggesting a sufficient compensation of limited O_2 supply by recruitment of ATP stores and increasing CBF. However, we identified a significant decrease in PCr/(ATP+ADP) ratio during breath-hold. This can be interpreted as a compensatory PCr decrease to provide ATP by PCr hydrolysis as a consequence of declining O_2 availability and reduced aerobic ATP production capacity in prolonged breath-hold.

Rising peripheral venous lactate levels during the breath-hold challenge in contrast to stable cerebral lactate stress the shift towards a preferred cerebral O_2 supply in breath-hold including CBF increase and simultaneously peripheral vasoconstriction. The rising glucose levels are likely a consequence of adrenaline-induced glucose mobilization and underline the exceptional metabolic and mental challenge of prolonged breath-hold[37]. The correlative venous pO_2 analyses documented pathologically low O_2 levels after five minutes of breath-hold (down to 60% SpO_2) and corroborate similarities of this breath-hold experiment with clinical settings of hypoxemia. On the other hand untrained persons may encounter life-threatening consequences under these circumstances, while freedivers face hypoxemia under voluntary and trained conditions.

Maximum breath-hold without contact to water is a particular challenge for freedivers because regular training sessions mostly include water immersion, which cause an augmented diving reflex. Only very few participants fulfilled the inclusion criteria and were able to perform sufficient breath-hold in the noisy MRI environment. Multiple candidates declined due to the lack of silence or they would not volunteer for additional MRS breath-hold experiments, which explains the limited cohort size. Further, participants did not tolerate a CO_2 mask, which interfered with their meditative state. We therefore decided to analyze venous CO_2. But while venous CO_2 can be used to confirm hypercapnia, it is however too variable to study the CO_2 influence on CVR due to known substantial deviations from arterial CO_2 in the brain[38]. For this reason we can also only assume that all normoventilating volunteers started at normal CO_2 blood levels into the experiment. The unavailability of this otherwise valuable data limits the evaluation of the physiological processes behind our observations.

CONCLUSIONS

In summary, this study revealed that experienced freedivers develop a CVR, which is sufficient to maintain a physiological cerebral energy metabolism even during a prolonged breath-old period of five minutes and severely diminishing blood O_2. Further, ASL parameters, which are determined by blood flow and vessel diameter alterations alike, serve as excellent candidate MR parameters to reveal this response, while ^{31}P-MRS revealed its utility to dynamically study acute changes in cerebral energy metabolism. ASL may also provide evidence for long-term adaptation of cerebral vasculature following repetitive hypoxic-hypercapnia. Imaging and metabolic findings of the present freediver study can be used to better understand CVR during hypoxic-hypercapnia in critical care and sleep apnea conditions.

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Tables and legends:

Table 1 Anthropometric data of freediver volunteers

Participant	Longest breath-hold until MRI (minutes)	Start of sport (years before MRI)	Estimated breath-holds >2.5 min. per session	Sessions per month	**Estimated cumulative lifetime breath-holds >2.5 min.**	Age (years)
1	10.6	11.3	30	0.4	**1,632**	31
2	5.5	1.5	7	8	**1,008**	36
3	5.4	2.0	1	1	**24**	20
4	5.4	11.0	~0	8	**4**	33
5	5.0	2.0	0.125	8	**24**	40
6	5.5	10.0	~0	4	**4**	38
7	5.0	8.0	5	8	**3,840**	61
8	5.5	1.5	0.5	4	**36**	34
9	5.0	7.0	6	16	**8,064**	47
10	6.2	9.0	6	7	**4,536**	26
11	5.3	27.5	15	16	**79,200**	64
12	6.5	10.2	12	28	**11,712**	51
13	5.5	11.3	10	16	**21,760**	35
14	5.5	1.6	18	10	**3,420**	32
15	5.0	5.0	0.083	4	**20**	50
Median	5.5	8.0	6	8	**2,526**	36.0
CI	5.3 – 5.5	2.0 – 11.0	0.14 – 12.0	4.0 – 9.96	**30 – 6,300**	32 – 50
Min	5.0	1.5	0	0.41	**4**	20
Max	10.6	27.5	30	16	**79,200**	64

Table 1. Participants were interviewed concerning the duration (start of sport) and intensity (freediving sessions per month) and the estimated frequency they keep breath for longer than 2.5 minutes per session. The latter could be near zero in freedivers specializing in short high-frequency breath-holds. Some participants held breath for longer than 2.5 minutes only as a qualification for this study, hence their cumulative lifetime breath-holds longer 2.5 minutes before MRI was four, while others very frequently underwent prolonged breath-hold.

