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CICLO XXXV

Title:

ADVANCED VIRTUALLY ASSISTED TELEMEDICINE in ADVERSE REMOTENESS (AVATAR)

Tesi redatta con il contributo finanziario di:

Diver Alert Network (DAN) Europe Foundation

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Summary

Abstract

The popularity of extreme sport and activities in remotes areas is steadily increasing together with the number of participant and correlated diseases per year.

Until today all the emergencies in remote areas are, at best, managed by a telephone interview, without any real time physiological data evaluation by the attending physician.

This kind of current service shows evident weaknesses:

1. It is based exclusively on a telephone contact.
2. There are no automatic mechanisms to intercept and prevent critical situations.
3. Is impossible to send automatic alarms in case of need.
4. It assumes that the injured has active telephone network coverage.
5. It assumes that the injured can actively interact with the emergency alarm center or that other bystanders can do so.
6. All the health information is collected by telephone interview without any "real" physiological data obtained by the patient.

The object of AVATAR: (Advanced Virtually Assisted Telemedicine in Adverse Remoteness), focuses on developing and assembling technologies allowing to monitor, record, transmit, process physiological data, and, as a consequence, provide assistance and guidance by remote control to: bystanders, rescuers, health-care professionals, as well as possibly patients themselves, in case of emergencies in extreme environment such as: diving, high altitude and space.

The project aims at providing real time physiological and environmental information from remote areas and to develop specific algorithms to elaborate this information to permit a more accurate management of accidents in remote areas, based on an advanced bidirectional telemedicine concept.

The final goal, and also the original challenge of AVATAR, could be to realize a dedicated international control center able to receive and manage physiological and environmental data received from remote areas and, supported by customized wearable technology, augmented and virtual reality and exposure monitoring electronic devices, to provide appropriate instructions to assist accident victims supported by real-time medical, environmental and exposure information.

During the three years of Ph.D we developing and assembling devices allowing to monitor, recording and transmit physiological and environmental data and developing the data base, the algorithms and the software allowing the AVATAR system function.
To permit a correct interpretation of remotely collected data we also developing physiological scientific protocols to investigate the body adaptation to extreme environment.

1 Ph.D. PROJECT

1.1 Introduction and scope

The popularity of extreme sport and activities in remotes areas is steadily increasing together with the number of participant and correlated diseases per year [1]. Only as concerning diving, our main field of interest, with a prudent approach the recent estimate of about 35 million amateur scuba and breath hold divers, can be considered as the minimum value, in fact it doesn't take into account the commercial divers, employed in companies specialized in underwater works, and the other kind of operators working in high pressure environments, such as caissons and hyperbaric chambers [2].
Diving, being the most hostile adverse remoteness for the difficulty in signal transmission and the impossibility for normal devices to work underwater, could be the core-test of the project because the effort made for underwater testing can be easily transferred to all the others extreme environments.
Until today all the emergencies in remote areas are, at best, managed by a telephone interview, without any real time physiological data evaluation by the attending physician.
This kind of current service shows evident weaknesses:

1. It is based exclusively on a telephone contact
2. There are no automatic mechanisms to intercept and prevent critical situations
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The object of AVATAR: (Advanced Virtually Assisted Telemedicine in Adverse Remoteness), focuses on developing and assembling technologies allowing to monitor, record, transmit, process physiological data, and, as a consequence, provide assistance and guidance by remote control to: bystanders, rescuers, health-

care professionals, as well as possibly patients themselves, in case of emergencies in extreme environment such as: diving, high altitude and space.

Due to the particular environmental conditions in which the AVATAR system is intended to work the project points at using or developing wearable devices for data acquisition.

The project aims at providing real time physiological and environmental information from remote areas and to develop specific algorithms to elaborate this information to permit a more accurate management of accidents in remote areas, based on an advanced bidirectional telemedicine concept.

The final goal, and also the original challenge of AVATAR, could be to realise a dedicated international control centre able to receive and manage physiological and environmental data received from remote areas and, supported by customized wearable technology, augmented and virtual reality and exposure monitoring electronic devices, to provide appropriate instructions to assist accident victims supported by real-time medical, environmental and exposure information. (Figure 1)

Experts know well the limits of assistance by phone-mediated communication and this prompts for more investment, dedicated projects and new solutions.

The participants in remote and adverse environment activities will be the first to profit from the AVATAR solution, as, in case of emergency, they can count on in-depth analysis of the environmental conditions where the emergency occurred, coupled with real-time physiological parameter analysis, with decreased diagnostic error possibility, less intervention delay, and improved treatment and emergency management quality.

Summing up the AVATAR project could:

1. **Improve** the quality of assistance for people in difficulty in remote areas
2. **Improve** a better differential diagnosis in case of symptoms of disease occurring in hostile environments and in the absence of medically trained persons on site
3. **Improve** the management of emergencies needing immediate treatment regarding both medical evacuation and cost optimization
4. **Reduce** the numbers of diagnostic errors due to non-correct information provided by the injured or bystanders
5. **Reduce** the serious consequences of any delay in proper diagnosis
6. **Reduce** the fatal cases and the disabling sequelae of remote emergencies and disease by a quick and correct diagnosis
7. **Reduce** the delay of appropriate treatment

During the three years of my Ph.D we developed the following aspects:

1. Developing and assembling devices allowing to monitor, recording and transmit physiological and environmental data
2. Developing the data base, the algorithms and the software allowing the AVATAR system function

3. Developing physiological scientific protocols to investigate the body adaptation to extreme environment and permit a correct interpretation of remotely collected data.

1.2 Project description

The AVATAR project aims at providing real time physiological and environmental information from remote areas, develop specific algorithms and to elaborate these information's to permit a more accurate management of accidents in remoteness. In this first phase the system is developed and test in diving activities, the most difficult hostile environment for data transmission, with the aim of using the acquired experience for subsequent development in less hostile environments.

The AVATAR approach developed for underwater use include: (Figure 1)

1. The wearable devices able to take physiological and environment data from the divers
2. A dedicate devices (dive sense) able to received data from wearable and transmit them to a dedicate data base using a nano acustic modem for underwater transmission and a surface buoy to collected the information and, by a GSM or satellite connection, transmit them worldwide
3. An data control center (AVATAR web portal) able to record big data, receive multiple information simultaneously and comparing all the peripheral information with the history of the diver and the entire diving community information available in the DAN Europe Data DB, ensuring a more in-depth decompression risk analysis as compared with the common diving computer by original dedicate algorithms that permit personalized analysis processes which can trigger emergency alerts, or give precious information to carry out accurate diagnosis if they were triggered automatically or by the diver him/herself or by the boat attendant.
4. An international medical diving emergency center 24/7 able to acquires real time emergency and to exchange information with the data base, to manage the emergency also assisted by an included underwater medical team. In addition to real time data available (which already represents an important opportunity to better manage diving remote emergency) the entire diving assistance can count on a bi-directional communication between divers and the medical diving emergency center,

guaranteeing an important additional support to management of the first aid process from differential diagnosis, correct hospitalization, optimizing it and minimizing the risk of mistakes.

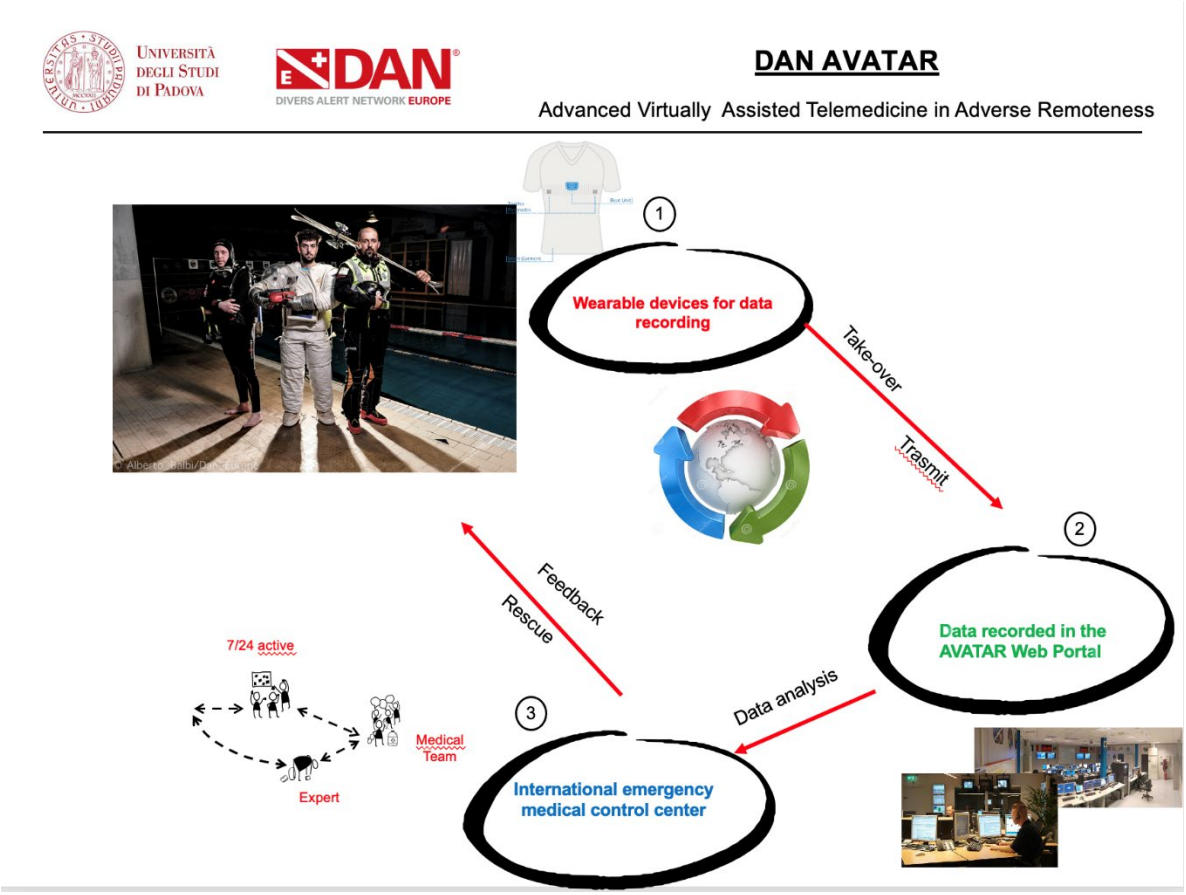

Figure 1 – AVATAR project scheme

Therefore, the AVATAR approach provides that the physiological and environmental information essential to start the process will be delivered through the use of innovative medical wearable devices and environmental sensors able to collect, transmit and manage data. The wearable monitoring systems based on smart garments, textile sensors, electronic devices and software is able to detect ECG, breath rate, blood oxygen saturation and position of body. (Figure 2). All data are available in real time through a dedicated application allowing the transmission from a smart phone directly to a control center on in case of underwater use using dedicate devices that including an acustic modem. (Figure 3)

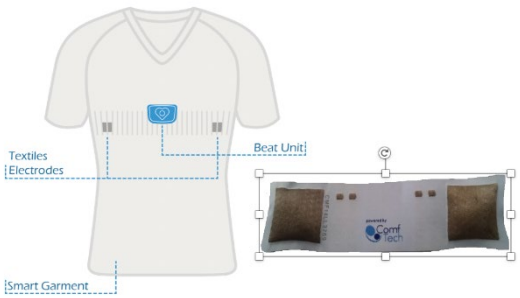

Figure 2 – Wearable T-shirt.

Environment data collection and physiological data transmission can be realized by different kinds of dedicated devices ensuring a preliminary data analysis to intercept risk condition and to produce automatic alert in case of need.

Figure 3- Dive sense prototype to record environment data.

Wearable devices can be defined as smart electronic devices (electronic device with micro-controllers) that are worn close to the surface of the skin, where they detect, analyze, and transmit information concerning physiological signals such as heart and breath rate, saturation, body temperature etc., and/or environmental data and that allow, in some cases, immediate feedback to the subject [3]. Wearable devices are already revolutionizing medicine through mobile and digital health by enabling continuous, longitudinal health monitoring outside clinical settings (telemedicine).

The use of wearable devices stimulated the development of algorithms for automated health event prediction, prevention and intervention. New wearables-based analytic platforms are developing online to improve quality and accessibility of healthcare everywhere from health care structures to home-care chronic disease management to resource-limited field settings [4]. Wearable sensors can be classified in three categories: mechanical, physiological and biochemical [5].

1. Wearable mechanical sensors (Figure 4) usually contain parts of inertial measurement units (IMUs) to estimate a subject's translational and rotational motion, applied forces and surrounding magnetic field. IMUs use biaxial or triaxial accelerometers to measure planar or 3D movement, respectively, gyroscopes to measure rotation and magnetometers to measure relative position. Accelerometers are most common in wearable devices, but gyroscopes are appropriate when it is necessary to differentiate acceleration due to gravity versus acceleration due to movement [6],

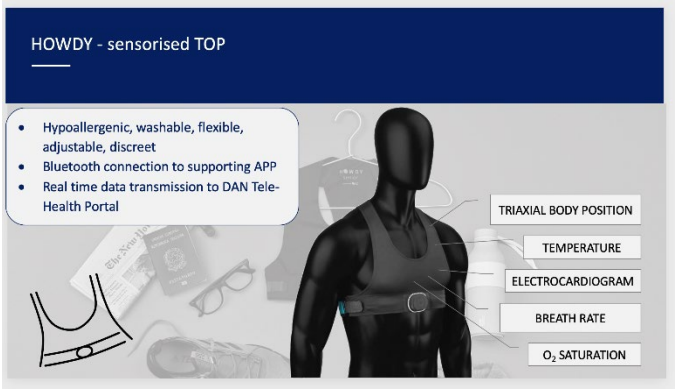

Figure 4 – Wearable mechanism sensors.

2. Physiological and biochemical sensors include biological recognition elements into the sensor operation (for example, enzyme, antibody, cell receptor or organelle). This element recognizes a specific target analyte and a physicochemical transducer (electrochemical, optical or mechanical) to translate this biorecognition event into a useful signal [7-11].

Wearable devices are useful tools to monitor health condition in several situations, from in-hospital and in-clinic care, to home ambulatory care as well as in remote areas including rural and low-resource environments.

Figure 5 – Data Transmission scheme.

One of the major difficulties is the elaboration and the management of large amounts of data: for this reason, specific algorithms are being developed to automatically process and interpret wearable-generated data in order to present evidence-based health insights such as detection of inflammation or prediction of cardiometabolic health based on activity habits [12, 13]. New analysis methods, especially machine learning, may contribute to expand the use of wearables into clinical applications ranging from detection of acute health events (infection/inflammation) to monitoring and management of chronic diseases (cardiovascular disease, diabetes) [12, 14]. The use of wearable technologies in healthcare structures can facilitate rapid triaging to provide immediate care to patients most in need, especially those in intensive care units (ICU) [15]. ICUs can benefit from algorithms that synthesize multimodal sensor data and tailor alarm systems and medical decision support systems. By continuously monitoring for dynamic changes of patient conditions, warning signs prior to health event escalation may provide the opportunity for early interventions to prevent/avoid critical health events [16, 17]. ICU monitoring techniques are often invasive such as intra-arterial continuous glucose monitoring (IA-CGM) and finger–stick glucose monitoring, may cause patient discomfort or result in adverse events [15]. Wearable technologies can reduce invasiveness and patient discomfort using, for example, a subcutaneous CGM electrochemical sensor, inserted in the abdomen adipose tissue [18]. Fitbit Charge devices have been tested to monitor heart rate (HR), arrhythmia detection and sleep measurements in postoperative ICU patients with results similar to gold standards [16, 19]. No ICU patients can be constantly kept “under watch” through multifunction monitors that record several parameters including BP, blood oxygen saturation (SpO_2), heart rate, respiration rate [20]. The wireless technology of new wearable sensors measuring vital parameters can improve patient comfort and mobility [21]. One of the most important challenges is the integration of the wearable technologies with the Clinical Decision Support (CDS) software [22]. CDS software are able to take clinical decisions [23]: an integration of wearables and CDS may improve the data sharing of clinical outcomes and analysis between clinicians and patients, providing information to remote physicians that enhances the clinical snapshot collected while the patient is in the hospital.

Outside the medical structures, wearable devices can also be useful tools to guarantee monitoring and real time analysis of physiological data, ensuring an appropriate remote management of patients [24] that are delocalized [25, 26] with respect to the medical team or with reduced mobility [27], finding application, in non-emergency related conditions, on old population (elderly care) often affected by chronic disease, thus ensuring a real time analyzed continuous monitoring [28]. In particular, elderly people show several risk

factors including chronic diseases [29-31], falls [32, 33], injuries [34] and cognitive impairments that range from dementia to Alzheimer disease [35]. However, elderly living in remote areas is in a less favorable environment for health care compared to those living in an urban environment. Healthcare facilities in remote areas are usually poor or have limited accessibility [36]. The benefits of wearable devices result in remote monitoring of physical activity, falls, or behavior of community-dwelling older adults and have been applied to change the lifestyle and reduce metabolic risk of older adults [37, 38]. Wearable devices are important instruments for healthcare professionals to continue to monitor those patients who have been discharged from hospital, especially during medical emergency such as the actual COVID-19 pandemic [39], as well as all those who result positive to SARS-CoV2 diagnostic test but who have symptoms that do not require hospitalization. This can relieve pressure on health care facilities and optimize hospitalization management.

The market of wearables for fitness and sport uses is continuously raising, especially for body weight and high-intensity interval training [40]. Prolonged and/or intense physical activity may lead to chronic fatigue, overreaching, overtraining and negative health effects [41]. For this reason, wearable devices can be useful noninvasive tools to monitor biological markers [41], analyzing and transmitting data concerning several internal and/or external variables to an external device and provide in some cases immediate biofeedback to the athlete. Among the great variety of relevant physical parameters, HR is one of the most important: this parameter can be split in HR during exercise (HRex), as well as recovery (HRR) and variability (HRV) of HR [42, 43]. Another important aspect of the physical activity is the hydration status since dehydration can compromise performance and is associated with several deleterious health consequences, including heat stroke [44] while overdrinking can result in hyponatremia, leading to fatigue, confusion, coma [45]. Athletes are exposed to temperature variations that can be associated to hyperthermia or hypothermia [46, 47]. Decrease of athlete's performance may be due to alteration of arterial blood saturation (SpO_2) [48], especially at altitudes where this value is lowered, and may also help to predict acute mountain sickness [49].

1.3 Project development

The AVATAR project was developed over three years; during which different aspects have been developed.

1.3.1 First year:

The beginning of the project was dedicated to investigate the state of the art regarding telemedicine in extreme environment and to developing the first prototypes of wearable devices and dive sense.

During the first year we also performed the first tests bout the use of wearable device to investigate system's strengths and weaknesses, to develop the preliminary dedicated algorithms and evaluate the accuracy of measurement.

On the first year we also started physiological tests about the body modification during scuba diving.

1.3.1.1 Experiments year 1

5/11/2019- 6/11/2019: the wearable devices tests during the dives were carried out in the deepest pool in the world, Y-40 The Deep Joy in Montegrotto Terme (PD). The device has been also tested in a special hyperbaric chamber up to a depth of 100m.

2/12/2019-9/12/2019: In the second test session, we inserted a 2G modem with dedicated SIM for data transmission and tested the system during a diving week in the Maldives that are by definition one of the most frequented remote destinations by divers and where the hospitals as well as the hyperbaric chambers are very far from the diving points. Seventeen subjects participated who made 3 dives a day for a week were tested. In this second phase, using the 2/3 G network coverage, the data were sent to the ALTEC control center, usually dedicated to logistic services and support operations for the International Space Station.

20/1/2020-24/1/2020: Given the plasticity of the system, it was natural to use avatars even in medical contexts and for this reason we study the possibility of using wearable T-shirts for screening subjects potentially affected by obstructive sleep apnea (OSA) (Figure 6) [50]. The t-shirts in their standard configuration are able to record the electrical activity of the heart (ECG), body movements in space, chest movements and respiratory rate. In particular, our attention focused on the study of the accelerometer included in the t-shirt as a possible actigraphy instrument specifically dedicated to recognize apnoea phenomena, aware that in the absence of other signals such as nasal flow or signals of abdominal movements it is not possible to establish the obstructive or central nature of apnoea but also aware that in the context of a screening this aspect can be delegated to a second phase of differential diagnosis by polygraphy. Actigraphy is a technique for measuring and recording motor activity used to determine rest-activity cycles and can therefore be applied to the study of circadian rhythms and the measurement and evaluation of certain aspects of sleep.

Figure 6 – Experimental OSA wearable system.

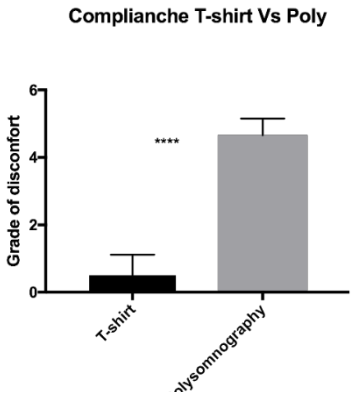

Figure 7 – Comparison of compliance of wearable T-shirt and traditional polysomnography

This specific study allowed us to investigate whether these instruments could generate a discomfort in subjects during slip and indirectly also in sport activates. The degree of discomfort of wearable instruments vs classic polygraphs was investigated. The t-shirts in fact also aim at a minimum interference with normal sleep due to the fact that the sensors integrated inside normal t-shirts are also textile in nature and therefore do not generate any kind of encumbrance being part of the fabric that constitutes it, on the other hand the t-shirts are very small in size to ensure, with their elasticity, the contact of the sensors with the skin of the patient and this aspect raises doubts about the

real comfort of the instrument. Finally, the absence of electromagnetic interference in the simultaneous use of the two devices was investigated.

1.3.1.2 Test on COVID-19 emergency

The AVATAR approach was also investigated during the current COVID-19 pandemic [51-53], AVATAR was tested in a real application for monitoring patients affected by COVID-19 in home isolation. Three subjects, all with moderate symptoms, were monitored for the entire period of isolation by healthcare professionals without any direct contact with the victim and reducing the infection risk. During the tests AVATAR provided an advanced physiological data transmission method permitting telemedicine assistance and allowing for more sophisticated analysis and customized medical care, ensuring an efficient and valuable service also perceived by patients as a direct line with emergency care services. In this first test AVATAR, albeit in a preliminary version, guarantee an effective monitoring and real time analysis of physiological data, ensuring an appropriate remote management of patients that are delocalized with respect to the medical team and/or with reduced mobility. This test indicates how AVATAR can also find application, in non-emergency related conditions, on old populations often affected by chronic disease ensuring continuous monitoring with real time analysis. One of the most important strengths showed by AVATAR during the test was represented by the possibility to interact in a bidirectional way with a control centre using a normal smartphone APP. Through this dedicated application it is possible to transmit, in addition to physiological parameters, real-time images and videos to facilitate the work of medical staff, reducing diagnostic errors and providing useful and timely information to the staff of the nearest hospital in case of need for hospitalization.