Table 2 Mean CBF and blood gas/heart rate dynamics during the breath-hold challenge

experiment phase	ASL CBF					oximetry		venous blood gas analysis			
	GM	**WM**	**ACA**	**MCA**	**PCA**	**heart rate**	**SpO_2**	**glucose**	**lactate**	**pO_2**	**pCO_2**
baseline	35.3 ± 12.3	8.9 ± 5.1	36.6 ± 12.2	37.9 ± 14.5	29.1 ± 9.9	79.9 ± 19.2	98.9 ± 1.2	98.4 ± 11.8	1.35 ± 0.37	37.6 ± 9.7	43.3 ± 7.5
early breath-hold	41.6 ± 15.1	12.3 ± 6.6	44.1 ± 15.9	44.9 ± 16.9	32.7 ± 12.6	63.7 ± 11.1	93.7 ± 3.6	98.8 ± 12.0	1.53 ± 0.37	33.1 ± 6.7	42.9 ± 5.7
late breath-hold	53.6 ± 12.5	20.6 ± 6.0	56.8 ± 12.0	57.3 ± 14.2	42.7 ± 10.8	66.9 ± 24.6	75.0 ± 9.0	100.4 ± 11.8	1.60 ± 0.32	26.8 ± 4.7	48.8 ± 10.8
early recovery	29.6 ± 11.6	6.6 ± 4.9	30.8 ± 11.0	32.2 ± 13.2	22.0 ± 10.9	65.1 ± 13.2	97.7 ± 1.4	103.5 ± 13.2	1.54 ± 0.37	40.0 ± 6.4	40.0 ± 6.1
late recovery	33.6 ± 12.1	8.0 ± 5.3	34.7 ± 12.0	36.0 ± 13.5	27.1 ± 11.0	61.2 ± 11.0	98.1 ± 1.3	-	-	-	-

Table 2. Baseline: last 2.5 minutes before breath-hold at normal ventilation; early breath-hold: first 2.5 minutes of breath-hold; late breath-hold: second 2.5 minutes of breath-hold; early recovery: first 2.5 minutes after breath-hold; late recovery: second 2.5 minutes after breath-hold. Data in mean ± standard deviation; GM: CBF in total gray matter ROI; WM: CBF in total WM ROI; ACA: anterior cerebral artery; MCA: middle cerebral artery; PCA: posterior cerebral artery; CBF in mL/100 g/min.; heart rate in /min.; SpO_2 in %; glucose in mg/dL, lactate in mMol/L, partial pressure of oxygen and carbon dioxide (pO_2 and pCO_2) in mmHg/Torr.