1.3.2 Second year:

During the second year we assemble the different part of the AVATAR system and we have set up the various communication systems in the different environments. Operative test in conditions of simulated emergency were performed related to the efficiency of hardware firmware, app and software.

1.3.2.1 Experiments 2 year

27 - 28 March 2021: *Santa Margherita Ligure; Field Test about Underwater Telemedicine application in technical divers (Global Underwater Explorers-GUE Group)*

24 - 25 April 2021: *Finale Ligure; Field Test about Underwater Telemedicine application in technical divers (Global Underwater Explorers-GUE Group)*

16 - 17 April 2021: *Y-40 the deep swimming pool; Field Test about diving after COVID-19*

14 - 15 May 2021: *Y-40 the deep swimming pool; Field Test about diving after COVID-19*

11 - 12 June 2021: *Y-40 the deep swimming pool; Field Test about diving after COVID-19*

09 - 10 July 2021 *Y-40 the deep swimming pool; Field Test about diving after COVID-19*

14 - 15 May 2021 *Y-40 the deep swimming pool; Field Test about real time underwater environmental data transmission*

09 - 10 July 2021: *Y-40 the deep swimming pool; Field Test about real time underwater physiological data transmission*

1.3.3 Third year:

In the last year we developed and actually made ten (10) complete working prototypes (Figure 8). These devices are able to record (in a solid-state memory) and transmit (via Acoustic Modem) both environmental (pressure, temperature and diving time) and physiological (heart rate, RR-Time and breathing rate) data.

Figure 8 – Working prototype for AVATAR system.

The system was tested several times on scuba divers equipped with a wearable device based on the smart garments, textile sensors, electronic devices and software able to detect ECG, breath rate, blood oxygen saturation and body position and transmit from the electronic device worn under a dry suit to a scuba unit outside the dry suit by BLE (Bluetooth Low Energy). The scuba unit in the vinal version has been developed to include a microcontroller, an acoustic modem, a pressure and temperature sensor, 64MB flash memory and a real time clock. The microcontroller integrates a dual-core 32 bits microprocessor and a 2.4GHz programmable transceiver. Sensor, memory and real-time clock are “off the shelf” components. The acoustic modem uses a piezoelectric ring and a special modulation to transmit and receive data. The range is up to 500m. The last unit is a buoy at the surface able to receive data from the scuba unit and send them via BLE to a smartphone through a custom-made APP.

This APP stores data (Figure 9) locally and sends information to a remote server using Wi-Fi or mobile data.

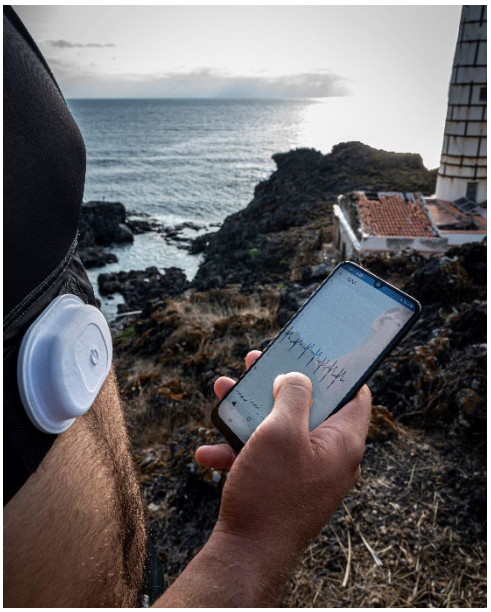

Figure 9 – Phone APP to send data.

The anchor can also work without smartphone and APP using UMTS transceiver or satellite modem.

The remote server uses a web application to follow scuba divers during diving and to show data to remote user (doctors, expedition chief, etc.). Every 10 seconds the scuba unit sends environmental and physiological data to the anchor in different data-packets.

With this system we have monitored 10 scuba divers from the first jump in the water until they reached the surface (Figure 10).

Figure 10 – Example of SCUBA diver monitoring.

The second effort in the third year was to perform physiological tests to acquire real time environmental and physiological information from a large number of divers to guarantee adequate knowledge and correct interpretation to achieve our goal of personalized assistance in case of emergency.

For this reason, a large part of our work over the last three years has been spent on research to improve knowledge about human physiological changes during exposure to extreme environmental conditions in Underwater Diving and in high altitude. In order to achieve the goal, we used the developed tele-monitoring system to collected data during several physiological tests. (Figure 11)

The system includes satellite geo-location.

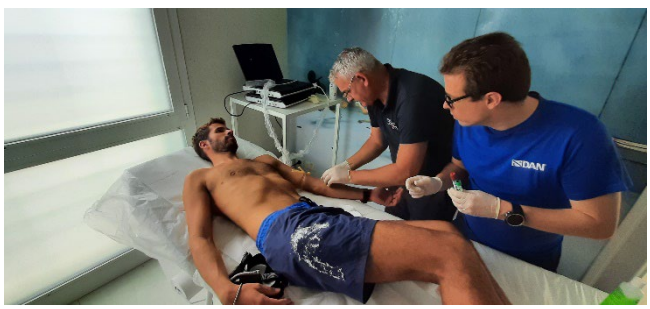

Figure 11 two moment of SCUBA diver monitoring.

During the last three months spent in the Motor Sciences Lab - Université Libre De Bruxelles, we performed several tests focuses on the aims of the project specially to include new sensors inside a full-face mask. We realized a new device able to record oxygen saturation and performed new physiological tests to compare NOx production and oxidative stress in subjects diving using normal masks Vs subjects diving with full-face masks. The results are really interesting and we wrote a new original paper "Endothelium disfunction and oxidative stress in full-face mask diving" (see below).

1.3.3.1 Experimental plan (3 years)

- 24-25-25 September 2021: *Abetone; field test about the use of real time monitoring at high altitude. Event: Mega-Cod ski patrol 118 Tuscany.*
- 15 e 16 October 2021: *Y-40 the deep swimming pool; field test about diving after COVID-19.*
- 19 e 20 November 2021: *Y-40 the deep swimming pool; field test about diving after COVID-19.*
- 2-3- 4-5-6 December 2021: *Dubai Deep Dive Dubai swimming pool; field test about Under water telemedicine application in technical divers (Global Underwater Explorers-GUE Group).*
- 4-5-6 January 2022: *Lavarone; field test about the use of real time monitoring at high altitude. Event: diving Under ice.*
- 13-17 May 2022: *Dubai Deep Dive Dubai swimming pool; field test about Under water telemedicine application in technical divers (Global Underwater Explorers-GUE Group). "During the visit abroad".*
- June 20 2022: *Y-40 the deep swimming pool; field test about underwater blood draw and lung echocardiography.*

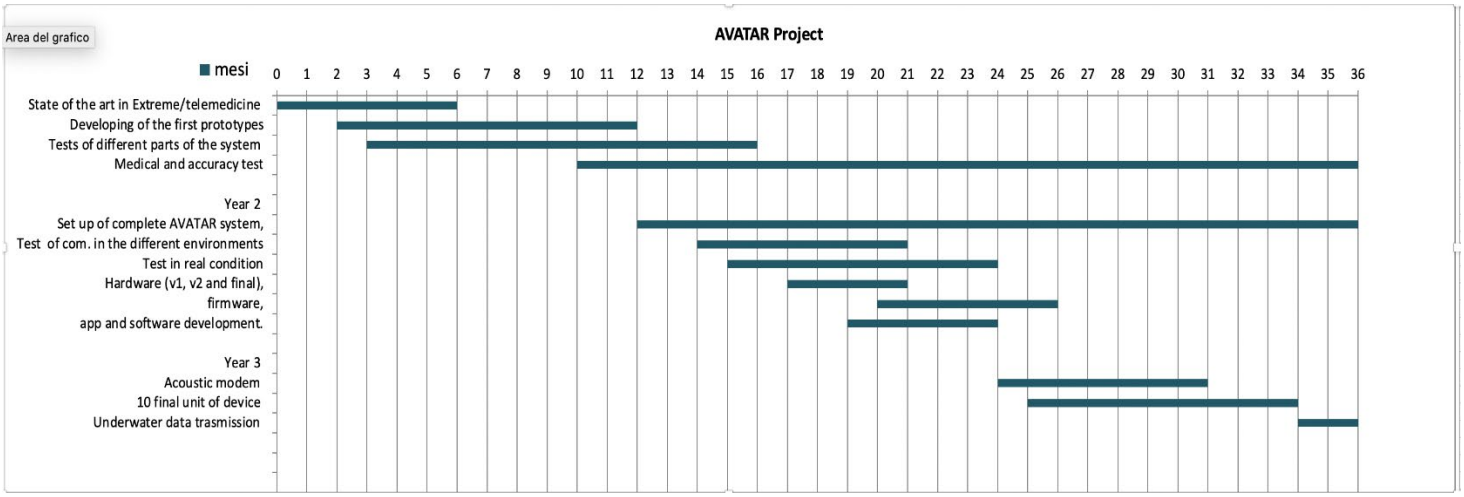

Figure 11 – Gantt chart of experimental plan of this Ph.D project

The preliminary final results of AVATAR project are included in the following papers under submission.

1.4 Advanced Virtual Assisted Telemedicine in Adverse Remoteness (paper) (Under submission)

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1.4.1 Introduction

Wearable devices can be defined as smart electronic devices (electronic device with micro-controllers) that are worn close to the surface of the skin, where they detect, analyze, and transmit information concerning signals such as vital signs (heart and breath rate, O_2 saturation), and/or environmental data and which allow in some cases immediate feedback to the subject [3]. Wearable devices are already revolutionizing medicine through mobile and digital health by enabling continuous, longitudinal health monitoring outside clinics or hospitals (telemedicine).

Furthermore, prolonged and/or intense physical activity may lead to chronic fatigue, overtraining and negative health effects [34, 41], this effect can have greater importance for athletic performance in extreme environments. For this reason, wearable devices could be useful noninvasive tools to monitor biological markers [41], analyze and transmit physiological and environmental data from subjects engaging in sports or activities in remote environments, thus guaranteeing an immediate biofeedback in case of accident and permitting remote assistance by a dedicated delocalized medical team. Among the great variety of relevant physical parameters, HR is one of the most important: this parameter can be split in HR during exercise (HRex), as well as recovery (HRR) and variability (HRV) of HR [42, 43]. Another important aspect of the physical activity in extreme conditions is the temperature variations that can be associated to hyperthermia or hypothermia [46, 47] and the arterial blood saturation (SpO_2) often linked to the athlete's performance reduction. [48] At altitude and in scuba diving this value can be very important and may help to predict acute mountain sickness or decompression sickness [49]. The AVATAR project aims at developing and assemble wearable devices able to record and send data in real time from the subject to a dedicated medical control center, BUT with the peculiarity to be also work in extreme environments, and particularly underwater, allowing for a real time wireless communication.

The scope of this paper is to show the results of the AVATAR approach related to the use of the wearable devices in the underwater environment, by assuring that data arrival to the control center is not affected by

any magnetic interferences and that received data can be used by medical teams for proper accident/disease management accident even in remote and hostile areas.

1.4.2 Materials and Methods

1.4.2.1 Subjects and Diving Procedures

A total of 10 expert SCUBA of different groups including military special task force divers belonging to Gruppo di Intervento Speciale (GIS) of Italian Carabinieri were investigated during a single dive in seven different diving conditions. All divers received an explanation of the study's purposes, risks and benefits, were familiarized with the experimental protocol and read and signed a specific informed consent form before the experiment. The study was conducted in accordance with the Helsinki Declaration and was approved by the Ethical Committee of Università degli Studi di Milano, Italy (Aut. n° 37/17).

No diver performed any compressed-gas diving or any breath- hold diving during 30 days before the day of the experiment. The diving parameters (depth, diving time and Gradient Factor) were recorded for each dive using a diving computer with sampling rate set at 10 s. The Maximum Gradient Factor value is generally reached at the end of the dive. The Gradient Factor measures the inert gas load in the diver's tissues, according to the selected decompression algorithm. In our protocol, the maximum Gradient Factor, expressed as a percentage of the M-value, is computed using the Buhlmann ZHL16 C model. This is a way to estimate inert gas supersaturation and to compare diving exposure in the different investigated subjects [54].

1.4.2.2 Materials and Protocol

We organized six tests, increasing the environmental difficulties and the depth from time to time and preceded by an identical test in a controlled room, repeated three times (control).

In particular we effected one test with the divers lying down on an examination bed, three dives at shallow depth (10 meters), one indoor and two outdoor, and three at increasing depth (40, 60 and 75 meters), two indoor and the final one outdoor.

In all cases divers used a dry suit and dived (or laid on the bed) for 60 minutes minimum, exceeded only to respect mandatory decompression time.

Diver tests were done in these different locations:

1. In the Y-40 swimming pool in an isolated room
2. In the Y-40 swimming pool in the 10 meters depth area
3. In the Venice lagoon, 10 meters depth
4. At Elba Island, 10 meters depth
5. In Y-40 swimming pool, 40 meters depth at the bottom
6. In the Deep Dive Dubai pool, 60 meters depth, at the bottom
7. In Sicily, 75 meters depth

A T-shirt with sensors to record ECG, breath rate and posture (Comftech srl) was worn by each diver under the dry suit.

The ECG, posture and respiratory rhythm signals were acquired by an electronic device, equipped with an electronic board, a microcontroller and a low energy Bluetooth transceiver (BLE).

The ECG signal is acquired through three textile electrodes, processed and digitally filtered by the microcontroller, the respiratory rate is measured by a "strain gauge" placed inside the T-shirt and the posture is read by means of a triaxial accelerometer: the signal is identified among the three positions where the gravitational acceleration is present.

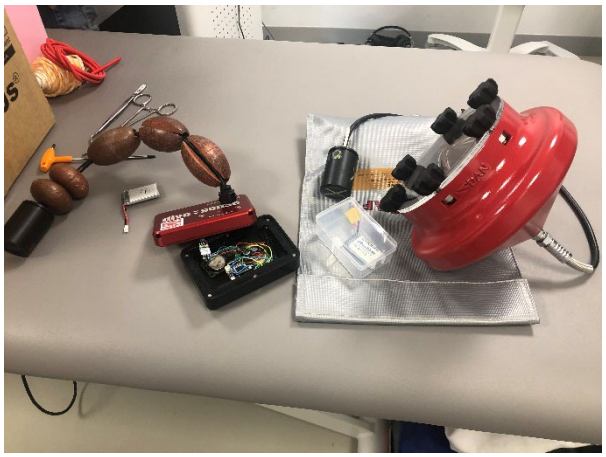

Figure 12 – Electronic equipment for underwater data transmission

Figure 13 – Military Task force divers at Elba Island

With our wearables we record 128 data every second, one data every 7.8125 milliseconds, ECG data are transmitted at every recording (7.8125 milliseconds), while both respiratory rate and position only when the parameter itself changes. At this point data were sent to a dedicated device called "Dive Sense" positioned outside the dry suit and including an acoustic modem to wirelessly send data to the surface.

The acoustic modem exploits the reverse piezoelectric effect (Lippmann effect): if a piezoelectric material is stimulated by an electrical source of, it deforms elastically and produces a vibration. This electrical stimulation induces a vibration at a frequency ranging from 24kHz to 32kHz which allows the modem to send data (Figure 12). The acoustic modem consists of a microcontroller circuit and a medium voltage generator (about 200V) used to vibrate a ring made of piezoelectric material. The maximum achievable data rate is 463bits/s and the range is up to 2km. The modulation used is a BPSK (Phase-Shift Keying two-phase).

At the surface the signal is received by a dedicated devices called “Boa”, using GSM or satellite connection to send data to a dedicated web portal called “DANA Health” that receives Real Time (streaming) Wireless Underwater Dive & Physiological Data and provides the possibility to show, analyze and elaborate data.

1.4.2.3 Signal quality evaluation

Two Electronic Engineers and two Emergency Medicine Specialists, unaware of the protocol and the nature of the received signals sat at the dedicated center and analyzed (in streaming) the arriving data and the quality of the signal for the entire duration of the test.

The 2 engineers were instructed to fill in a grid where to indicate correct signal reception every 30 seconds (yes or not), while the two medical doctors were instructed to indicate the presence of inconsistent physiological or possible artifacts every 30 seconds (e.g. heart rate or respiratory data outside normal physiological possible ranges).

1.4.3 Results

During all the test the signals were properly recorded and sent, all the steps of the protocol were respected and the data were constantly available - in streaming - at the medical control center.

As reported in Figure 14, We did not find statistically significant differences in signal reception and physiological congruity between the control groups and the six different investigated diving conditions. When also adding the received diving data and comparing to the control groups no significant differences were found. Concerning signal reception, and considering the double rating, only 55 moments (110/2) on a total of 720 recordings, were not received in streaming while regarding physiological congruity only (36/2) 18 data were considered not congruous.

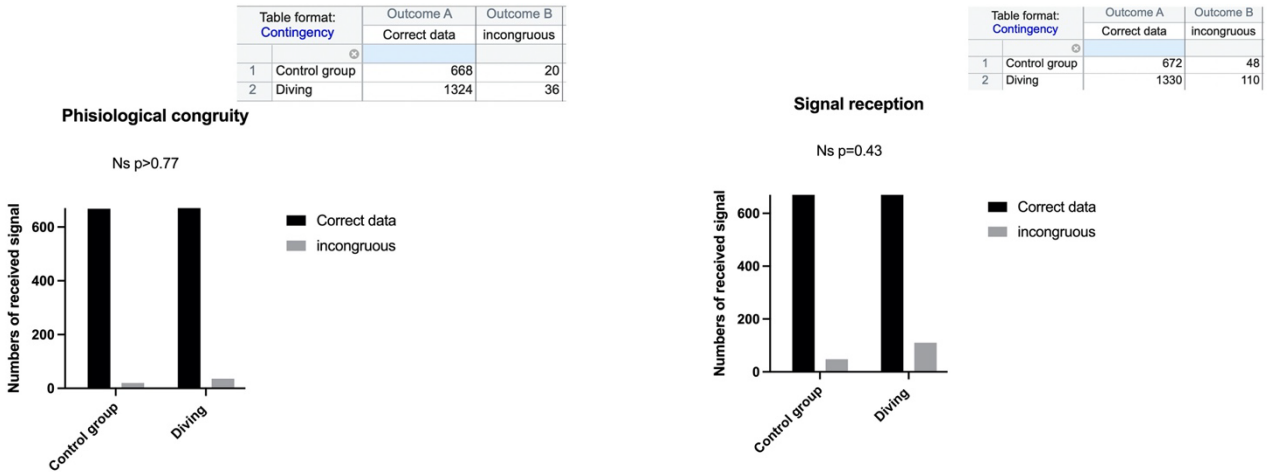

Figure 14 – Diving Data and Signal reception.

1.4.4 Discussion

Our preliminary data seem to indicate a high accuracy in the streaming transmission of physiological and environmental data recorded during SCUBA diving and sent to a control center with 4 different devices. Data were recorded by wearable devices characterized by textile sensor (first step), sent to an underwater device called Dive Sense (second step) transmitting to the surface by an acoustic modem, where data were received by a "Boa" (third step) that finally sent data by GSM or satellite connection to a web portal (fourth step) capable of diving and physiological data analysis, and to provide immediate feedback to the diver and to assure real time availability for consultation by a dedicated medical control center. Despite the real difficulty to manage data in such a complex context (water) and the difficulty to use four different devices to guarantee the data transmission from depth to surface and from there to the AVATAR web portal, data constantly arrived with a very high frequency to the final web portal. Only the 7,6% of data (monitored in packages of ten second) did not arrive, but this percentage was similar (6.67%) to the unreceived data not arrived when recording in a dedicated room with divers lying on a bed and the four devices all positioned close together with ease of transmission. Also, the physiological data, heart rate, breath rate and body position, as evaluated the two medical doctor, showed discrepancies only in 2.65 % of packages, thus assuring the possibility to use real time underwater data transmission to manage accidents occur in adverse / remote environments where victims cannot be reached rapidly.

This new holistic approach, that we called AVATAR, also opens a new path to assure advanced telemedicine support for sport activities in extreme areas and will permit to:

- Improve a better differential diagnosis in case of diving injuries, even if they happen in hostile environments and in the absence of medically trained individuals on site
- Improve the management of diving emergencies needing immediate treatment for both medical evacuation to a hyperbaric facility and cost optimization
- Reduce the numbers of diagnostic errors due to non-correct information provided by bystanders
- Reduce serious consequences of any delay in proper diagnosis by improving rescue management and first aid quality.
- Reduce fatal outcomes and disabling sequelae of decompression illness by a quick and more correct diagnosis
- Reduce the delay of appropriate treatment
- Reduce social and legal costs of improper diagnosis

The evolution of this service, enhanced by real time data collection of physiological parameters transmitted by innovative telemedicine protocols will enable more sophisticated analyses, customized medical care, and

therefore an even more efficient and valuable service as perceived by divers as well as by participants in extreme activities and involved healthcare personnel.

It is well known that the greatest limit of the current Emergency Medical Services assistance in remote areas is due to the fact that the entire emergency can only be initially handled only counting on information communicated by telephone. The development of the AVATAR project's solution is also now possible thanks to the development of the efficiency of satellite networks, allowing for worldwide information also in case of poor GSM coverage and this can really open a new era in diving and remote emergency management.

AVATAR could also relieve divers', and free divers' anxiety and fear of suffering diving related permanent damages, with special attention to the riskiest one: decompression sickness and its counterpart Taravana for free divers, that can often result in a fatal outcome if not promptly and correctly managed. Epidemiologically speaking, all this can imply a positive impact on a potential of 35 million people all over the world, when only considering divers.