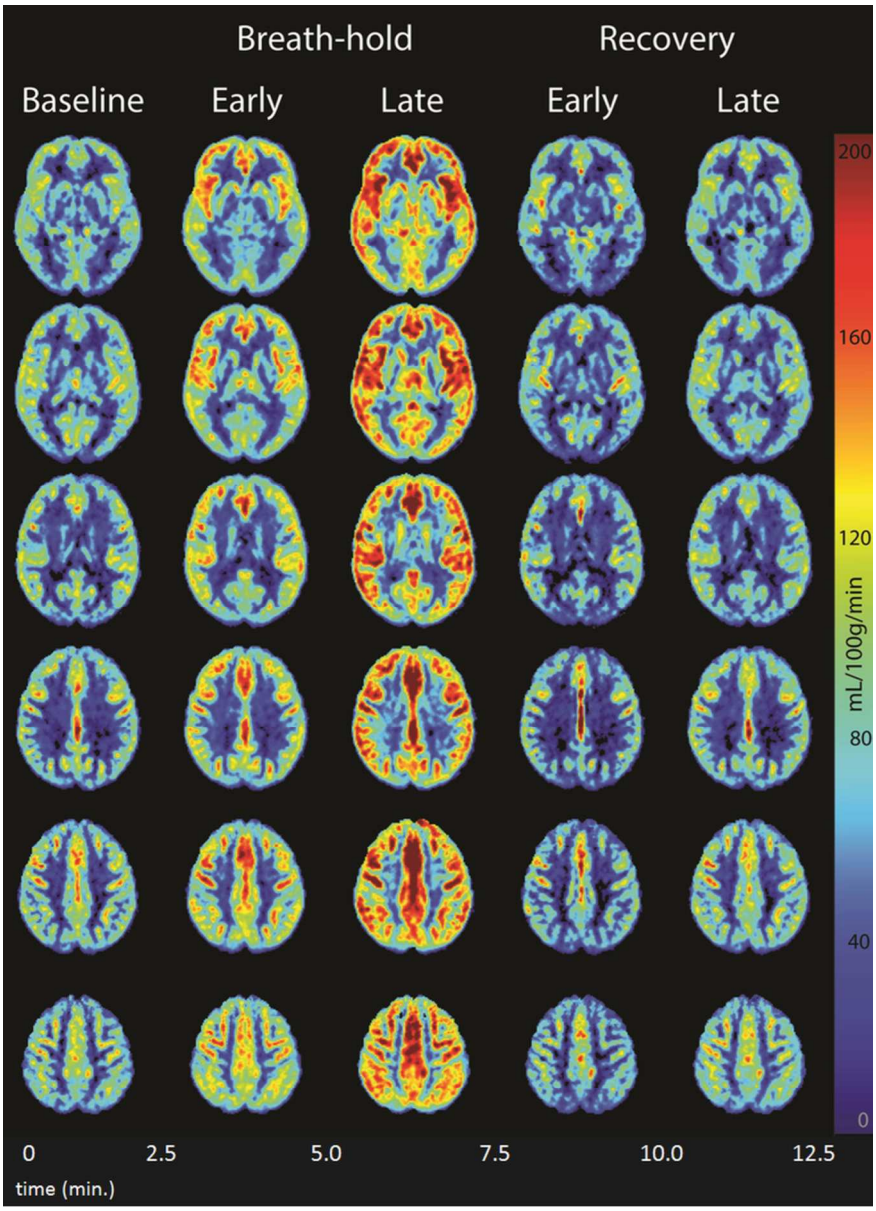

Fig. 1 Mean CBF mean during the breath-hold experiment
False color maps of cohort mean cerebral blood flow during five-minute breath-hold. Selected transverse brain sections of the cohort mean CBF before, during and after the five minute breath-hold challenge. The mean total GM CBF over all participants and over all phases was scaled to 60 mL/100 g/min. Likewise, the mean total WM CBF for all participants over all phases was scaled to 20 mL/100 g/min. for WM.

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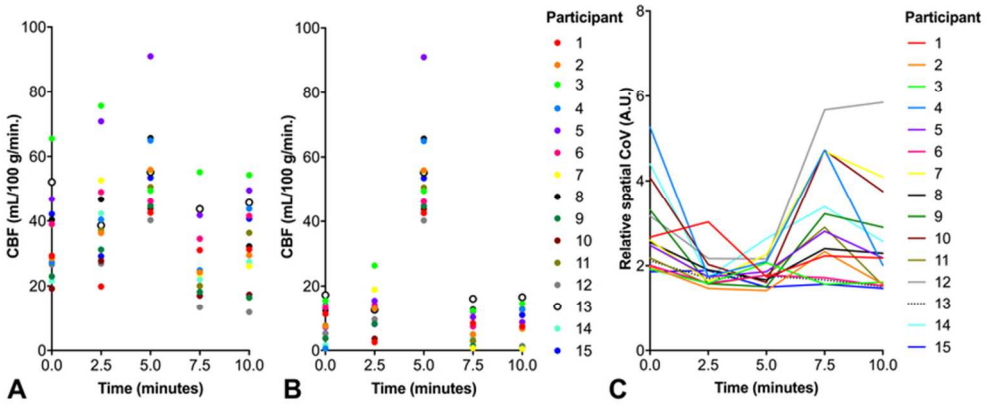

Fig. 2 Cerebral blood flow and partial volume corrected relative spatial coefficient of variation dynamics

Baseline scan with normal ventilation starts at 0 minutes; the second and third measurement points represent 2.5 and 5 minutes of breath-hold, the fourth and fifth scan phase at 7.5 and 10 minutes represent the recovery phase.**A:** CBF in gray matter voxels only (n=15 participants; each represented by a color dot).**B:** The same for white matter only.**C:** Partial volume corrected relative spatial coefficient of variation dynamics in gray matter. Y-axis: Relative CBF spatial CoV defined as the ratio of the actual spatial CoV divided by the spatial CoV expected based on anatomy (in arbitrary units, A.U.).

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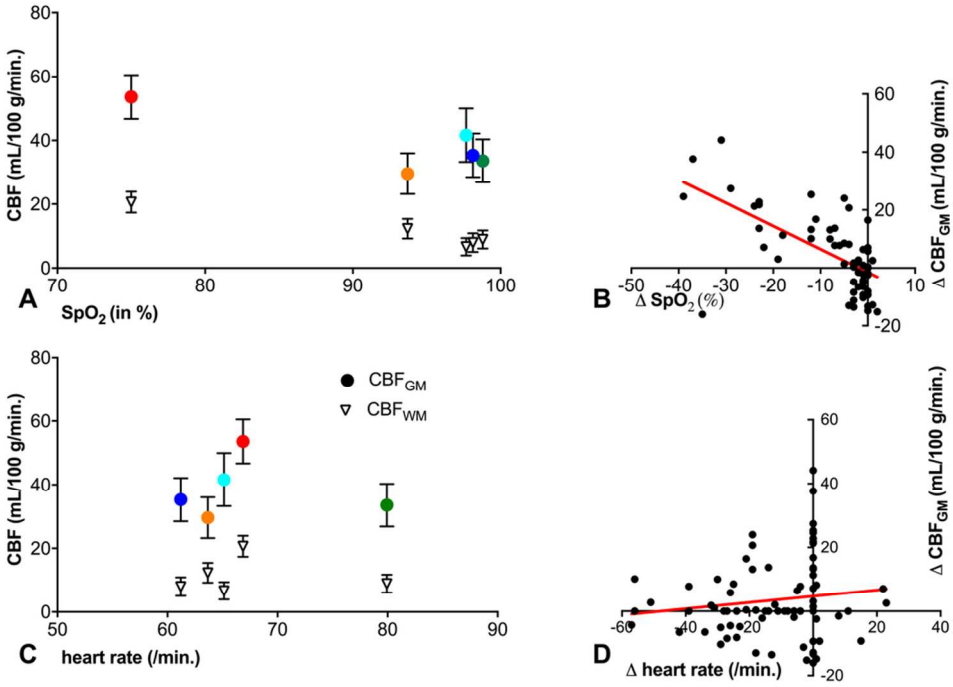

Fig. 3 Relationship of SpO_2 and heart rate with CBF

A: Mean GM CBF±standard deviation (colored dots) and mean WM CBF±standard deviation (triangles) were both strongly correlated with absolute SpO_2. Color encoding for **A** and **C:** green for baseline; orange for 2.5 minute breath-hold; red for 5 minute breath-hold; turquoise after 2.5 minutes of recovery, blue after 5 minutes of recovery; WM same time points right below.**B:** ΔCBF (here measured in GM) similarly correlated well to ΔSpO_2 (all 75 single values).**C** and **D:** There was no strong correlation between heart rate and CBF in absolute values (**C**, mean±standard deviation) or ΔCBF and ΔSpO_2 **(D)**.