1.5 Return to SCUBA diving after COVID-19 (Paper) (Under submission)

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1.5.1 Introduction

COVID-19 is a disease, known as severe acute respiratory syndrome, caused by SARS-CoV-2, a virus that targets not only the lungs but also the cardiovascular system, and has resulted in a global pandemic. COVID-19 alters microcirculatory disturbance associated with thrombus formation contributing to respiratory dysfunction and leading to endothelial dysfunction [55]. Normal endothelial functions are typically regulated by nitric oxide (NO) and include vascular tone, permeability, cell adhesion, and anticoagulation [56]. SARS-CoV-2 can suppress endothelial nitric oxide synthase (eNOS) production leading to NO deficiency [57] and thrombus generation [58]. NO seems to be an important intra- and extracellular mediator in asthma, but also in lung fibrogenesis [59]. Exhaled NO fraction (FeNO) has been proposed as marker to evaluate the potential persistence of alveolar and bronchiolar inflammation in post COVID-19 patients: some authors found higher

FeNO value in subjects who had COVID-19 with respect to those who did not [60]. NO is an important signaling molecule involved in the modulation of inflammatory tone in airways and may also be produced through the inducible NO synthase (iNOS) expressed by alveolar macrophages and pneumocytes [61].

NO reacts with some ROS to produce other free radicals that inactivate some NO synthases [62], reducing NO availability and exacerbating oxidative stress condition. To control oxidative stress due to increased O_2 level associated with hyperbaric conditions during the dive, divers can activate the endogenous antioxidant system [63, 64]. COVID-19 may cause low oxygen saturation (SpO_2)[65]. This reduced saturation promotes ROS production [66], especially hydroxyl radical ($OH^{\bullet}$), an extremely toxic agent that increases the formation of cell membrane lipid peroxides and protein oxidation, triggering cell death by apoptosis and/or necrosis [67]. Mitochondria impairment leads to cytopathic hypoxia with ROS generation and lower ATP production [68, 69]. ROS exacerbate the expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα, and iNOS via activation of the NF-κB pathway [70, 71]. These cytokines activate leucocytes to generate more free radicals [70]. This interaction between ROS and cytokines leads to a self-sustaining cycle between cytokine storm and oxidative stress that may bring COVID-19 patients to multiorgan failure [72].

SCUBA diving exposes the human body to environmental stress including physical effort efforts and breathing resistance [73].

The cardio-respiratory examination may reveal signs suggestive of costochondritis or musculoskeletal pain. Musculoskeletal symptoms are common in patients with COVID-19: myalgia, arthralgia, and fatigue are the most common musculoskeletal symptoms [74-76]. CK values have been found to raise in subjects as a consequence of Sars-CoV-2 infection, with strong relation with disease severity, markers of inflammation [77], and myopathic involvement of skeletal and respiratory muscles [78]. . In COVID-19 subjects, AST increases can be fully explained by summing the effects of hepatocellular injury with muscle damage. but it is quite difficult to establish whether the nature of the liver injury is directly mediated by the virus or caused by concurrent factors including immune-mediated damage due to the hyperinflammatory response caused by the SARS-CoV-2 infection, respiratory failure related anoxia or liver damage by drug treatment [79]. ALT, a specific liver injury marker, raises in survivors with slightly more elevated values than non-survivors, probably as consequence of side effect of pharmacological treatments [79]. Severe COVID-19 infection can release great amount of LDH from lung tissue as a severe form of interstitial pneumonia, often evolving into acute respiratory distress syndrome. Elevated LDH levels might reflect that the COVID-19 related multiple organ injury/failure seems to play a more prominent role in this pathology influencing the clinical outcomes in patients [80]. Subjects with severe COVID-19 show hemodynamic instability, as well as several cardiovascular complications including cardiogenic shock [81], myocardial injury and myocarditis or signs of heart failure [82]. In these patients, CK-MB, myoglobin, and troponin I are more significantly elevated in long-term hospitalization than short-term hospitalization [83]. Cortisol values raise in severe COVID-19 illness and it has been suggested as a potential risk factor for severe outcome and death [84]. Raised cortisol

seems to be related to immune system regulation: similarly to Sars-Cov [85], Sars-Cov-2 might activate an immunogenic response to adrenocorticotropic hormone: an insufficient cortisol release may increase the risk of morbidity and mortality [86].

It has been proposed that SARS-CoV-2 induces mitochondrial dysfunction, NADPH-oxidase activation and oxidative stress, and that therefore can initiate a feedback loop promoting a chronic state of inflammation and/or endothelial dysfunction [87].

At the moment, there is no clear, evidence-based way to guide return to SCUBA diving, but some guidelines based on a prudent approach that should be gradual, personalized and based on subjective tolerance.

The following "advice" guidelines have been issued the Divers Alert Network (DAN) Europe:

«COVID» Divers, as long as not suffering impairing disease-related sequelae, although not presenting particularly serious contraindications to Scuba Diving, appear to be:

1) more susceptible to diving related oxidative (possibly also inflammatory) stress
2) less efficient with compensatory chemical reduction mechanisms (NO recovery)

COVID divers might prefer conservative Dive Profiles, such as the Low Bubble Profiles suggested for PFO, at least until oxidative stress markers return to normal levels [88].

The aims of this study were:

1) To investigate pulmonary function, NO derivatives (NOx), oxidative stress and cardio muscular markers and physiological parameters (such as Electrocardiogram, breathing rate, body position, oxygen saturation and body temperature) in divers who had COVID-19 to produce guidelines for return to diving safety.
2) To record the physiological parameters requested by the protocol using the new AVATAR system, testing the system in its final configuration.

1.5.2 Material and methods

1.5.2.1 Subjects

A total of 24 expert SCUBA divers (20 male and 4 female), who suffered COVID-19, were studied during three No-Decompression (no mandatory decompression stops) dives: the first at -10m, the second at -20m and the third at -40m. Nine male divers who had not suffered from COVID-19 served as control group. No subject reported previous episodes of decompression illness (DCI), historical or clinical evidence of arterial hypertension, cardiac, pulmonary or any other significant disease, none of them took prescription drugs, suffered any acute disease during the 15 days before the experiment, or reported assumption of anti-inflammatory drugs and exposure or high altitude in the 7 days before the experiment. All the divers received an explanation of the study's purposes, risks and benefits, were familiarized with the experimental protocol and read and signed a specific informed consent form before the experiment.

The diving parameters (depth, diving time and Gradient Factor) were recorded for each dive using a diving computer compatible with the AVATAR system, with a sampling rate set of 10 s (Ratio IX3M2). All the dive

profiles were downloaded automatically by a dedicated App upon surfacing, according to the AVATAR project protocol, and immediately sent to the AVATAR web portal so to calculate the Maximum Inert Gas Supersaturation Gradients (Gradient Factor - GF) reached during the dive and at Surfacing. The Gradient Factor measures the inert gas load in the diver's tissues, according to the selected decompression algorithm. In our protocol, the Maximum Gradient Factor, expressed as a percentage of the M-value, is computed using the Buhlmann ZHL16 C model. This is a way to estimate inert gas supersaturation and to compare diving exposure in the different investigated subjects [54].

Data are recorded in the AVATAR web portal where new decompression algorithms, developed during the AVATAR project, were tested for their abilities to identify new decompression sickness (DCS) or decompression stress markers.

1.5.2.2 Blood sample analysis protocol

We collected blood samples in three different tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, United States):

- one containing EDTA
- one containing Lithium heparin
- one containing citrate gel for serum analysis

Blood collection and physiological data were recorded as follows:

- Basal: 30 min before the dive
- T1: 30 minutes after surfacing
- T2: 30 minutes after T1

For each time point, 3 test tubes were used, as described above.

Plasma/serum was separated from the cell component by centrifugation (3,000 rpm for 10 min) immediately after the blood draw and was refrigerated at -20 °C until use, according to the recommendations by Firuzi et al [89].

1.5.2.3 Physiological analysis protocol

1.5.2.3.1 Exhaled nitric oxide Fraction (FeNO)

FeNO: Exhaled FeNO was measured (Basal, T1, T2) using a FeNO Breath portable analyzer (Bedfont Scientific, Maidstone, UK). Divers expired into a disposable mouthpiece connected to the instrument for 10s. Results are expressed as FeNO ppb.

1.5.2.3.2 Nitrate + nitrite (NOx) determination

Divers were investigated for the NOx plasma level repeated for the three times specified in the protocol. Before the analysis, 400 ml of sample were treated with 400 ml of acetonitrile [90] to precipitate proteins, and

then centrifuged at 12,000 rpm for 10 min. NOx were measured in the de-proteinised plasma. The method for detection of plasma NOx was based on Griess's reaction as index of NO
concentration [91]. Standard solutions of $NaNO_3$ in a concentration range from 5 to 200 mM were placed in a 96 wells polystyrene microplate to build the standard curve. The diluting medium was used as the standard blank. After loading the plate with standard solution or sample, addition of 100 ml 51 mM VCl_3 to each well was followed by 25 ml of 87 mM sulfanilamide and 25 ml of 1 mg/ml N-(1-Naphthyl) ethylenediamine. After 50 min incubation at 55 °C, the optical density was read at 540 nm in a Sunrise microplate reader (TECAN, Salzburg, Austria). Plasma NOx levels were obtained by interpolation of standard nitrate curve.
As concerning saturation divers and sailing subjects, NOx concentrations were assessed in urine via a colorimetric method based on the Griess reaction, using a commercial kit (Cayman, BertinPharma, Montigny le Bretonneux, France) [92].

1.5.2.3.3 Reactive oxygen species (ROS) generation
An X-band electron paramagnetic resonance spectroscopy (9.3 GHz) (E-Scan, Bruker Co., MA, USA) was used to detect ROS production and TAC values. Saliva samples were stabilized at 37 °C using a Temperature and Gas Controller "Bio III" unit (Noxigen Science Transfer & Diagnostics GmbH, Germany), interfaced with the E-Scan. ROS production and TAC assessment methods were previously described [92, 93].
1.5.2.3.4 Total antioxidant capacity (TAC) evaluation
TAC value changes were evaluated using Trolox equivalent antioxidant capacity (TEAC) assay according to Re et al. [94] with slight modifications. The TEAC Test is based on the reaction with the colored and relatively persistent 2,20- azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\circ}$) radical cation, which has a strong absorption band at 734 nm. The antioxidant activity is defined as the amount of $ABTS^{+\circ}$ quenched after a fixed time and is compared with that produced by Trolox [94]. ABTS was dissolved in 10 mM phosphate buffer (pH = 7.4) to give a 14 mM solution. Potassium persulfate was dissolved in water to give a 4.9 mM solution. ABTS radical cation ($ABTS^{+\circ}$) was produced by mixing the same volumes of ABTS and potassium persulfate stock solutions and allowing the mixture to stand in the dark at room temperature for 16 h before use. Standard solutions of Trolox in a concentration range from 5 to 100 mM were prepared to build the calibration curve. The $ABTS^{+\circ}$ solution was diluted with phosphate buffer to an absorbance of 0.700 ± 0.02 at 734 nm. After addition of 1.5 ml of diluted ABTSC solution (A734 = 0.700 ± 0.02) to 30 ml of plasma or Trolox standard (final concentration 0.5–20 µM), the absorbance was taken after 20 min of incubation at 25 °C, using a Uvikon 931 UV-VIS Spectrophotometer (Northstar Scientific, Bardsey, United Kingdom). The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of the concentration of antioxidants and of Trolox for the standard reference data. Results were expressed as Trolox equivalents (µmol/l).

1.5.2.3.5 8-Isoprostane

Lipid peroxidation was assessed by competitive immunoassay measuring 8-isoprostane concentration (8-iso-PGF2α) (Cayman Chemical, USA). The method was previously described [95].

1.5.2.3.6 Cardio muscular markers

After 15 min and before 30 min from collection at room temperature, blood samples were centrifuged (3000 rpm for 10 min) to separate serum from cell fraction and was frozen at -20°C. Then, the serum samples were delivered to the laboratory for analysis and kept at -20 °C until analysis.

Creatine kinase (CK), aspartate transaminase (AST), alanine aminotransferase (ALT), creatine kinase isoenzyme (CK-MBm), cardiac troponin I (cTnI) and lactate dehydrogenase (LDH) were measured in serum samples. Serum samples were de-frozen, at room temperature shacked with the Vortex for 5 s for homogenization before the analysis. Briefly, 500 µl of each sample were placed in a plastic test tube. The test tubes were allocated in the auto sampler of a VITROS® 5600 (Ortho-Clinical Diagnostics, High Wycombe, United Kingdom). The reported total imprecision was <2.8 %, while the intra assay CV % was <1.8 %. The reference value of the laboratory tests is the following: CK 30-135 U/L, AST 17-59 U/L, ALT <50 U/L, CK-MBm <6.73 ng/mL (male) and <3.77 ng/mL (female), LDH 120-146 U/L.

1.5.2.3.7 Echocardiography Protocol

Echocardiography was done before the dive and within 30 min maximum after the dive. All echocardiographies were made using a commercially available instrument (MyLab 5, Esaote SPA, Florence, Italy) with a cardiac probe (2.5–3.5 MHz). All echocardiograms were performed with the subject lying on the left side and breathing normally: recording time was 20 s, and all frames were saved in the hard drive for subsequent analysis. Bubbles were graded according to the Eftedal and Brubakk (EB) scale, and with the consensus guideline of ultrasound use in diving research, as follows [96, 97]:

0 – no bubbles;

1 – occasional bubbles;

2 – at least one bubble per 4 heart cycles;

3 – at least one bubble per cycle;

4 – continuous bubbling;

5 – “white out”; impossible to see individual bubbles.

After grading the divers, they were divided into two groups: subjects not showing bubbles or only solitary bubbles, nonbubblers (NB), and subjects showing consistent bubbles degree 2 or higher (B).

Ultrasound lung comets (ULCs) were detected by chest sonography according to Frassi et al. [98].

1.5.2.3.8 Wearable Sensors Protocol

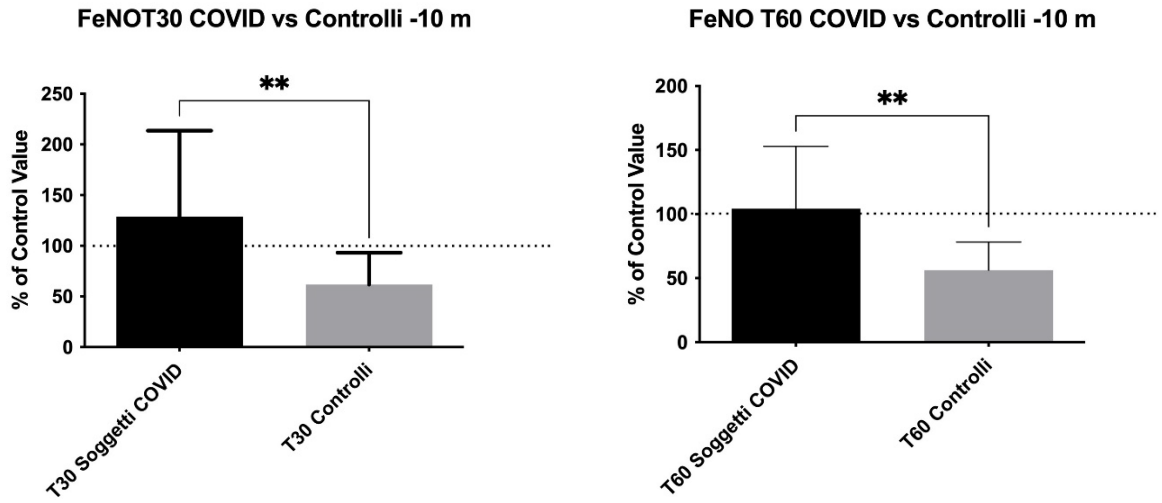
A T-shirt with sensors to record ECG, breath rate and posture (Comftech srl) was worn by each diver before and after each dive. The ECG signal acquisition system, posture and respiratory rhythm consists of an electronic board equipped with a microcontroller and a low energy bluetooth transceiver (BLE). The posture

is detected by means of a triaxial accelerometer: the signal is identified among the three positions where the gravitational acceleration is present. The respiratory rate is measured by a "strain gauge" inside the T-shirt. Measurement is performed through a Wheatstone bridge and finally the reading of the analogue signal is done using the Analogue-Digital Converter (ADC) inside the microcontroller. The ECG signal is acquired through three electrodes, processed and digitally filtered within the microcontroller itself. ECG data are transmitted every 8 ms for the ECG signal, while those of respiratory rate and position only when the parameter itself changes.

1.5.3 Results

A total of 24 experienced divers (20 male and 4 female), who suffered COVID-19, mean age 50.6 ± 12.6 years, mean height 178.2 ± 8.0 cm; mean weight 84.2 ± 14.4 kg and BMI 26. ± 4.1 were investigated. The control group (subject who did not suffer COVID-19) was composed by 9 male divers, mean age 43.6 ± 14.2 years, mean height 182.3 ± 5.7 cm, mean weight 87.8 ± 13.5 kg and mean BMI 26.4 ± 4.0. All the volunteers completed the experiment without DCS episodes, evidence of pulmonary and/or ear barotraumas or other health trouble. All the divers were studied during three dives in the Y-40 "The Deep Joy" swimming pool.

As concerning FeNO changes (Figure 15), we found statistically significant differences between COVID-19 and control groups in both T30 and T60 after the first (-10 m) and the third dive (-40 m) while we didn't find any difference in the second dive (-20).



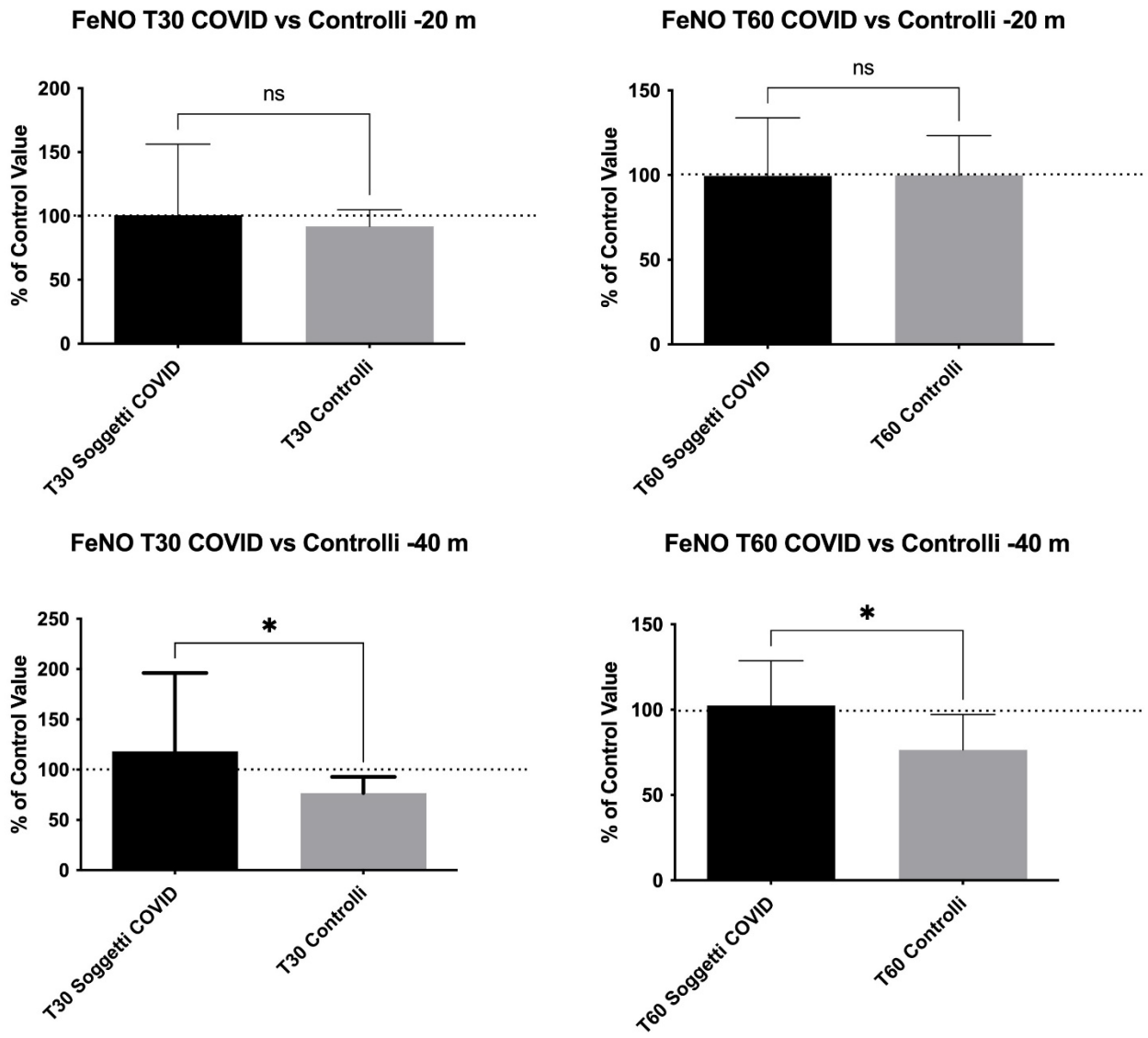


Figure 15 – FeNO differences between COVID-19 and control group

About NO derivatives (NO_x), we observed statistically significant differences at T60 after the second and the third dive (Figure 16). No differences were observed in the first dive and at T30.

As concerning ROS (Figure 17), we found statistically significant differences between COVID-19 and control group in both time point after surfacing in any dive.

We found statistically significant differences in TAC values at both T30 and T60 in the first dive while the other dives showed statistically significant decrease in the control group only at T30 (Figure 18).