93x67mm (300 x 300 DPI)

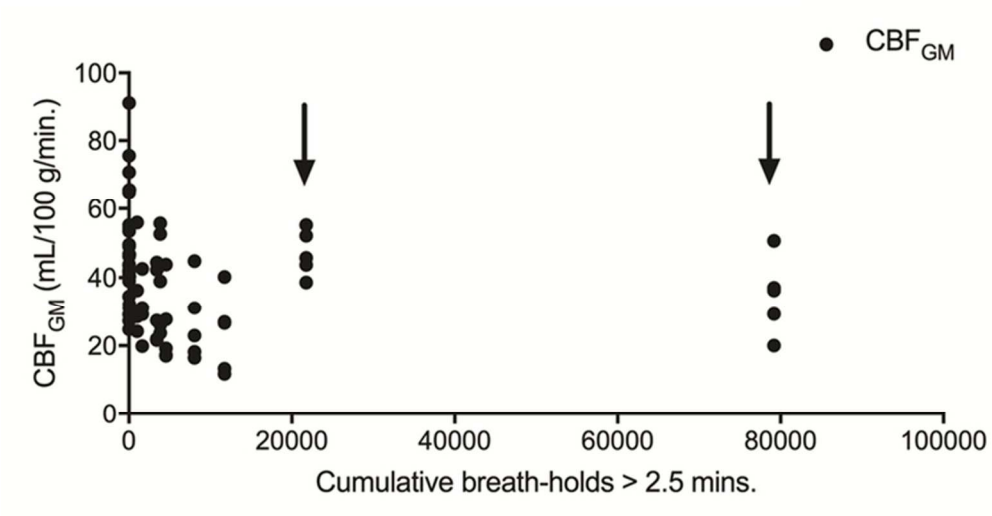

Fig. 4 Scatter plot illustration of the relationship between cumulative breath-hold experience and CBF during the experiment

With the exception of two participants (diver 11 and 13, marked with arrows) there was a lower CBF during the experiment with more previous prolonged breath-hold events (cumulative breath-hold >2.5 minutes).

64x34mm (300 x 300 DPI)

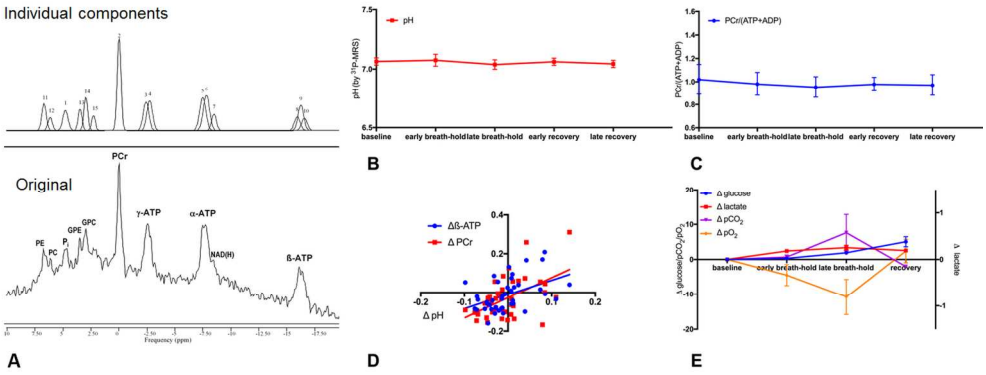

Fig. 5 Energy metabolites and blood gases in brain and venous blood A: Example ^{31}P-MR spectrum of one participant averaged over 2.5 min of the late breath-hold phase (above), displayed together with AMARES-fitted spectral components (below) **B:** Brain pH as assessed by ^{31}P-MRS is near constant during the entire breath-hold experiment **C:** The PCr/(ATP+ADP) ratio measured by ^{31}P-MRS slightly decreased during breath-hold. **D:** PCr and ß-ATP differences (Δ) to individual baseline values correlated with the pH differences from baseline indicating that small tendencies towards acidosis and ATP depletion occurred during breath-hold. **E:** Venous blood analyses revealed significant hypoxemia and hypercapnia development (left axis in mmHg) during breath-hold with fast recovery. Blood glucose (in mg/dL, left axis) and lactate (in mMol/L, right axis) rose during breath-hold and did not return to baseline. All values are expressed as differences to baseline.

270x110mm (300 x 300 DPI)