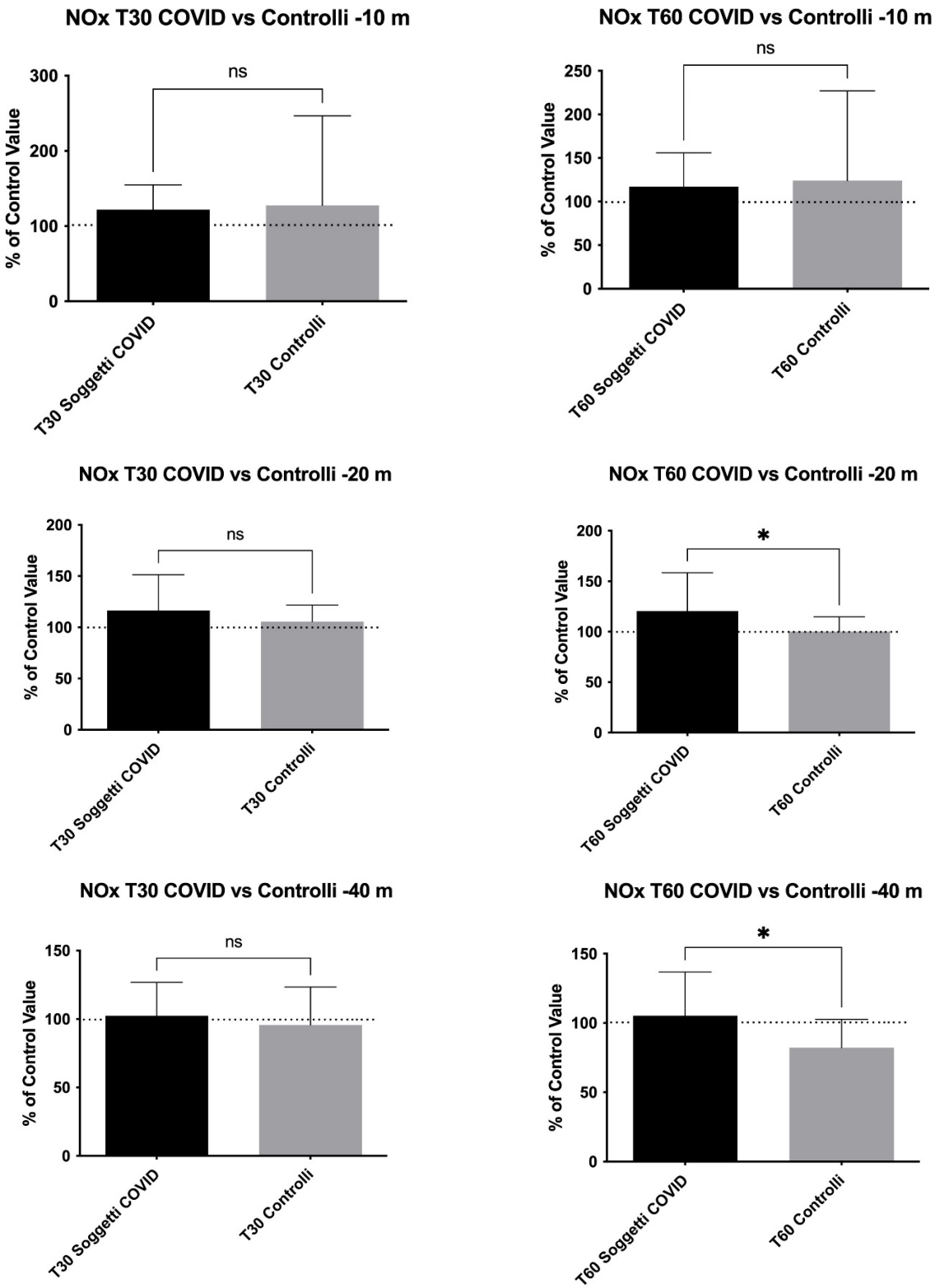


Figure 16 – NOx changes in COVID-19 and control groups

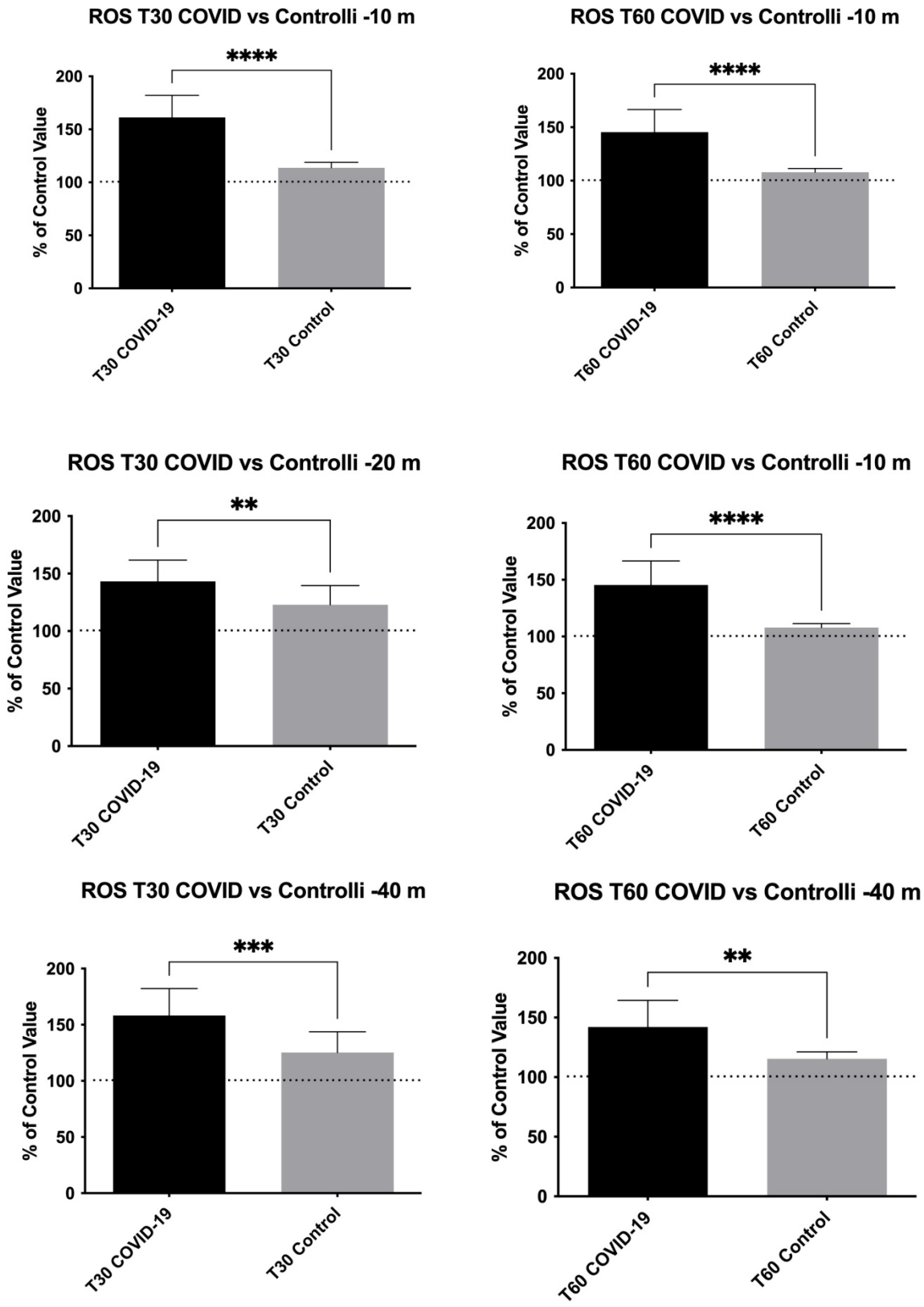


Figure 17 – ROS changes between COVID-19 and control groups

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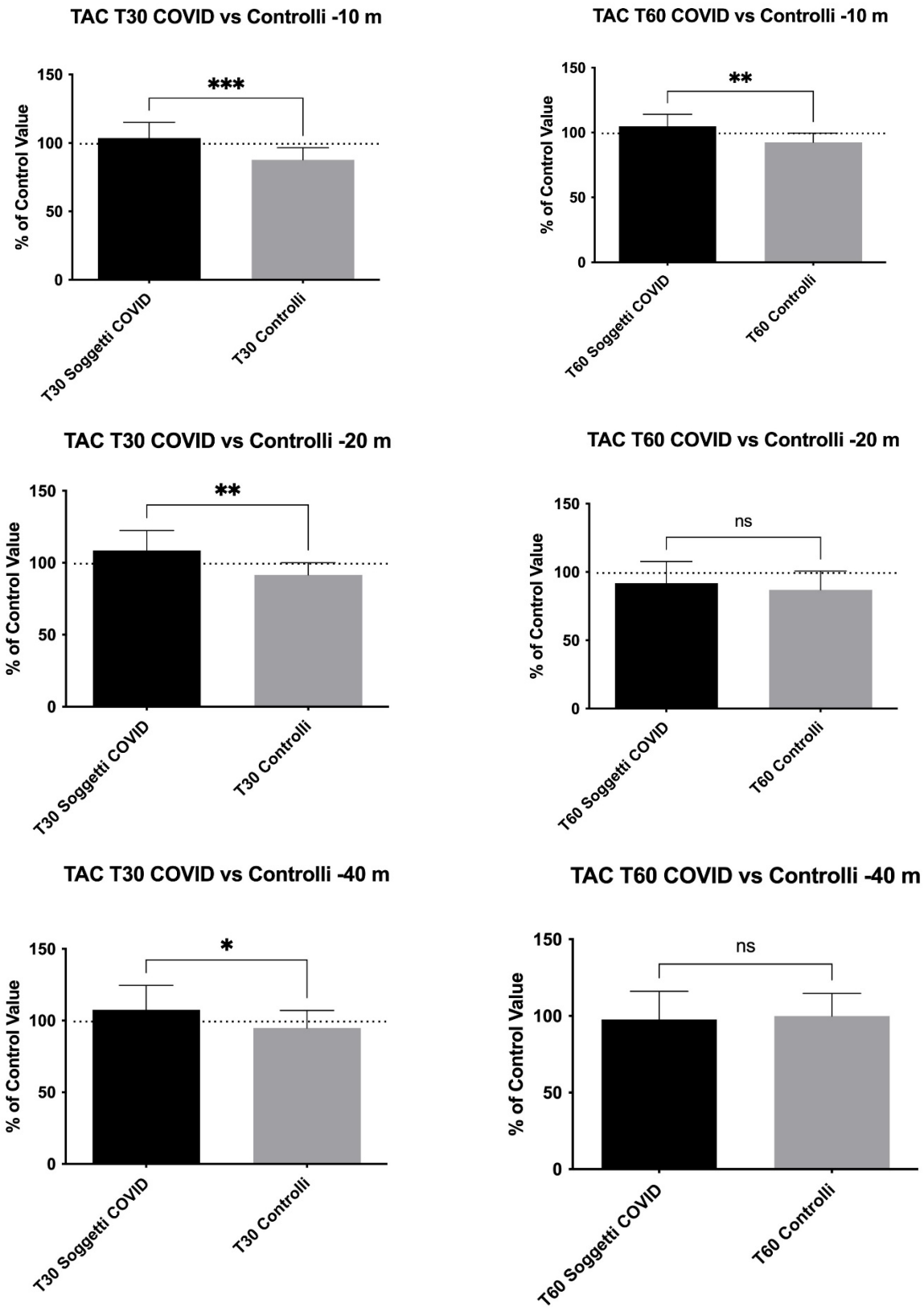

Figure 18 – TAC values in COVID-19 and control group.

As concerning 8-isoprostane (Figure 19), only the first dive showed statistically significant differences between the groups in both T30 and T60 while the other dives showed significant differences only at T60.

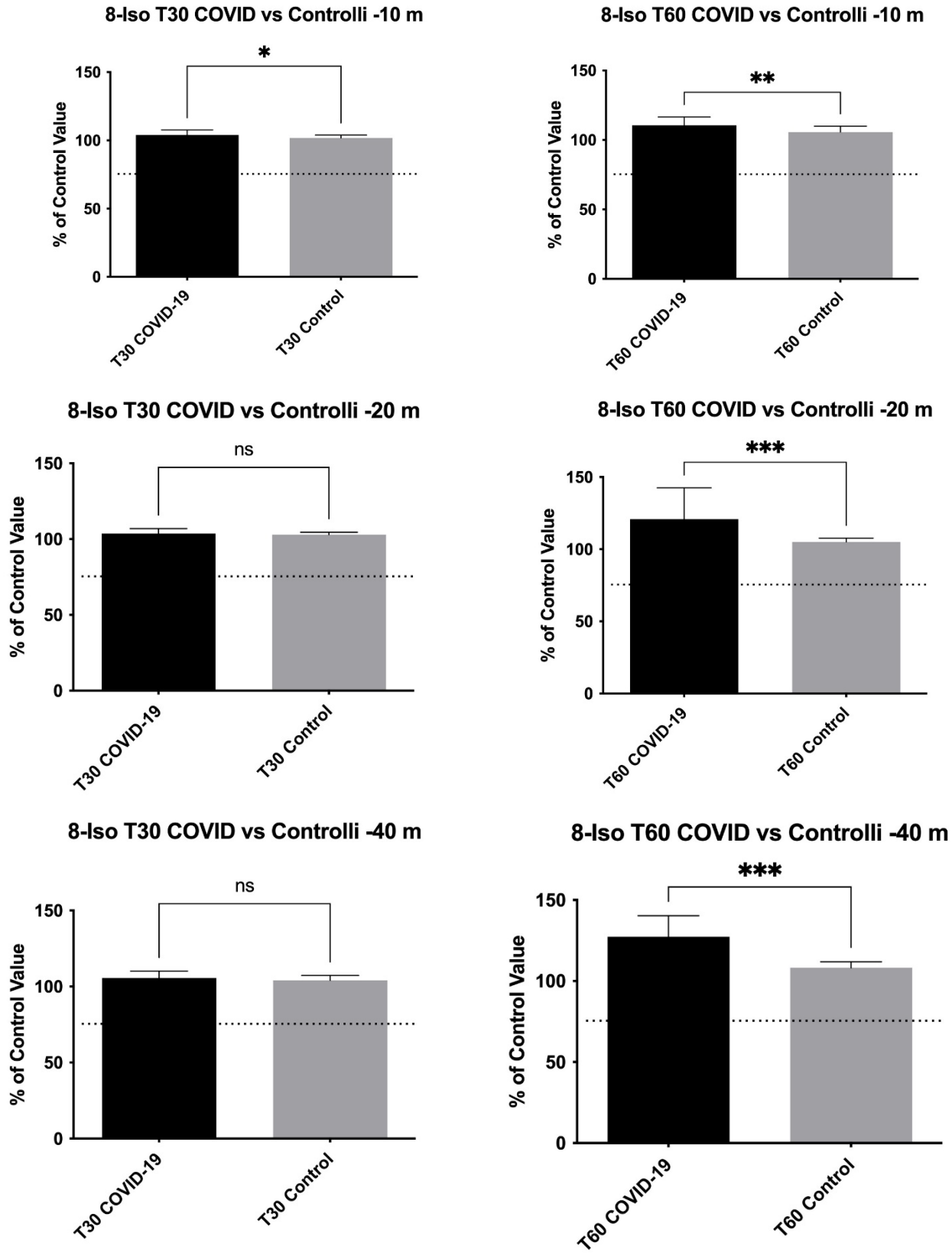

Figure 19 – 8-isoprostane changes between COVID-19 and control groups.

About the cardiopulmonary biomarkers, only CK showed statistically significant differences between the groups in the first dive (Figure 20) without significant changes in the other dives.
CK-MBm, AST, ALT, LDH and Cortisol didn't show any statistically significant differences between the groups in any dive. We did not find any changes in cTnI levels between pre-dive and post-dive samples.

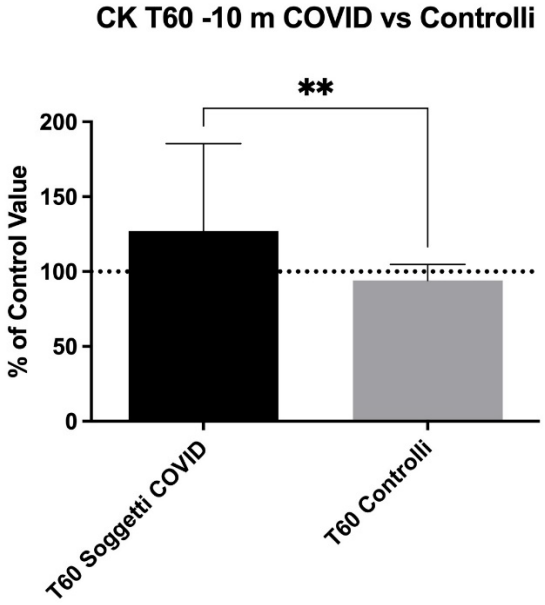

Figure 20 – CK changes between COVID-19 and control groups.

All the others parameters did not show any statistical differences between pre-T1 and T2. All the data were recording using the final configuration of AVATAR project without any problem in acquisition of signal by wearable, transmission and recording in the AVATAR web portal.

1.5.4 Discussion

This study aimed to investigate whether divers who have contracted COVID-19 can return to dive by evaluating pulmonary conditions, NO derivatives (NOx), oxidative stress and cardio muscular parameters before and after three dives at increasing depth (-10, -20 and -40 m).
In two of the three dives, we observed significantly higher values of FeNO in COVID-19 subjects with respect to controls. NO is an important mediator in the amplification and modulation of inflammatory response of the airways, produced through iNOS [61] and as an intra- and extracellular mediator in lung fibrogenesis [59]. Lung fibrosis was clinically confirmed up to 3 months after healing in patients who experienced moderate COVID-19 symptoms and in patients with severe symptoms, [99, 100]. However lung fibrosis was also observed in subjects with very mild symptoms including dyspnea, intermittent cough, and lingering fatigue [101]. In SCUBA diving, NO is an important signaling molecule for the adaptation to the hyperbaric environment regulating the vasoconstriction/vasodilation mechanism [102]. Observing higher FeNO values in divers who had COVID-19 may indicate that these subjects require more NO to adapt to the hyperbaric

environment, this probably being related to a reduced efficiency of the vasoconstriction/vasodilation mechanism. These evidences may suggest the persistence of alveolar and/or bronchiolar inflammation and/or mild impairment of the alveolar-capillary membrane in these divers as consequence of interstitial/alveolar lung damage.

We found lower NOx level in control group with respect to COVID-19 subjects. As observed in our previous works [103-105], NOx levels come back to pre-diving values to restore NO consumed during the dive trough NO_x reduction by several enzymes, including xanthine oxidase [106] and xanthine oxidoreductase [107, 108]. NOx values found in the post diving reflect the NO availability after diving and are not influenced by a NOx accumulation related to diving confirming a rapid metabolization of the NO derivatives. In subjects who had COVID-19, this NO regeneration is less efficient probably due to a eNOS dysfunction in impaired endothelium during SARS-CoV-2 infection [109]. In addition, ROS/RNS can reduce eNOS activity oxidizing tetrahydrobiopterin (BH4), a fundamental cofactor of NOS enzyme family [110] reducing NO generation.

In our experiment, we observed higher ROS level in COVID-19 group respect to those of control group. The immune system activation in response to SARS-CoV-2 infection can induce the onset of ROS production and inflict oxidative damage leading to impairment of several tissues [111]. ROS may facilitate viral entry and infectivity promoting viral growth during the acute stage [112]. ACE-2 converts angiotensin II (Ang II) into Angiotensin 1-7 (Ang 1-7), limiting Ang II effects via Ang II type 1 receptors (AT1R). On the other hand, AT1R activates NADPH oxidase (NOX)-2, increasing ROS generation and reducing NO availability [113], leading to endothelial dysfunction well-known in SCUBA diving. Similarly, to ROS levels, we observed TAC values in COVID-19 group higher than those of control subjects. TAC is a biochemical index to evaluate the overall antioxidant status of plasma against free radicals. Oxidative stress may also be favored by the high pO_2 due to hyperbaric pressure, which increases the amount of free O_2 that may favor mitochondrial uncoupling and represent an independent source of ROS/RNS [114]. SCUBA divers can activate the endogenous antioxidant defenses to control vascular oxidative stress due to increased O_2 level associated with hyperbaric conditions to protect from excessive antioxidant depletion and oxidative stress [103, 115]. In COVID-19 subjects, there was a slower return to TAC basal levels than in the control group, probably due to persistence of oxidative stress condition and a less efficient reaction of the endogenous antioxidant defenses toward ROS and RNS. To contrast oxidative stress, the levels of some endogenous antioxidant biomarkers and/or TAC components can increase [116]. 8-isoprostane (8-iso-PGF2α), a well-known marker of oxidative stress [117], exhibited also values in COVID-19 higher group than control group. Also in this case, an elevation of 8-iso-PGF2α may be associated to the ROS generation while some authors hypothesized a relation with oxidative anxiety, a symptom of depression [118].

Among the investigated cardio muscular markers, only CK showed a statistically significant difference between the groups but only in a dive. A direct infection of voluntary muscle is uncertain or rare (and still unproven), transient muscular dysfunction is common during COVID-19 [119]. On the other hand, CK might

be associated to a myopathic involvement of skeletal and respiratory muscles during COVID-19 infection [78]. In our case, increased CK may be associated to fatigue where the conversion of adenosine triphosphate (ATP) to and adenosine diphosphate (ADP) with energy release is reduced. Some authors reported evidence of impaired ATP production from O_2, glucose, fatty acids, and amino acids in some cell types where glycolysis, but possibly Krebs and Urea Cycles, may be less efficient [120, 121]. On the other hand, we did not find any significant difference in other cardio muscular biomarkers.

Taken together, our data seem to indicate a greater effort in the regulation of the vasoconstriction/vasodilation mechanism and in the antioxidant response against ROS/RNS, generated by increased pO_2, after repetitive dives. Increased NO is well-known in SCUBA and BH-diving adaptation to hyperbaric environment but it can act as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations including diseases [122]. Further investigation will be oriented to understand if raised NO level could result in nitrosative stress associated with hypernitrosylation (increased NO or nitroso binding) [123]. Our data showed an imbalance between oxidative stress toxicity and antioxidant defenses with a persistent antioxidant response toward ROS/RNS, related to vascular adaptation and to response to oxidative stress during diving deep phase, as observed by SCUBA underwater blood draw study [103].

On the others hand we are satisfied about the perfect functioning of the AVATAR protocol that permit to acquired physiological and environmental data during the test using wearable devices and sending data in real time to a medical control center without any signal loss.

1.6 NO derivative and oxidative stress marker changes in Full face vs half mask dives

This section describes the experimental phase of this Ph.D. carried out abroad, at the department Environmental & Occupational (Integrative) Physiology laboratory, University of Brussels.

1.6.1 Introduction

NO has a vaso- and bronchodilator properties and the NO inhalation, even in very low concentrations, has been shown to reduce the resistance in the lower airways [124]. For these reasons, NO can be used therapeutically in subjects with pulmonary disease [125], newborns with pulmonary hypertension [126] and patients with acute respiratory distress syndrome (ARDS) [127]. NO has a direct action on bronchial tone as well as on the blood perfusion only in the ventilated areas. For this reason, NO could contribute to an improved match between perfusion and ventilation in the lung (V/Q) [128]. Lung perfusion distribution play a pivotal role not only in terms of gas exchange but also for body defenses against infectious diseases, as poorly perfused lung areas are more vulnerable [129, 130]. On the other hand, NO has an active role in the regulation of pulmonary perfusion, making the pulmonary circulation more uniform despite gravity, and, most likely, an evolutionary phenomenon since the quadruped stage. For this reason, another regulating mechanism may be autoinhalation of NO, which, among mammals, is released in high amounts from the nasal passages in humans [131]. Some authors found NO autoinhalation may redistribute blood within the lungs to counteract the effects of gravity, improving the influence of gravity on pulmonary blood flow distribution [132]. This could be very important also for SCUBA divers, especially for commercial divers that use typically the full face mask (FFM). In SCUBA diving, NO is very studied in SCUBA diving for its role in the flow arterial regulation [133] and adaptation to hyperbaric condition [103].

FFM is a model of diving mask that seals the whole of the diver's face including eyes, nose and mouth in a single volume, differing from the traditional “half-mask” which covers only the eyes and nose. FFM contains a demand valve or constant flow gas supply that supplies the diver with breathing gas mixture [134]. Because a mouthpiece is not needed, the diver can breathe from the nose and speak with other divers and the surface team when FFM is equipped with communicators, as those of commercial divers [135]. An important advantage of FFM is its protection from cold: FFM provides some thermal insulation of the face and might reduce both heat loss and reflexes induced by facial cooling [136, 137].

Despite this knowledge, there are not data available regarding the advantage of FFM use related to the nasal NO generation and effect on lung perfusion.

The aim of this study was to compare NO and oxidative stress markers changes in full face mask divers vs half mask divers.

1.6.2 Material and Methods

1.6.2.1 Subjects and diving protocol

A total of 8 expert male SCUBA divers were investigated during two single recreational dive in Y-40 "The Deep Joy" swimming pool (Montegrotto Terme, PD), 42 meters depth, in two different day. During the first day, volunteers wore the half mask while they wore the full face mask during the second dive.

Venous blood samples were obtained from the antecubital vein of divers in heparin containing tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, United States). Blood samples were collected 30 min before the diving session and 30 min after surfacing (post diving).

Plasma was separated from the cell component by centrifugation (3000 rpm for 15 min) and was frozen until the analysis.

Divers were invitated to reach a bottom time of 7 minutes (descent and permanence at the bottom) so not to exceed the no-decompretion limit. After the dive, all the diving profile were downloaded into the Divers Alert Network (DAN) database using a common export fomat called Universal Dive Data Format (UDDF) and the Gradient Factor (GF) was calculated by a dedicated DAN Software using the Buhlmann ZHL16 C model and used to ascertain uniformity of hyperbaric exposure in addition to the analysis of compliance with the suggested diving profile.

The Maximum GF value is generally reached at the end of the dive. The GF measures the inert gas load in the diver's tissues, according to the selected decompression algorithm. This is a way to estimate inert gas supersaturation and to compare diving exposure in the different investigated subjects [54].

1.6.2.2 Analysis

NO derivatives (NOx) and oxidative stress (TAC) were measured as reported in paragraph *1.5.2.3*.

1.6.3 Results and Discussion

The results of this part of the Ph.D are still in evaluation, a second step of data acquisition is planned in October 2022 and for this reason the results are at this moment not available. Anyway, a preliminary statistical analysis of the data recorded seem to indicate a better NO production and oxidative stress menagment in the FFM group. This result, if confirmed, coul rappresent un interessant aspect to improve the safety of diving considering that the quicly development of ecquipment, mask including.

On the others hand this aspect could be very interesting in the menagment of the higher numbers of commercial divers that usually diving using FFM.

1.7 Conclusion of Ph.D Project

This work towards a Ph.D qualification aimed at studying and implementing methods and tools to provide real time physiological and environmental information from remote areas and to develop specific algorithms to elaborate this information and permit a more accurate management of accidents or medical emergencies in remote areas, by developing an advanced bidirectional telemedicine concept. (Figure 21)

Figure 21 – AVATAR system structure.

The final goal, that is also the original challenge of AVATAR, is to realize a dedicated international control center allowing to manage physiological and environmental data as received from remote areas, supported by customized wearable technology, augmented and virtual reality and dedicated electronic devices and to provide correct and appropriate instructions to assist remote victims as supported by accurate real-time medical and environmental information.

With regard to Diving activities, in particular, data recorded by wearable devices (heart rate, breathing frequency) have also been integrated with physiological parameters, as obtained by blood draw, that represent the current gold standard to investigate human physiology in the underwater environment.

The project was completely carried out from a technical point of view, by in-house customization of the wearable devices for underwater use, production of the devices needed for underwater data transmission and creation of the original web portal and the algorithms allowing to analyze the received data and identify possible risk conditions. However, a large part of the time of the Ph.D was spent on physiopathology tests essential to increase the knowledge of the human body behavior during diving. Without this information it would not be possible to create a personalized profile for each diver which, even if it was not the initial aim of the project, represents now an essential final purpose of the project.

During this physiological testing we found:

- That NOx values return to basal conditions immediately after SCUBA and BH-diving, showing a very rapid control mechanism.
- Oxidative stress is contrasted by endogenous antioxidant defenses.
- Increase of CK, AST, ALT, LDH, and CK-MBm is probably due to physical exercise, but other factors such as increased ambient pressure, hypoxia, and diving response could also play a role.
- A sensible decrease in AA related to energy supply, fatigue adaptation, antioxidant and NO synthesis.
- HR variability can represent an interesting marker of diving stress.

Furthermore, a preliminary study in which the blood physiological parameters studied during the AVATAR project and the wearable-related data acquired using the AVATAR web portal, was realized to study the return to dive after COVID-19.

Taken together, our data seem to indicate a greater effort in the regulation of the vasoconstriction/vasodilation mechanism and in the antioxidant response against ROS/RNS, generated by increased pO2, after repetitive dives in subjects previously affected by COVID-19.

1.8 Future perspective

The continuation of this project will aim at obtain a personalized algorithm for safer SCUBA and BH-diving decompression.

A crossover between real-time acquired data and physiological parameters study will lead us to build a new customizable algorithm taking into account the individual physiology response to the diving stress and to reduce decompression sickness and diving related pathologies risk. This study indicates how the approach proposed by this Ph.D is very plastic and adaptable to many pathological conditions to optimize assistance for delocalized patients by medical centers and that this approach can also be used to conduct scientific study protocols using wearable devices and a web portal to collect and analyze data.

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If you can do it underwater you can do it anywhere!

Thank you

2 Ph.D. PAPER PRODUCTION (Appendix)

Below we reported the most important scientific publications from the three years of PHD work; we remind that all the algorithms support the correct function of the AVATAR project need detailed physiological information related to the body response in extreme environment.

For this reason, we have devoted a lot of energy to realize scientific protocols aiming, in particular, at performing the tests not only before and after the extreme environment exposure but, using the wearable devices and the AVATAR approach, also during the investigated extreme activities; in our case, at the moment, mostly SCUBA diving activities.

2.1 Complete List of paper

1. **Danilo Cialoni** , Andrea Brizzolari, Michele Samaja, Massimo Pieri, Alessandro Marroni. Altered Venous Blood Nitric Oxide Levels at Depth and Related Bubble Formation During Scuba Diving. Front Physiol. 2019 Feb 21; 10:57.
2. Claudio Marabotti, **Danilo Cialoni**, Alessandro Pingitore, Acute Pulmonary Edema in Healthy Subjects. A. Aerosp Med Hum Perform. 2020 Aug 1;91(8):662 668.
3. Simona Mrakic-Sposta, Alessandra Vezzoli, Federica D'Alessandro, Matteo Paganini, Cinzia Dellanoce, **Danilo Cialoni**, Gerardo Bosco. Change in Oxidative Stress Biomarkers During 30 Days in Saturation Dive: A Pilot Study. Int J Environ Res Public Health 2020 Sep 28;17(19).
4. **Danilo Cialoni**, Andrea Brizzolari, Michele Samaja, Gerardo Bosco, Matteo Paganini, Massimo Pieri, Valentina Lancellotti, Alessandro Marroni. Nitric Oxide and Oxidative Stress Changes at Depth in Breath-Hold Diving. Front Physiol 2021 Jan 7;11.
5. **Danilo Cialoni**, Andrea Brizzolari, Michele Samaja, Gerardo Bosco, Matteo Paganini, Nicola Sponsiello, Valentina Lancellotti, Alessandro Marroni, Endothelial Nitric Oxide Production and Antioxidant Response in Breath-Hold Diving: Genetic Predisposition or Environment Related? Front. Physiol. 2021, 12:692204
6. Andrea Brizzolari, Michele V. Dei Cas, **Danilo Cialoni**, Alessandro Marroni, Camillo Morano, Michele Samaja, Rita Paroni, Federico Maria Rubino, High-Throughput Griess Assay of Nitrite and Nitrate in Plasma and Red Blood Cells for Human Physiology Studies under Extreme Conditions, Molecules 2021, 26, 4569.
7. Pierre Lafère, Kate Lambrechts, Peter Germonprè, Ambre Balestra, Faye L. Germonprè, Alessandro Marroni, **Danilo Cialoni**, Gerardo Bosco, Costantino Balestra, Heart Rate Variability During a Standard Dive: A Role for Inspired Oxygen Pressure? Front Physiol 2021 Jul 26; 12:635132.
8. **Danilo Cialoni**, Andrea Brizzolari, Nicola Sponsiello, Valentina Lancellotti, Cesare Lori, Gerardo Bosco, Alessandro Marroni, Alessandra Barassi, Serum Cardiac and Skeletal Muscle Marker Changes in Repetitive Breath-hold Diving Sports Medicine - Open (2021) 7:58.
9. **Danilo Cialoni**, Andrea Brizzolari, Nicola Sponsiello, Valentina Lancellotti, Gerardo Bosco, Alessandro Marroni, Alessandra Barassi, Serum Amino Acid Profile Changes After Repetitive Breath-Hold Dives: A Preliminary Study, Sports Medicine - Open (2022) 8:80.

10. **Danilo Cialoni,** Andrea Brizzolari, Alessandra Barassi, Gerardo Bosco, Massimo Pieri, Valentina Lancellotti, Alessandro Marroni, White Blood Cells, Platelets, Red Blood Cells and Gas Bubbles in SCUBA Diving: Is There a Relationship? Healthcare 2022, 10, 182.
11. Sergio R. Schirato, Vitor Silva, Katia Iadocicco, Alessandro Marroni, Massimo Pieri, **Danilo Cialoni**, José G. Chaui-Berlinck, Post-decompression bubble and inflammation interactions: a non-extensive dynamical system model, Undersea Hyperb Med, 2022 Second-Quarter;49(2):207-226.
12. Tommaso A. Giacon, Gerardo Bosco, Alessandra Vezzoli, Cinzia Dellanoce, **Danilo Cialoni**, Matteo Paganini, Simona Mrakic-Sposta, Oxidative stress and motion sickness in one crew, during competitive offshore sailing. Sci Rep 2022, 12:1142.
13. Claudio Marabotti, **Danilo Cialoni**, Alessandro Pingitore, Edema in Healthy Subjects, 2020 Aug 1;91(8):662-668.

2.2 Paper endothelial dysfunction and oxidative stress related

As well known the majority of diving related disease find in the endothelial dysfunction and in oxidative stress one of the most important triggers for their pathogenetic mechanism. As consequence we decided to develop some scientific protocols to investigate these aspects in real time during diving and others extreme conditions, looking to up-date the knowledge in this specific field. Related to this aim we produced six studies:

1. **Danilo Cialoni** , Andrea Brizzolari, Michele Samaja, Massimo Pieri, Alessandro Marroni. Altered Venous Blood Nitric Oxide Levels at Depth and Related Bubble Formation During Scuba Diving. Front Physiol. 2019 Feb 21; 10:57.
2. **Danilo Cialoni**, Andrea Brizzolari, Michele Samaja, Gerardo Bosco, Matteo Paganini, Massimo Pieri, Valentina Lancellotti, Alessandro Marroni. Nitric Oxide and Oxidative Stress Changes at Depth in Breath-Hold Diving. Front Physiol 2021 Jan 7;11.
3. **Danilo Cialoni**, Andrea Brizzolari, Michele Samaja, Gerardo Bosco, Matteo Paganini, Nicola Sponsiello, Valentina Lancellotti, Alessandro Marroni, Endothelial Nitric Oxide Production and Antioxidant Response in Breath-Hold Diving: Genetic Predisposition or Environment Related?, Front. Physiol. 2021, 12:692204
4. Andrea Brizzolari, Michele V. Dei Cas, **Danilo Cialoni**, Alessandro Marroni, Camillo Morano, Michele Samaja, Rita Paroni, Federico Maria Rubino, High-Throughput Griess Assay of Nitrite and Nitrate in Plasma and Red Blood Cells for Human Physiology Studies under Extreme Conditions, Molecules 2021, 26, 4569.

Below a description of the paper endothelial dysfunction and oxidative stress related: Introduction and discussion are presented only one time, being similar for the six papers, while a dedicate up-date is proposed for any paper as concerning material and method and results.

2.3 NOx and oxidative stress

2.3.1 Introduction

Nitric oxide (NO) is a signaling molecule involved in many physiological and pathological processes [133, 138], particularly in the control of the cardiovascular system, and the blood flow and pressure [139]. However, the role of NO in human physiological and pathological responses is more complex, including its nature as a

signaling molecule in several physiological mechanisms such as: central nervous system transmission and neuroprotection [133, 140], immune system response [141], vascular remodeling [142], and several other conditions. NO is largely released from the arterial endothelium that can be considered a real autocrine and paracrine organ, able to produce many other substances such as Prostacyclin, Endothelium Derived Relaxing Factor, Platelet Activating Factor, Angiotensin-Converting Enzyme and several other [56]. As a consequence, NO is involved in many physiological processes such as: platelet activity, leukocyte adhesion, thrombosis, and in the development of atherosclerosis [58, 143].

Due to its short half life (0.05–1.8 ms) [144], NO availability is ensured by a family of NO synthases (NOS), composed of at least three different isoforms [145]. The endothelial isoform (e-NOS) is mainly involved in modulating the vasodilator tone, vascular integrity preservation, and regulation of arterial blood pressure [133]. eNOS also inhibits platelet aggregation and adhesion and enhances vascular permeability [146]. e-NOS levels are regulated by many factors, such as hypoxia and local substrate availability [147], vascular shear stress [148], and, most important, by different genetic variants (polymorphisms) [149, 150]. Indeed, functional variants in the endothelial NOS3 gene might alter the expression of the enzyme [151]. Typically, it is preferable to measure NO derivatives, nitrates and nitrites (NOx) [145]. Specifically, NO is oxidated to nitrite (NO_2) or, when oxyhemoglobin is available, to nitrate (NO_3), with NO_3 being predominant in blood circulation [152]. Healthy individuals produce approximately 1 mmol of NO_3 daily due to the oxidation of endogenously synthesized NO [153]. If necessary, NO_3 can be reduced to NO_2 by several enzymes, such as xanthine oxidase [106] and xanthine oxidoreductase [107]. NO_2 is further reduced to NO by different pathways, including hemoglobin [154], myoglobin [155, 156], xanthine oxidoreductase [108], and ascorbic acid [157]. These pathways are significantly enhanced during hypoxia and acidosis to ensure NO production when the oxygen-dependent NOS enzyme activities are compromised [147, 158]. In addition, NO_2 reduction to NO during physiological hypoxia seems to contribute to physiological hypoxic signaling, vasodilation, and modulation of cellular respiration [154, 156, 159, 160].

Being NO a free radical, it is also expected that higher- than-normal NO levels in the circulation may combine with superoxide anions (O_2^{2-}) giving rise to aggressive reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) that, besides inactivating some NO synthases [62], triggers the formation of reactive oxygen species (ROS) leading to oxidative stress. Oxidative stress may also be favored by the high pO_2 due to high pressure, which increases the amount of free O_2 that may favor mitochondrial uncoupling and represent an independent source of ROS [161]. Oxidative stress has been investigated in diving [162, 163], both in SCUBA [102] and BH-diving [164]. Oxidative stress is exacerbated during saturation diving, the human body is subjected to severe/extreme environmental conditions, exposing divers to higher risks and accidents [165]. Specifically, divers are exposed to increased partial pressure of oxygen (pO_2), potentially toxic gases, bacteria, and bubble formation during decompression. Hyperoxia increases ROS production [166] , leading to a macromolecule damage, specifically proteins, lipids, and nucleic acids [92]. Little is known about the balance between pro-

oxidant effects and antioxidant responses related to saturation diving. Antioxidant defenses are known to increase when the production of ROS [166, 167].

Oxidative stress can also be the consequence of strenuous physical activity characterized by high intensity and anaerobic bursts, with increases in oxygen consumption and heart rate [168, 169]. Such activity often leads to heat loss and dehydration [170, 171], and produces muscle damage and oxidative stress. Oxidative stress levels have been investigated in other endurance sports, such as triathlon, ultra-endurance races [172], and swimming [173], revealing an overproduction of ROS and a depletion of total antioxidant capacity (TAC). The redox status—namely, the equilibrium between ROS and TAC—deeply affects intracellular function. Maintaining ROS homeostasis is crucial for normal cellular responses, while overproduction is deleterious and can damage cell structures leading to progressive organism's disfunction [174].

Recently more and more confirmations suggest a primary role of the increase of oxidative stress as the cause of endothelial dysfunction [175]. Endothelial dysfunction and oxidative stress have been also investigated in diving [162, 163] with particular regard to NO-related endothelial changes both in SCUBA diving [102] and in BH-diving [147, 164].

Endothelial dysfunction has also been demonstrated in BH-diving [162, 163] and can be explained by two hypotheses. First, the BH-diving-related transient hypoxia and accumulation of CO_2 induce an increase in ROS levels, causing higher oxidative stress [92, 164] and NO-related endothelial changes [102]. Second, the development of venous gas embolism, frequently observed in self-contained underwater breathing apparatus diving (SCUBA) despite correct decompression procedures [176], has also been recently demonstrated in BH-divers [177] and could play a role in the pathogenesis of BH-diving-related endothelial dysfunction. Since NO is primarily released from arterial endothelium along with many other regulatory substances [56], any condition causing endothelial dysfunction inevitably affects NO levels and leads to cardiovascular diseases, such as coronary artery disease, peripheral arteriopathy [178, 179], and atherosclerosis [58, 143]. On the other hand, recent observations seem to indicate the existence of a genetic predisposition in developing BH-diving-related injuries [180] or diving reflex related adjustments [181], but there is no clear evidence in the published literature whether NO availability and oxidative stress occurring in BH-diving are related to extreme environmental conditions (such as ambient pressure, salinity, and water temperature) rather than genetic predisposition. However, we also need to take into account that in niche sectors, such as diving, in which it is very difficult to plan genetic protocols in higher numbers of subjects, the recommendation in these conditions is to use biallelic markers [such as single nucleotide polymorphisms (SNPs)] to obtain indicative data even in lower numbers of subjects [182].

Furthermore, most available data have been obtained on land (be it laboratory of field conditions) before and after the dive, but, as far as we know, no data is available on what happens during the water immersion phase of the dive.

The study aims to investigate:

1. the changes in NOx and antioxidant response [plasma total antioxidant capacity (TAC)] before during and after a single SCUBA dive
2. The change in NOx and TAC concentration before, during, and after repetitive BH-dives in healthy volunteers.
3. NOx concentration and oxidative stress markers before and after repetitive BH-dives in healthy volunteers in different environmental and hyperbaric exposure conditions. We also show the preliminary results related to the NO and oxidative stress response in BH-divers with different genetic variants of 10 selected polymorphisms.

2.3.2 Material and Methods

2.3.2.1 SCUBA and BH-diving subjects for underwater blood draw

A total of 15 expert SCUBA divers, 13 male and 2 female, were studied during a single dive. No subject reported previous episodes of decompression illness (DCI), historical or clinical evidence of arterial hypertension, cardiac, pulmonary or any other significant disease, none of them took prescription drugs, suffered any acute disease during the 15 days before the experiment, or reported assumption of anti-inflammatory drugs and exposure to high altitude in the 7 days before the experiment. All the divers received an explanation of the study's purposes, risks and benefits, were familiarized with the experimental protocol and read and signed a specific informed consent form before the experiment. Subjects were asked to avoid food rich in nitrate, such as red meat [183] and leafy green vegetables [184] and moderate or intense exercise during the 48 h before the experiment. The study was conducted in accordance with the Helsinki Declaration and was approved by the Ethical Committee of Università degli Studi di Milano, Italy (Aut. n° 37/17). No Diver performed any compressed-gas diving or any BH-Diving during 30 days before the day of the test. The diving parameters (depth, diving time and Gradient Factor) were recorded for each dive using a diving computer with sampling rate set at 10 s. All the dive profiles were downloaded using the Subsurface software, exported in UDDF format and imported into the DAN Europe Diver Safety Guardian (DAN DSG) original and proprietary program to calculate the Maximum Gradient Factor reached during the dive. This Maximum value is generally reached at the end of the dive. The Gradient Factor measures the inert gas load in the diver's tissues, according to the selected decompression algorithm. In our protocol, the maximum Gradient Factor, expressed as a percentage of the M-value, is computed using the Buhlmann ZHL16 C model. This is a way to estimate inert gas supersaturation and to compare diving exposure in the different investigated subjects [54]. We recruited 14 healthy BH-divers, who were investigated during a series of deep dives at "Y-40 The Deep Joy" pool (Montegrotto Terme, PD, Italy) with the same exclusion criteria of SCUBA divers. The selected volunteers are labeled "expert" because they are affiliated to the "Apnea Academy" Training Agency as instructors or high-level BH-divers, and are able to reach −42 m depth in variable weight, 4 min static apnea

(at the surface), 75 m dynamic BH-diving (horizontal) in a swimming pool (distance). The dives were performed in teams of two subjects each as per the no-limits BH-diving technique (using a dedicated machine to facilitate descent and ascent). All the subjects performed a gradual warm-up, with a determined number of dives at increasing depth: 1st dive at −15 m; 2nd dive at −25 m; 3rd dive at −35 m. The volunteers were however allowed to adapt the warm-up protocol to their needs. When ready, they made one dive to the bottom of the swimming pool (−42 m) where the "bottom blood draw" was obtained. To ensure the safety of the divers, two SCUBA divers were stationed near to the surface during the descending and ascending phases, ready to take action in case of potential troubles. The following diving parameters were also recorded for each dive, using a free-diving computer (UP-X1 Omersub, Spa, Monza Brianza, Italy): depth; diving time; bottom time; surface interval and numbers of dives. This computer measured and recorded diving data every 2 s.

2.3.2.2 BH-diving subjects for genetic polymorphisms

50 Expert healthy BH-Divers were studied in two settings: the first group during a series of deep dives at the swimming pool. Y-40 "The Deep Joy " (Montegrotto Terme, Italy) (42-m-deep); the second group during an open water training session at the Elba Island (Italy). All the divers were informed about the risks and benefits of this study and read and signed a specific, informed consent form before the experiment. All the participants also signed a dedicated genetic informed consent allowing the genetic analysis.

All the divers performed their usual training with a freely determined number and time of warm-up dives, bottom time, and surface intervals. As per the "Apnea Academy" standard procedures, the training session involved a gradual approach to the maximum daily personal depth and an unrestricted number of deep dives, at the end of which all the divers returned to the laboratory for the post diving test protocol. Diving profiles were recorded using a UP-X1 free-diving computer (Omersub S.p.a., Sovico, Italy), including mean depth, maximum depth (MD), and number of dives (ND). The freediving computers measured and recorded data every 2 s. The included divers were stratified into several groups to be analyzed. First, divers were interviewed and divided by diving level (LD) in medium or high experience (ME vs. HE) considering their BH-diving skills, defined by years of activity, personal depth record, number of weekly training sessions, and certification level. Another stratification considered three parameters achieved on the day of the experiment, namely: average depth (AD), an average of MD reached, and an average ND; subjects were thendivided into those who dived above (AD-above, MD-above, and ND-above) and those who dived below the calculated averages (AD-below, MD-below, and ND-below).

2.3.2.3 SCUBA diving underwater Blood draw

A 2-way peripheral venous catheter was placed in the antecubital vein before the dive, wrapped in waterproof bandage and fixed with waterproof plaster. A 3-way stopcock was connected to the catheter and a IV. Cannula needle. A vacutainer tube with EDTA (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ,

United States) as anticoagulant was used to collect blood samples at each phase of the blood draw protocol. Blood samples were collected 30 min before diving (basal), at the pool's bottom (-42 m) (diving 1) (Figure 22), during the safety stop at -5 m (diving 2), immediately after the end of the dive (post 0), 30 min (post 30) and 60 min after surfacing (post 60). The collected blood was immediately treated to obtain plasma; as far as the blood sample taken at bottom is concerned, it was put into a dry box and immediately taken from the bottom to the surface, by means of a rope hoisted in about 1 min by a dedicated person. The same procedure was applied for the blood collected at -5 m. Plasma was separated from the cell component by centrifugation (3000 rpm for 15 min) and was frozen until the analysis.

2.3.2.4 BH-diving underwater blood draw

The catheter was the same used for SCUBA divers. We collected blood samples in one EDTA containing tube per each of the following time steps:

• Basal: 30 min before the start of the warm-up;

• Bottom: at −42 m;

• T0: Immediately on arrival at the surface (head out of the water, while normally breathing);

• T30: 30 min after the deep dive;

• T60: 60 min after the deep dive.

Except for the "basal" test (when the blood sample was taken immediately after the cannula placement), 5 ml of blood was drawn and discarded before collecting the sample in the tube at each step to reduce the interference of blood clotted at the inner side of the cannula. After each sampling, the cannula was flushed with normal saline (NS) to prevent clotting. At the "bottom" sampling, the stopcock hosted the tube adapter and a 10 ml syringe filled with 5 ml of NS. The subjects waited about 10 s at the bottom, needed to draw 5 ml of blood in the syringe, fill the tube, and then flush the cannula. Finally, each diver brought the tube back to the surface and delivered it to a researcher.

Plasma samples were centrifuged and frozen as described above.

2.3.2.5 Polymorphism vs environment protocol

The protocol was the same in both the swimming pool and the sea tests. Venous peripheral access was obtained from the antecubital vein of each subject, and blood samples were collected 30 min before the start of the diving series (basal). Blood samples were then collected 30 min (T30) and 60 min (T60) after the end of the BH-diving session, after discarding 5 ml of the blood to remove any clots. Plasma was obtained by centrifugation (3,000 rpm for 10 min) and was refrigerated at -20.

Plasma samples were then delivered to the Laboratory of Biochemistry of the Department of Health Sciences (DISS) of the Università degli Studi di Milano for analysis. Epithelial oral cells were also obtained using two buccal swabs from each volunteer. DNA was isolated using the Charge Switch kit (Invitrogen Corp., Carlsbad, CA, United States), following the instructions of the manufacturer, and both buccal swabs were suspended in 100 ml of elution buffer.

Figure 22 – Underwater blood draw on the bottom of Y-40 Swimming pool.

We investigated for the following:

1. Differences in plasma concentration of NOx and TAC for the following diving risk factors:
    1. BH-LD (ME vs. HE)
    2. Environmental (swimming pool vs. sea)
3. Differences in plasma concentration of NOx and TAC in the following recorded diving risk factors, between those above and those below the calculated average:

1. AD (AD-above vs. AD-below)
2. MD (MD-above vs. MD-below)
3. ND (ND-above vs. ND below)
4. Differences in plasma concentration of NOx and TAC (before and after the dives) in genetic variants of 10 investigated polymorphisms (as explained in the following sections).

2.3.3 Analysis

2.3.3.1 Nitrate + nitrite (NOx) determination

All SCUBA and BH-divers s were investigated for the NOx plasma level repeated for the three times specified in the protocol as described in paragraph 1.5.2.3.

2.3.3.2 Plasma Total Antioxidant Capacity (TAC)

TAC changes in SCUBA divers and BH-divers investigated for generic polymorphisms was measured using the ferric reducing antioxidant capacity (FRAP) assay [185]. Plasma samples were added to 3 mL of a freshly

prepared FRAP solution in glass test tubes in triplicate and the absorbance measured at 593 nm in a Uvikon 931 UV-VIS Spectrophotometer (Northstar Scientific, Bardsey, United Kingdom). After 5 min of incubation at 37 °C against a blank of FRAP solution. Aqueous solutions of $FeSO_4$ $7H_2O$ (100–1000 mM) were used for the calibration and the TAC results were expressed as FRAP value [μM Fe (II)] of the Samples [186].
TAC value changes in BH-diving underwater blood draw using Trolox equivalent antioxidant capacity (TEAC) assay according to Re et al. [94] as described in paragraph 1.5.2.3.

2.3.3.3 DNA Polymorphisms

BH-divers were examined with 10 different genetic polymorphisms related to the investigated risk factors (available NO and oxidative stress) were analyzed, especially, 2 involved with NO availability, 4 with the anti-inflammatory activity, and 3 with the antioxidant capacity. Also, angiotensin-converting enzyme polymorphisms were analyzed. The polymorphisms were analyzed using a real-time PCR (RT-PCR) technique. Specific primers and probes for the SNP rs1799983 were designed according to the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, United States), while SNP rs2070744 was analyzed using primers and probes designed according to the Kaspar genotype assay (KBIoscience [B]). Both SNPs were analyzed on ABI 7900 following the instructions of the manufacturer. In all the investigated polymorphisms, the NOx and TAC levels before the diving and at follow-up (T30 and T60) for the different genetic variants were analyzed.

2.3.3.5 Echocardiography Protocol

Echocardiography was done before the dive and within 30 min maximum after the dive. All echocardiographies were made using a commercially available instrument (MyLab 5, Esaote SPA, Florence, Italy) with a cardiac probe (2.5–3.5 MHz) as described in paragraph 1.5.2.3.7.

2.3.3.6 Statistical analysis

Data are presented as the mean ± standard deviation (SD) for parametric data and median and range for non parametric data. Taking the pre diving value of NOx and TAC as 100% the percentage of changes were calculated in each measurement foreseen by the protocol. They were analyzed by means of one sample t-test after the D'Agostino and Pearson normality test to assume a Gaussian distribution. Differences between non-bubblers and Bubblers were investigated using the Mann–Whitney U-test for non-parametric data and two-sample (unpaired) t-test for parametric data both after Shapiro–Wilk normality test. A probability lower than 5% was assumed as the threshold to reject the null hypothesis ($p < 0.05$).

2.3.4 Results

2.3.4.1 BH-diving underwater blood draw

All the subjects respected the warm-up protocol, except for two who requested an adjunctive dive to −25 m to fully achieve correct ear equalization. All the volunteers completed the experiment without Taravana

episodes, evidence of pulmonary and/or ear barotraumas or other health trouble. The diving profile showed a mean of dives of 4.1 ± 0.4; a mean depth of 29.0 ± 9.7 m; a mean of diving time of 118.7 ± 20.0 s and a mean surface interval of 384.0 ± 40.7 s. All the subjects reached the bottom of the swimming pool for the blood draw at 42 m. Descent time was typically 35–40 s and the blood draw at depth was performed immediately after. Divers spent 30–40 s at maximum depth for sampling, then the ascent phase took between 40 and 45 s. We found a statistically significant increase in NOx plasma concentration at the bottom, in terms of percentage of basal value (410.5% ± 194.1; $p < 0.001$), the NOx value returned to normal at T0 and remained unaltered at T30 and T60 We observed a statistically significant decrease (−60%) in TAC at the bottom ($p < 0.0001$). Also, the TAC value returned to normal at T0 and remained unaltered at T30 and T60 (Figure 3). We did not find any correlation between the underwater results and AGE or BMI.

2.3.4.2 SCUBA diving underwater blood draw

A total of 15 experienced SCUBA divers, 13 male and 2 female, mean age 47.9 ± 10.7 years; mean height 176.7 ± 6 cm; mean weight 78.9 ± 13.2 kg, and BMI 25.18 ± 3.6 were studied. The Diving profile showed a mean depth of 41.2 ± 0.6 meters a mean diving time of 42.5 ± 3.5 min with bottom time of 8.6 ± 1.9, ascent time 1.7 ± 0.3 m/s and a mean of GF of 0.86 ± 0.02. Diving was performed in the swimming pool Y-40 (42 m depth), in thermal water at 34 °C, and mean temperature, as recorded by diving computer, of 33.4 ± 0.7 °C. We found a statistically significant increases of NOx plasma concentration in the bottom blood draw, +195.2 ± 58% of basal value (44.7 ± 14.2 μM) $P = 0.0001$, and in the safety stop blood draw +239.5 ± 149% of the basal value (50.25 ± 21.3 μM) $P = 0.01$, compared to the basal condition pre diving taken as 100% (24.6 ± 11.3 μM). We did not find any difference in NOx plasma concentration between the basal value and the post diving samples $P > 0.05$ (T0 = 29.1 ± 13.8 μM; T30 = 26.4 ±13.3 μM; T60 = 23.6 ± 14.4 μM). NOx values found before the diving exposure were in the normal plasma levels as compared to the no-diver population (20–40 μM) [183]. TAC was successfully investigated only in 9 of the 15 investigated subjects, on these we did not find any significant statistical difference in the bottom blood sample, while the safety-stop and the post-dive samples showed higher TAC values compared with the basal value (safety stop +127 ± 52.06%; T0 +109 ± 6.8%; T30 +109 ± 5.0%; T60 +110 ± 10.3%). Differences in T0, T30, and T60 were statistically significant.

We found four B (including two grade 2 and two grade 3 at EB Scale) vs. 11 NB.

We did not find any difference in NOX and TAC mean between non-bubblers and Bubblers (NB vs. B).

Also, we did not find any correlation between NOx AGE and BMI.

2.3.4.3 BH-divers investigated for genetic polymorphism vs environmental variables

A total of 50 healthy BH-Divers (40 male and 10 female; mean age 43.24 ± 9.8; mean height 176.3 cm ± 7.1; mean weight 74.4 kg ± 10.4; and BMI 23.8 ± 2.4) were studied in two different environmental conditions: 22 at the swimming pool and 28 at sea. The overall diving profiles showed an AD of 22.2 ± 8.5 m, n = 23 AD-above, and n = 27 AD-below; MD of 33.2 ± 8.2 m, n = 24 AM-above, and n = 26 AM-below; and an ND of 16.5 ± 5.8, (n = 26 ND-above vs. n = 24 ND-below). Subjects were also classified as HE of n = 28 and ME of n = 22. The groups obtained by dividing the sample in above and below the average (AD, MD, ND-above vs. AD, MD, ND below, respectively) showed significant differences between the more performant subjects as compared with the less performing ones in terms of AD (AD-above vs. AD-below); MD (MD above vs. MD-below) and ND (ND-above vs. ND-below) confirming a different diving exposure in the two groups (above vs. below). Similar significant differences were also found between more experienced and less experienced divers [ME vs. HE]. We did not find any statistical differences in BMI and age in the groups selected as the MD and ND were not statistically different in the two investigated environments (swimming pool vs. sea). We only found a higher mean of depth in the swimming pool group as compared with the sea group.

Regarding overall plasma NOx concentration, a significant decrease of –27.6% at T30 (73.5% of the control value, $p<0.0001$) and a significant increase of C24.1 at T60 (124.1%, of the control value, $p < 0.012$) were found. All these differences (decrease at T30 and increase at T60) were statistically significant in terms of the percent of the pre-diving control value. Regarding the differences in blood NOx concentration among the groups, a significantly lower decrease was found at T30 in experts (AD, $p = 0.002$; MD, $p = 0.01$; ND, $p = 0.01$; DL, $p = 0.03$). The difference, if any, in NOx plasma concentration was detected comparing the swimming pool vs. the sea setting ($p = 0.81$). At T60, a higher increase of NOx value was found in subjects with higher diving exposure in terms of MD ($p = 0.018$) and LD ($p = 0.006$). In contrast, the mean depth and the ND were not associated with significant changes in NOx levels. Finally, a higher NOx increase at T60 was found in the sea group than in the swimming pool group ($p = 0.014$). No statistical difference was detected in TAC levels between pre- and post-dive values. Similarly, no differences were found at T30 in the groups above vs. below average for the four-diving risk levels (AD, MD, ND, and LD) and in the two different environmental conditions (sea vs. swimming pool). Only the follow-up at T60 demonstrated higher oxidative stress levels in divers who performed BH-diving above the mean of MD and in HE divers HE ($p = 0.01$ and $p = 0.03$, respectively). No significant difference was found as well between the swimming pool and the sea BH-dives. Finally, no significant relationship was found in the NOx and TAC levels in the different genetic variant.

2.3.5 Discussion

Scuba diving exposes the human body to environmental stress conditions implying increased ambient pressure, pO_2, physical efforts and breathing resistance [73].

NO [187] plays an essential and complex role as a signaling molecule in several human physiological and pathological responses, including diving and hyperbaric related adaptations [102, 188]. NO (and its behavior) is a complex molecule to be studied, for its multifaceted actions and the short half-life [144], especially, when adding the challenging test conditions of an extreme environment.

A standard method to investigate plasma NO changes is by looking at the variations of its oxidation products, NOx, because the half-lives of NO_3 and NO_2 in blood circulation are 5–8 h and 20–40 min, respectively [152, 189]. Physical exercise increases eNOS activity resulting in a higher level of circulating NOx [190, 191]. Under particular conditions, such as hypoxia, NOx can be reconverted to NO through different pathways as previously described. This effect results from two mechanisms: the availability of deoxyhemes (reaction substrate) to bind NO_2, which is maximal in deoxygenated hemoglobin, and the amount of oxygenated hemoglobin tetramer, which increases the intrinsic reactivity of the heme with NO_2 [152].

Data obtained from an underwater blood draw (-42 m) carried out on expert SCUBA and BH-divers clearly showed the NOx kinetics in diving. It is particularly interesting to note that the increased NOx values found at the bottom and at the safety stop were not observed at post dive sampling (T0, T30, T60), showing a very rapid return to the pre-dive values. These changes could not have been identified by the usual pre and post dive tests, suggesting the need to further develop underwater real-time monitoring protocols. These data indicate a significant underwater increase in plasma NOx concentration and an immediate return to baseline values after reaching the surface [192]. These data confirmed a significant use of NO during SCUBA and BH-diving, compatible with the well-known underwater-related circulatory adaptations, but unexpectedly showed a swift return of circulating NOx to basal levels at the surface. This last aspect confirms that the NOx measured after diving reflects the availability of NO in real-time, without any diving-related “accumulation” effect in tissues. This observation helps to understand the results reported in this new protocol performed after a BH-diving training session, where we found a decrease of NOx 30 min after the training session, followed by an increase at T60. These data partially confirm a previous study where a similar increase was found, although without any initial decrease [102]. This difference could be easily explained by the different diving protocols of the previous test compared with that proposed in this research, especially concerning the ND and the descent technique. Therefore, the T30 post-diving reduction of NOx found in this experiment can be ascribed to the lower NO availability in the first few minutes after the dives caused by the higher use of this molecule during diving [192] to ensure the BH-diving related vascular adaptations. On the other hand, an increase at T60 could be a rebound of the efforts of the body to restore basal conditions after exceptional stress exposure.

Hyperbaric exposure-related oxidative stress is the second aspect taken into account due to the consequences potentially affecting SCUBA and BH-divers. Scuba diving can activate the antioxidant defenses to control

vascular oxidative stress due to increased O2 level associated to hyperbaric conditions and change the vascular endothelial growth factor metabolism, with the activation of a signaling cascade resulting in a stimulation of tissue resistance to diving derived oxidative stress [115]. Similarly, to SCUBA divers, BH-diving results in higher ROS production and oxidative stress, as confirmed by several authors [92, 102, 192], along with the activation of endogenous antioxidant defenses [64, 193]. As recently suggested [192] oxidative stress is transitory, increasing in the underwater phases but returning near pre-dive levels after reaching the surface. TAC did not show any difference between pre- and post-diving in the present experiment suggesting the absence of an oxidative stimulus at the end of the training session, despite the hyperbaric exposure. The data, in this study, indicate significant differences in NO consumption only when stratifying the divers into the two groups of high or low hyperbaric exposure or in the two groups of more expert vs. less expert subjects.

It is also intriguing to note that the lower NO consumption was observed in expert divers (HE) and those with higher hyperbaric exposure on the day of the experiment. We can hypothesize that a possible adaptation effect was undergone in these subjects who trained more intensely or had higher performances on the day of the investigation, with respect to the BH-divers that dived below the average. This aspect can also be explained by the "relax and comfort" training and diving techniques adopted by expert BH-divers, most likely decreasing the NO necessary to support the BH-diving induced hyperbaric-related physiological changes. However, this variable was not explicitly investigated and is worthy of further assessment in the future.

Another important observation concerns the changes in NOx and TAC when comparing swimming pool and sea exposures. Data at T30 were similar in the two different environments indicating a low influence of variables such as temperature (34 vs. 24 °C) and salinity (freshwater vs. seawater). Therefore, NOx level changes are probably more influenced by the magnitude of hyperbaric exposure. An in-depth analysis of this aspect showed that the rebound effect noted at T60 is significantly higher in the sea subjects than in the swimming pool subjects (183.9 ± 112.9 vs. 113.5 ± 84.3). This could be related to the characteristics of sea BH-diving, requiring complex logistics for the training sessions, the use of a boat and a diving suit, more time inside the water (even if the ND is similar in the two groups), and more demanding environmental conditions (e.g., colder temperatures, waves, and currents). However, with all the limits that our genetic investigation shows, it is intriguing to note that the data did not show any differences in NOx and TAC values in the single nucleotide variant in all the 10 polymorphisms investigated. If this data will be confirmed by future studies, more focus on the genetic aspect could be indicated to confirm that the genetic predisposition is less critical as compared with hyperbaric exposure when concerning BH-diving related NOx and oxidative stress.

2.4 Papers markers of critical conditions related

One of the most important challengers in the prevention of extreme sport accident is related to the possibility to intercept some premature markers able to prevent or intercept possible critical health condition. As

consequence we decided to develop some scientific protocols to investigate these aspects, and up-date the knowledge in this specific field. Related to this aim we produced five studies:

1. Danilo Cialoni, Andrea Brizzolari, Nicola Sponsiello, Valentina Lancellotti, Cesare Lori, Gerardo Bosco, Alessandro Marroni, Alessandra Barassi, Serum Cardiac and Skeletal Muscle Marker Changes in Repetitive Breath-hold Diving Sports Medicine - Open (2021) 7:58.
2. Danilo Cialoni, Andrea Brizzolari, Nicola Sponsiello, Valentina Lancellotti, Gerardo Bosco, Alessandro Marroni, Alessandra Barassi, Serum Amino Acid Profile Changes After Repetitive Breath-Hold Dives: A Preliminary Study, Sports Medicine - Open (2022) 8:80.
3. Danilo Cialoni, Andrea Brizzolari, Alessandra Barassi, Gerardo Bosco, Massimo Pieri, Valentina Lancellotti, Alessandro Marroni, White Blood Cells, Platelets, Red Blood Cells and Gas Bubbles in SCUBA Diving: Is There a Relationship?, Healthcare 2022, 10, 182.
4. Sergio R. Schirato, Vitor Silva, Katia Iadocicco, Alessandro Marroni, Massimo Pieri, Danilo Cialoni, José G. Chaui-Berlinck, Post-decompression bubble and inflammation interactions: a non-extensive dynamical system model, Undersea Hyperb Med, 2022 Second-Quarter;49(2):207-226.
5. Pierre Lafère, Kate Lambrechts, Peter Germonprè, Ambre Balestra, Faye L. Germonprè, Alessandro Marroni, Danilo Cialoni, Gerardo Bosco, Costantino Balestra, Heart Rate Variability During a Standard Dive: A Role for Inspired Oxygen Pressure? Front Physiol 2021 Jul 26; 12:635132. doi: 10.3389/fphys.2021.635132. eCollection 2021.

Below a description of the paper related to the markers of critical diving condition, some part of the paper description similar in the different manuscript are presented only one time, while a dedicate up-date is proposed for any paper as concerning material and method and results.

2.4.1 Introduction

BH-divers are exposed to extreme environmental conditions such as increased hyperbaric pressure and low temperature that cause changes in arterial blood gases [194-196], inducing the "diving response" which includes bradycardia, reduced cardiac output, increased arterial blood pressure and peripheral vasoconstriction [197]. This complex adaptation mechanism is caused by the simultaneous activation of the sympathetic and parasympathetic nervous system that seems to reduce O_2 consumption in peripheral tissues to ensure enough O_2 supply of the vital organs [198]. Blood is rerouted to brain, heart [199, 200], liver [201] and the active muscles [202] as a result of the diving response to optimize O_2 management and help prolonging apnoea duration [203, 204]. BH-diving carries several risks related to physiological stressors that arise in the extreme environment as a consequence of the increased hydrostatic pressure, hypercapnia, hypothermia and strenuous exercise [195]. A prolonged strenuous physical activity may result, also in not extreme environments, in an increased exercise-induced muscle fatigue, in both skeletal and cardiac muscles [205] that can induce an increase of muscles injury markers such as: creatine kinase (CK), aspartate transferase (AST) and alanine transferase (ALT) when concerning the skeletal muscle and cardiac creatine kinase isoenzyme (CK-MBm), and cardiac troponin I (cTnI) when concerning the cardiac muscle [206, 207]. Lactate dehydrogenase (LDH) could also be used as a marker of muscle stress [208].These enzymes are commonly studied as stress markers of specific organs or tissue (i.e., muscle) where they are more represented.

CK is an enzyme expressed by various tissues, especially those that need high amounts of ATP such as skeletal muscle, brain, heart [209], where it catalyzes the reversible conversion of creatine and uses adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP). CK is also involved in smooth muscle energetic system but with relatively low concentrations as compared to that of skeletal muscle [210]. CK is also considered a qualitative marker for skeletal muscle microtrauma [211]: its changes during physical activity depend on individual physiology, training level, exercise duration, with peak values recorded after endurance events [212].

Aspartate transferase (AST) and alanine transferase (ALT), are commonly considered liver damage markers [213]. ALT is found mainly in the liver but also, in smaller amounts, in the kidney, heart, muscle, and pancreas while AST is present in the liver as well as in considerable amounts in other tissues including muscles [214]. AST and ALT are released from activated muscles, and levels can increase after acute physical exercise: increase in AST and ALT activity is related to the type, intensity and duration of physical effort [215]. The increase in AST activity during intense exercise is more likely connected to the release of the enzyme from the muscle cells than to liver pathologies [216-219]

CK-MBm is the CK isoenzyme present in cardiac muscle. Similar to CK, the influence of physical exercise on CK-MBm is related to the type, intensity, and duration of muscular activity. The elevation of serum CK-MBm is not unique to athletes but is a common occurrence among individuals who perform eccentric exercise [220, 221]. Prolonged endurance exercise may be associated with increase in serum CK and CK-MBm levels that are comparable to those of a myocardial infarction [222].

A cardiac biomarker typically seen with myocardial infarction is cardiac troponin I (cTnI) [206, 207]. Troponins (T and I) are commonly used diagnostic markers for cardiac ischemia and infarction. Increased circulating troponins can be related to strenuous physical exercise such as marathon competition [223].

In skeletal and smooth muscle, LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+ to meet the energy demand of the cell. A closer, mechanistic analysis of lactate production under anaerobic conditions shows that there is no biochemical evidence for the production of lactate through LDH contributing to acidosis. While LDH activity is correlated to muscle fatigue, the production of lactate by means of the LDH complex works as a system to delay the onset of muscle fatigue [224].

Furthermore, endothelial dysfunction, well investigated in BH-diving [102, 225] may lead to smooth muscle alteration. The release of Amino Acids (AA) and their skeletal muscle catabolites in the blood circulation, and their reuptake by other tissues are parts of complex metabolic pathways aimed at maintaining energetic homeostasis [226]. As it is well known, AA are involved in several metabolic activities and particularly:

a) <u>fuel metabolism:</u> to ensure enough energy during exercise through the involvement of Alanine (ALA) and the three branched-chain amino acids (BCAA): Leucine (LEU), Isoleucine (ILE) and Valine (VAL). ALA is synthesized in skeletal muscle and released in the bloodstream [227] to be transported to the liver where to be

regenerated into pyruvate. BCAA are the most relevant AA metabolized during physical exercise for energetic request (because physical activity needs energy substrates) [228]. BCAA may protect from protein degradation and muscle enzyme release [229] reducing skeletal muscle damage during prolonged physical effort [230], mitigating central fatigue [231] and promoting recovery of muscle function [232]. During physical activity also Hystidine (HIS), Threonine (THR), Lysine (LYS) and Methionine (MET) are involved in catabolic processes (Krebs Cycle) [233] to produce the necessary energy to sustain the effort. Proline (PRO) can be converted in α-ketoglutarate, a Krebs cycle intermediate to sustain the energy request. Furthermore, PRO may be involved in to the free fatty acids (FFA) catabolism [234, 235].

b) Improved exercise fatigue tolerance: Physical activity increases catecholamine levels in athletes, depending on duration and intensity of exercise [236]. Some aromatic amino acids, Tyrosine (TYR), Phenylalanine (PHE) can influence the levels of catecholamine precursors [237]: plasma catecholamine precursors are associated to improved tolerance during prolonged physical exercise [238]. Ornithine (ORN) promotes lipid metabolism, activates the urea cycle, and improves fatigue tolerance ameliorating physical performance [239].

c) Nitric oxide (NO) production: NO is produced by the conversion of Arginine into Citrulline (CIT) by nitric oxide synthase enzymes (NOS) and it reflects NO synthesis [240]. During physical exercise also the catecholamines stimulate the production of NO [241] that plays a key role in skeletal muscle contractile function [242], increasing in response to acute sessions of exercise [243-245] to adapt the body to exercise training [246]. NO plays a key role in the response to hyperbaric exposure, modulating the endothelial adaptation during BH-diving [104, 105].

d) Antioxidant defences: a prolonged strenuous physical activity can lead to oxidative stress with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [247] reducing NO availability [62]. Human body can activate antioxidant defences to protect tissue from the toxic effects of free radicals [248]. Cystine (CYST), Glutamate (GLU) and Glycine (GLY) are necessary for the glutathione biosynthesis [249], a component of the body antioxidant defences. Endogenous antioxidants protect macromolecules from vascular oxidative stress due to increased O_2 level associated with hyperbaric condition [64] and hyperoxic exposure, as observed by data obtained from an underwater blood draw (-42 m) carried out on SCUBA [103] and BH-divers [104].

e) Hypoxic response: There is little knowledge on the release of AA in case of hypoxic exposures due to environmental variations or diseases (e.g. cancer and chemical hypoxia); Serine (SER) [250] and Taurine (TAU) [251] seem involved in hypoxia conditions. Changes in AA in BH-diving were studied at high altitude showing an increase of ALA and BCAA, representing an potential adaptation mechanism to hypoxia [252].

During underwater activities, a decreased heart rate seems a well-established steady-state response to pressure[253]. This change may be of primary importance and could even account for some diving accidents [254]. It seems thus important to further assess diving related changes in heart rate and in their ANS triggers to enforce divers' safety. The cardiovascular system is challenged by various combinations of the direct effects

of water immersion, thermal strain, exercise, depth pressure, hyperoxia, or apnea. Cold and hyperbaric pressure contributing to increase parasympathetic activity when diving in very cold water under constant hyperbaric pressure [255]. During thermo-neutral immersion hydrostatic pressure restrains the vascular compliance, which leads to a redistribution of blood volume from peripheral to central Circulation [256]. During this condition of repleted vasculature, the need for sympathetic activity maintaining smooth muscle tone in the vascular wall is lessened [257]. On the other hand, the redistribution of regional blood flow is responsible for an increase in heart volume, stroke volume, and cardiac output [258] accompanied by bradycardia, resulting from vagal activation. In addition to hydrostatic pressure per se and immersion, real diving (submersion) may exert its effects on the diver's cardiovascular system by mechanisms, such as increased partial pressures of inhaled gases. Cardiovascular sympathetic influence is lowered by normobaric and hyperbaric hyperoxia [259] while conversely cold-water immersion causes an increased systemic sympathetic activity [257]. The use of enriched air can result in oxidative stress affecting cardiorespiratory and vascular systems as it has been reported during CCR diving [95]. In general, in chronic disorders associated with oxidative stress (diabetes, cardiovascular (ischemic) disorders, etc.), a depression of aerobic metabolism is observed, reflected by low HRV [260, 261]. On the contrary, high intensity of redox reactions (more efficient autonomic regulation) is usually reflected by high HRV [262].

However, regarding diving conditions, the possible effects of acute exposure to oxygen-enriched diving oxidative state in humans remain unclear and only few studies addressed the effects of diving on ANS [263]. Recently, some authors showed that parasympathetic activation was increased during nitrox diving, and this increase was marked enough to reduce heart rate during the dive [264]. During a standard air dive, Dalton's Law of Physics implies that the partial pressure of oxygen (PpO2) increases during the descent phase, remains elevated during the bottom phase, and decreases again with decreasing environmental pressure during the ascent phase at the end of the dive. In contrast, during (electronic) closed-circuit rebreather (CCR) diving, a certain PpO2 is selected by the diver and remains constant through automatic addition of oxygen by the diving apparatus.

Despite all the available information in normobaric condition, very few data are known about cardiac and skeletal muscle serum markers in BH-diving. In particular, there is no information whether the possible variations of the mentioned biomarkers could be related to the physical effort and the hypoxia or a combination of both.

2.4.2 Materials and methods

2.4.2. Serum Cardiac and Skeletal Muscle Marker and Amino acid subject

A total of 12 expert healthy BH-divers, were investigated during an open sea training session at Elba Island, Italy. All the divers were informed about risks and benefits of this study, read and signed a specific informed consent form before the experiment and provided personal anthropometric parameters. Apnea Academy standards and exclusion criteria were described above.

As per Apnea Academy standard procedures, all the divers were asked to perform a free number of dives at increasing depth with a surface interval at least 3/4 the diving time do to a gradually approach to the maximum daily personal depth. When ready, they performed the last dive reaching the maximum depth of the training session. All the dives were performed as constant weight bi-fins discipline (CWTB). Divers wore a 5 mm wetsuit.

Diving profiles, including mean depth, maximum depth, and number of dives, were recorded using a free-diving computer (UP-X1 Omersub Spa, Monza Brianza, Italy). This computer measured and recorded diving data every 2 seconds.

The diving profile showed a mean number of dives of 17.7 +/- 3.2; a mean depth of 32.5 m ± 6.1 meters; a mean of maximum depth of 42.9 m +/- 1.7meters.

2.4.2.2 HR variability subjects

18 males, 12 using OC (30 mfw for 20 min) and 6 using CCR (30 mfw for 40 min.) were recruited. y. They were at least certified as "autonomous diver" according to European norm EN 14153–2 or ISO 24801-2, and each of them counted at least 50 logged dives. They were selected from a large sports/technical diver population to obtain a group of homogenous age (30–40 years), similar body composition (BMI between 20 and 25), and similar health status: non-smokers with regular but not very high physical activity (aerobic exercise one to three times a week).

2.4.2.3 Protocol details

A butterfly needle (21Gx34 0,8x19 mm Green) was placed in the antecubital vein to collect 5 ml of blood using 5 ml serum containing tube (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). We collected blood samples per each of the following time steps:

- Basal: 30 minutes before the start of the warm-up;
- T0: 30 minutes after the dive sessions;
- T1: 4 hours after the dive session.

After 15 min and before 30 min from collection at room temperature, blood samples were centrifuged (3000 rpm for 10 min) to separate serum from cell fraction and was frozen at -20°C. Then, the serum samples were then delivered to the laboratory for analysis and kept at -20 °C until the analysis.

Each diver of group 1 performed a 30 m depth dive into fresh water (mfw) for 20 min in a pool environment (Nemo33, Brussels, Belgium) in a 33°C water, which normally do not require any thermal protection suit. However, each diver wore a 5 mm neoprene suit. This depth-time profile is embedded within the "no-decompression limits" of the US Navy dive tables, 2008 edition (NAVSEA, 2008). Descent speed was 15 meters per minute and ascent speed to the surface 10 meters per minute, with no safety stop (none required according to the NAVSEA, 2008 dive table used).

Because CCR diving was not allowed in the Nemo 33 pool CCR divers performed a 30 m depth dive in a flooded quarry with fresh water. Using a Megalodon rebreather (InnerSpace System Corp, Centralia,

Washington, United States), a constant partial pressure of oxygen was set at 1.3 ATA with air as diluent gas; this yielded an equivalent air depth (EAD) of 24.2 mfw. Bottom time was set to 40 min so that the depth-time profile also lies within the same "no-decompression limits" (NAVSEA, 2008). Nonetheless, for safety reasons, a 6 min safety stop at 6 m was added. Descent and ascent speeds were kept similar to those in the pool experiment. As the water temperature was measured at 17°C (Scubapro-Uwatec digital depth gauge, Hallwil, Switzerland), divers wore a trilaminate drysuit equipped with dry gloves, a 5 mm neoprene semi-dry hood with face and neck seals so that only the mouth area was exposed to the water. They also wore a Thinsulate thermal underwear and a battery-powered heated vest system (Silent Planet, Portland, Dorset, England). Before and after the CCR dive, cervical-supraclavicular area skin temperature was evaluated using a forward-looking infrared (FLIR) camera, confirming the absence of significant skin cooling during the dive (Pre-dive: 36.9 ± 0.5°C vs. post-dive:37.5 ± 0.7°C; $p > 0.05$).

2.4.2.4 Cardio Muscular Markers

After 15 min and before 30 min from collection at room temperature, blood samples were centrifuged (3000 rpm for 10 min) to separate serum from cell fraction and was frozen at -20°C. Then, the serum samples were delivered to the laboratory for analysis and kept at -20 °C until analysis. The sample analysis was carried out as described in paragraph 1.5.2.3.6.

2.4.2.5 Amino acid Markers

The concentrations of the following AA were measured in serum:

- ALA, HIS, ILE, LEU, LYS, MET, PRO, THR, VAL, as concerning the energy need;
- TYR, PHE, ORN as concerning the tolerance to the prolonged physical activity;
- CIT as concerning NO production;
- CYST, GLU, GLY as antioxidant synthesis;
- SER, TAU as concerning the BH-diving related hypoxia.

Serum AA concentrations were determined by a Biochrom30plus Amino Analyzer (Biochrom Ltd., Cambridge, UK, EU), a cation-exchange chromatography system [265]. Briefly, AA were purified mixing the serum samples 1:1 v/v with 10 % sulfosalicylic acid (Sigma Aldrich Corp., St. Louis, MO, USA) containing the internal standard norleucine 500 μmol/L (Sigma Aldrich Corp.) and adding 2 volumes of Lithium Citrate Loading Buffer pH 2.20 [265]. After strong agitation and cooling at 4 °C for 5 min, the samples were centrifuged 8 min at 14,000 rpm. Supernatants were filtered and 100 μL were the operative injection volumes. Post-column derivatization with ninhydrin allowed the detection of AA at the wavelength of 570 nm, while 440 nm for Pro. Standard Lithium Citrate buffers with pH 2.80, 3.00, 3.15, 3.50, and 3.55 and ninhydrin reagents utilized during separation were provided ready to use by Biochrom Ltd. Briefly, 125 μmol/L AA standard solution was prepared by mixing physiological basis with acids and neutrals and internal standard solution (Sigma Aldrich Corp.). Data analysis was performed by EZChrome software (Agilent Technologies, Santa Clara, CA, USA). Areas of the peaks were used to determine AA concentrations and they were

expressed in terms of μmol/L. All divers were let loose on their usual diet without any AA supplementation or conditioning in food, we measured the serum AA value before and after the training session studying the relative delta regardless of differences in diet.

2.4.2.6 HR Variability Markers

HRV was recorded using a polar recorder. Four samples of R-R intervals representing the dive were saved for HRV analysis. Standard deviation of normal-to-normal intervals (SDNN), square root of the mean squared differences between successive RR intervals (rMSSD), and average RR intervals (RR) in time-domain; low frequency (LF) and high frequency (HF) in frequency domain were investigated. Nonlinear analysis included fractal dimension (FrD).

2.4.3 Results

2.4.3.1 Cardio Muscular Markers results

A total of 12 experienced breath-hold divers, 9 male and 3 female, mean age 41.6 ± 5.6 years, mean height 178.6 ± 9.8 cm; mean weight 76.5 ± 12.8 kg and BMI 23.8 ± 2.2 were investigated.

The diving profile showed a mean of maximum depth of 33.3 meters +/- 6.3. a mean of numbers of dives of 14.8 +/- 3.2 and a mean of average depth of 18.9 +/- 4.6. All the subjects respected the warm-up protocol. All the volunteers completed the experiment without Taravana episodes, evidence of pulmonary and/or ear barotraumas or other health problems.

Diving was performed at Elba Island, Italy, in salt water at 21±0.5 °C mean temperature, as recorded by diving computer.

As reported in Figure 23, we found statistically significant increases of CK (T0: 136.1% ± 15.8 $p<0.0001$; T1: 138.5% ± 20.1, $p<0.0001$), CK-MBm (T0: 145.1% ± 20.1, $p<0.0001$; T1: 153.2% ± 23.1, $p<0.0001$) LDH (T0: 110.4% ± 6.4, $p=0.0003$; T1: 110.1% ± 7.4, $p=0.0013$) in both T0 and T1 blood samples, as compared to basal value. AST showed a statistically significant increase only at T0 (106,8% ± 4.6, $p=0.0007$) while ALT did not show any statistical difference in T0 and T1. None of subject show an increase of cTnI levels in post diving samples, in this marker all subjects show the same results before and after the BH-diving session (< 0.012 ng/mL) and the statistical analysis was not applicable.

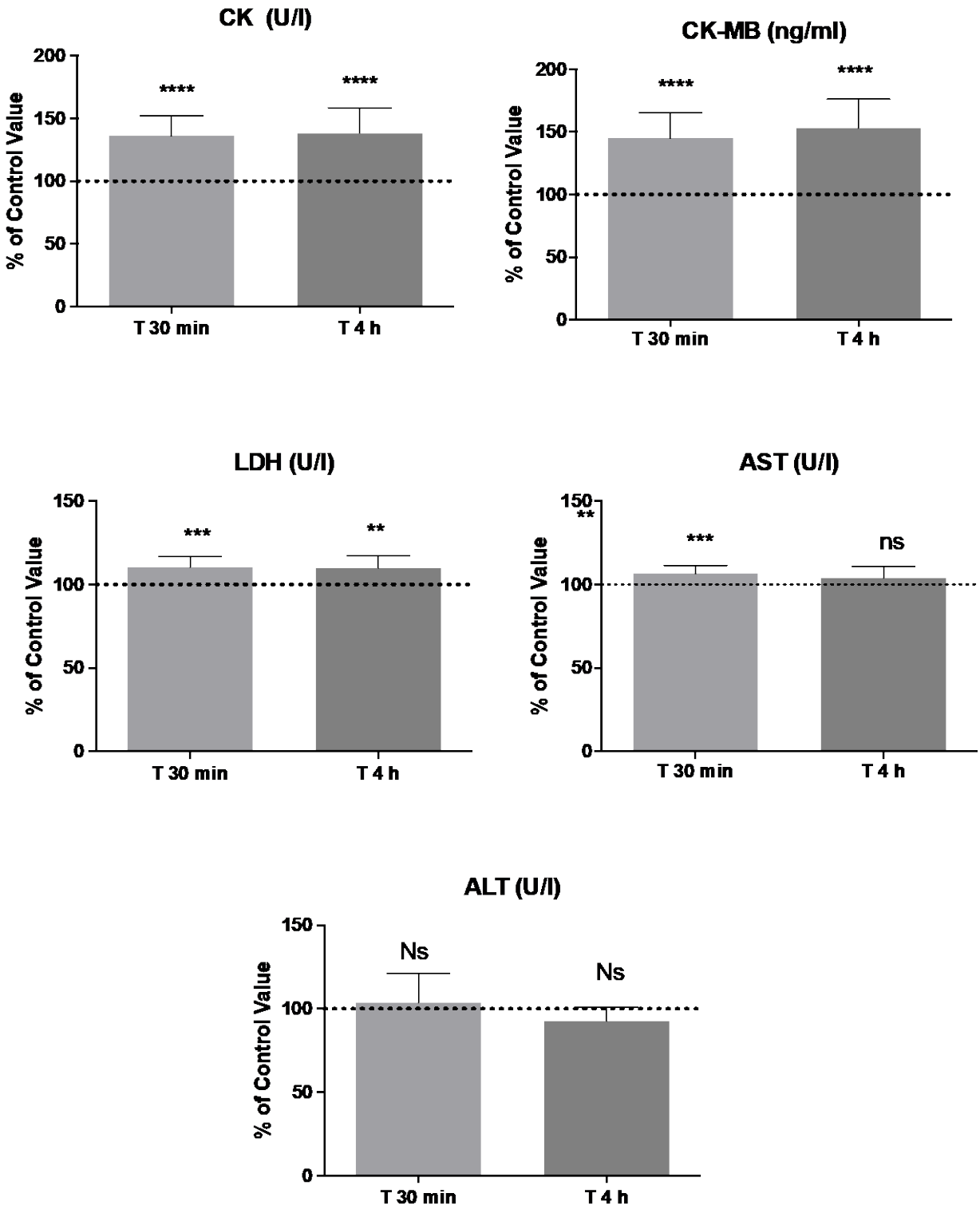

Figure 23 - Serum levels of cardiac and skeletal muscle stress biomarker changes after a session of BH-diving training. Data are presented as mean ± standard deviation (SD). The height of the columns represents means and the error bars represent sd's. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.4.3.2 Amino acid profile results

A total of 12 experienced BH-divers, 9 male and 3 female, mean age 41.6 ± 5.6 years, mean height 178.6 ± 9.8 cm; mean weight 76.5 ± 12.8 kg and BMI 23.8 ± 2.2 were investigated. All the volunteers completed the experiment without Taravana episodes, evidence of pulmonary and/or ear barotraumas or other health problems. Diving session was performed at Elba Island, Italy, in salt water at 21 ± 0.5 °C mean temperature, as

recorded by diving computer. Almost all the investigated AA showed a significant reduction after diving, some of them returned at pre diving value at T2 (4 hour). Only CYS, involved in endogenous antioxidant synthesis, showed a significant increase after diving (T1 and T2). The detailed results are showed in the figures 24-28 while Table I shows the details for each investigated AA. We did not find any differences in blood volume between pre and post diving value ($p>0.05$).

We found a significant correlation between several AA involved in energy supply and mean of depth, especially at T1 with THR ($p = 0.01$; r: 0.80) and VAL ($p = 0.01$; r: 0.72).

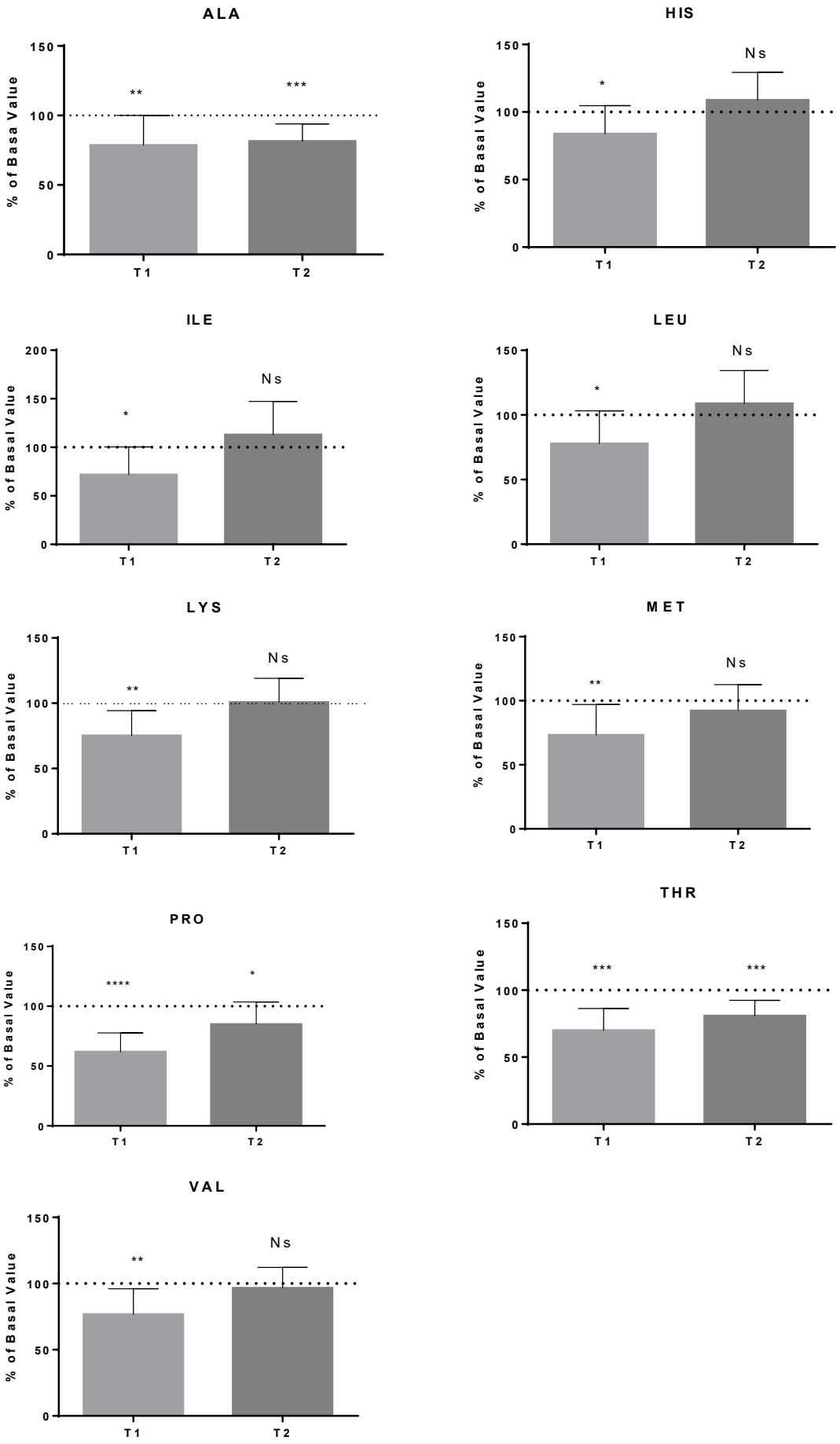

Figure 24: AA changes related to energy need. Data are expressed as percentage of control value. Asterisks indicate a value significantly different compared to basal (* p<0.05, **p<0.01, ***p<0.001, **** p<0.0001).

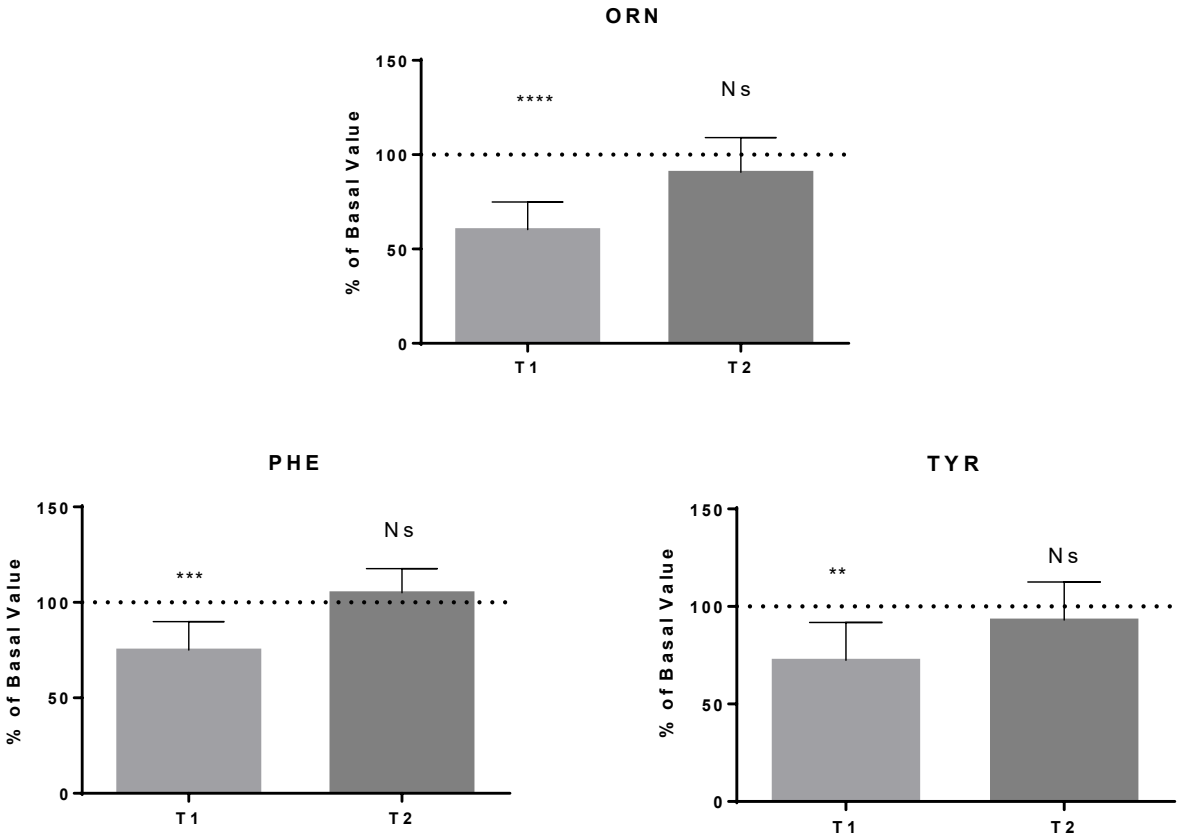

Figure 25: AA changes related to tolerance of physical effort.

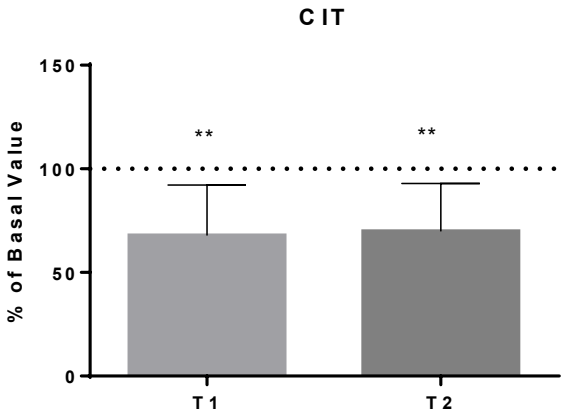

Figure 26: AA changes related to NO production.

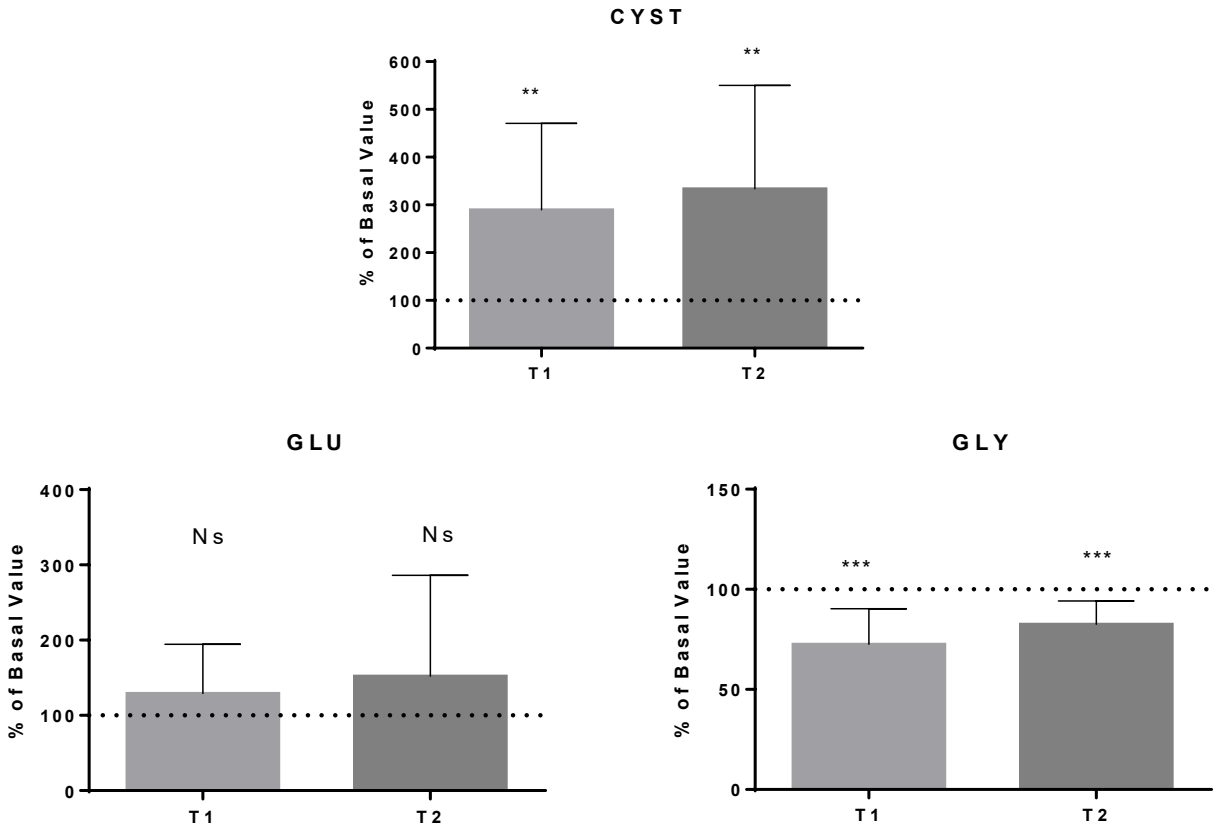

Figure 27: AA changes related to antioxidant synthesis

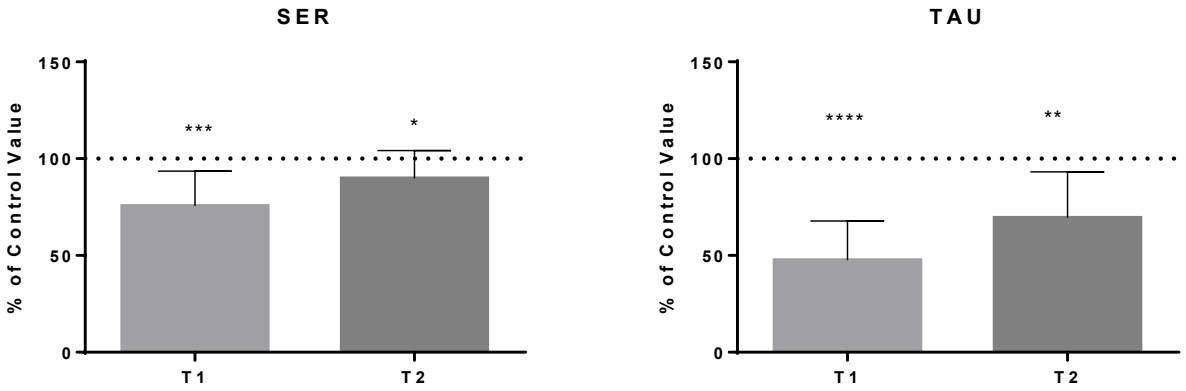

Figure 28: AA changes related to hypoxia tolerance

Table 1: AA Table 1: AA changes in BH-divers. Data are expressed as percentage of control value. Asterisks indicate a value statistically significantly different compared to basal value (* p<0.05, **p<0.01, ***p<0.001, **** p<0.0001).

Amino Acid	Basal Value (µmol/l)	T1 Value (µmol/l)	T1 % of basal value	T2 Value (µmol/l)	T2 % of basal value	P=Value
Energy Need						
Alanine	454.0 ± 97.1	343.1 ±69.1	78.7 ± 21.4	364.1 ± 64.2	81.5 ± 12.4	T1:**/T2:***
Histidine	70.5 ± 11.6	57.6 ±11.2	83.9 ± 20.8	75.4 ±10.1	108.9 ± 20.4	T1:*/T2: Ns
Isoleucine	70.7 ± 24.8	46.2 ±14.5	71.9 ± 28.6	75.6 ± 21.2	113.0 ± 34.3	T1:*/T2: Ns
Leucine	149.5 ± 40.8	110.1 ± 28.5	77.9 ± 25.2	157.3 ± 34.5	108.9 ± 25.4	T1:*/T2: Ns
Lysine	175.8 ± 26.5	129.8 ± 28.7	75.3 ± 19.1	175.6 ±32.0	100.8 ± 18.3	T1:**/T2: Ns
Methionine	28.3 ± 6.7	19.7 ±4.7	73.4 ± 23.8	25.9 ± 7.6	92.3 ± 20.2	T1:**/T2: Ns
Threonine	167.1 ± 39.9	114.5 ± 27.8	70.1 ± 16.3	134.1 ± 31.9	81.0 ± 11.4	T1:***/T2:***
Proline	234.1 ± 76.2	143.8 ±57.2	62.0 ± 15.6	198.8 ± 74.6	85.0 ± 18.5	T1:****/T2:*
Valine	257.1 ± 43.9	194.1 ±48.0	76.8 ± 19.2	246.5 ± 47.8	96.6 ± 15.6	T1:**/T2: Ns
Fatigue Tolerance						
Ornithine	85.6 ± 25.7	49.7 ±13.8	60.4 ± 14.5	76.4 ± 24.8	90.7 ± 18.4	T1:****/T2: Ns
Phenylalanine	73.1 ± 14.3	53.3 ± 7.4	75.1 ± 14.9	76.7 ± 18.1	105.0 ± 12.7	T1:***/T2: Ns
Tyrosine	77.6 ± 18.1	54.9 ±13.4	76.4 ± 19.4	70.2 ± 12.3	93.0 ± 19.6	T1:***/T2: Ns
Nitric Oxide production						
Citrulline	25.8 ±16.7	21.1 ± 5.4	68.1 ± 24.2	19.3 ± 9.6	70.0 ± 22.9	T1:**/T2:**
Antioxidant synthesis						
Cystine	8.7 ± 5.8	18.6 ±7.1	289.6 ± 181.3	21.2 ±6.8	333.9 ± 216.7	T1:**/T2:**
Glutamate	19.0 ±11.4	19.6 ± 8.0	129.2 ± 65.3	22.2 ± 14.5	152.1 ± 134.2	T1: Ns/T2: Ns
Glycine	227.7 ± 59.0	160.4 ± 36.8	72.6 ± 17.7	186.1 ± 46.4	82.6 ± 11.6	T1:***/T2:***
Hypoxia Tolerance						
Serine	138.4 ± 27.2	101.7 ± 16.6	75.8 ± 17.8	123.5 ± 26.5	90.1 ± 14.0	T1:***/T2:*
Taurine	121.2 ± 28.1	54.2 ± 17.2	47.8 ± 20.1	80.0 ± 16.0	69.6 ± 23.6	T1:****/T2:**

2.4.3.3 HR variability results

SDNN and rMSSD were significantly increased during descent and at depth with OC, not with CCR. Mean RR interval was longer at depth with OC, but only during ascent and after the dive with CCR. HF power was higher than baseline during the descent both with OC and CCR and remained elevated at depth for OC. The LF/HF ratio was significantly lower than baseline for descent and at depth with both OC and CCR. After 30 min of recovery, the LF/HF ratio was higher than baseline with both OC and CCR.

Nonlinear analysis detected differences at depth for OC and CCR.

2.4.4 Discussion

The 12 subjects, 9 males and 3 females gave consent to the protocol and the blood sampling at the noted times: all were an elite group of "experienced" free divers performing similar exposures regularly: their body characteristics distinguish their excellent physical status.

Increase of CK may be aimed at protecting tissues from hypoxia, as demonstrated by some authors [266]. On the other hand, increased CK can be considered a consequence of increased anaerobic respiration stimulated by muscular activity and pressure-induced peripheral hypoxia [267]. The CK raise might confirm the main role of "diving response" for its intervention in the regulation of hypoxia-inducible factor (HIF-1) expression and activity. CK can be also related to a hyperactivity of breath muscles and hypoxic stress induced by BH-diving [268]. During mild hypoxia, CK probably raises for energetic needs because physical activity requires energy substrates to support aerobic glycolysis. CK is used to sustain ATP production, a condition related to all the kinases involved in energy production [269].

We observed a small elevation in serum AST, however far from levels indicating liver or skeletal or cardiac muscle injuries [270]. The increase in AST levels is a consequence of a strenuous exercise, which can cause an aminotransferase raise in the absence of liver injury [271]. AST can be used as markers of oxygen desaturation: in hypoxia conditions, Norman et al found a strong correlation between hypoxia and aminotransferase levels [272]. Since the BH-divers didn't perform an intense smooth muscle activity, AST increase may be only due to the myocyte oxygenation. These changes are not confirmed by ALT that on the contrary did not show any change BH-diving related.

CK-MBm isoenzyme, located in cardiac muscle, may be increased due to the stretching of cardiac atria, induced by the increased hydrostatic pressure induced blood shift [102].

The greater CK-MBm concentration facilitates production of ATP from creatine phosphate for muscle contraction, suggesting that the increased CK-MBm may be due to the muscle disruption as consequence of the prolonged physical activity [273, 274]. After exercise, elevated CK-MBm values seem to be associated with the regeneration of new muscle fibers from muscle fiber necrosis caused by exercise [222, 275-277].

cTnI level are unchanged after breath-hold diving training session. Some authors described troponin changes related to electrographic abnormalities or stroke volume decrease [278-280], observed in BH-divers and

attributed this to the effect of pressure and/or hypoxemia. However, cTnI level can be also related to the BH-diving technique: Marlinge et al showed an increase in cTnI during apnoea session, probably a consequence of prolonged cardiac workload and hypoxemia [281]. However, we didn't observe these variations: a possible explanation may be due to the individual physiology, similar to a training induced adaptation. Other factors may be dive activity years, personal depth record, number of weekly training sessions, different BH-diving techniques.

LDH increased after the BH-diving training session, as a consequence of prolonged physical activity. Lactate degradation is fast: a persistence of LDH value higher than pre diving condition may be related to the experience and training of the divers. In hypoxic conditions, the CK raise may be due to energy request to sustain aerobic glycolysis: the creatine phosphate cleavage supports the energy production from ATP. Any similar condition is related by to an increase of the kinase enzymes involved in energy production [269]. In BH-divers, it's possible that the combination of physical activity with hypoxia could raise the CK level.

BH-diving related physical effort can request an increase of catabolic metabolism to produce adequate amount of ATP. We observed a statistically significant decrease of several AA that can be used as substrate for energy need including ALA, LEU, ILE, VAL, HIS, THR, LYS, MET. ALA reduction can be the consequence of pyruvate production that is converted in acetyl-CoA to produce ATP (Krebs Cycle). ALA release should also lead to the muscle protein synthesis but, in this case, the Cahill cycle did not occur for the inhibition of proteosynthetic cascade [282].

During physical activity, BCAA (LEU, ILE, VAL) release, splanchnic bed rises and is accompanied by an elevated BCAA uptake by contracting muscles and by an enhancement of BCAA oxidation therein [228]. In skeletal muscle, BCAA oxidation is catalysed by branched-chain α-keto acid dehydrogenase (BCKDH) [283] to use them as energetic substrate [284] providing about 3–6% of the total energy demand [285], according to some authors that observed a decrease in BCAA after prolonged effort such as a tennis tournament [286], a marathon [287] and a cyclist race [288], and LEU decrease in sprinters and jumpers when the muscles work in anaerobic conditions [289].

HIS decrease can be explained because it is converted into GLU, then in α-ketoglutarate to go into the Krebs cycle. THR is converted to pyruvate via threonine dehydrogenase. An intermediate in the THR catabolism can undergo thiolysis with coenzyme A (CoA) to produce acetil-CoA.

LYS is the precursor for carnitine [290] which transports fatty acids to the mitochondria, where they can be oxidised to produce acetil-CoA, involved in tricarboxylic acid (TCA) cycle [291]. Finally for this group, according to data obtained by other authors [292], MET decreased after prolonged physical activity. This reduction may reflect increased transmethylation in which DNA, histones and other macromolecules are methylated in response to exercise [292]. PRO may also be related to the free fatty acids (FFA) release because some authors found a correlation between the decrease of PRO and the increase of FFA [235] as energy source during prolonged physical activity.

Physical activity led to release of antifatigue molecule precursors to improve the tolerance to physical effort [238]. TYR, obtained from PHE, is decomposed to give acetoacetate and fumarate that go into the TCA cycle. Its slow recovery is due to the PHE reduction: PHE is used to produce catecholamines, as observed by Sponsiello et al: urine catecholamine levels increased immediately after the dives, while we would have expected despite the participants were very expert BH-divers [293].

TYR decrease after the BH-diving session: this may be related to the stimulation of catecholamines (dopamine, norepinephrine, epinephrine) synthesis [294]. Prolonged repetitive physical exercises may activate signaling testosterone and brain-derived neurotrophic factor (BDNF) dependent pathways, leading to a raise of tyrosine hydroxylase activity and increasing catecholamine levels [295].

ORN seems to have an antifatigue effect increasing the efficiency of energy consumption and promoting the excretion of ammonia [239, 296]. Some authors found that ORN promotes fatty acid and protein catabolism improving physical performance and fatigue tolerance, especially in female athletes [239]. Also, we observed a reduction of CIT, used with aspartic acid to synthesize arginine-succinate that is a precursor for arginine, the primary substrate for NO biosynthesis. This data could be explained by the significant increase of NO production in BH-divers [104]. Indeed, NO plays a key role in the adaptation of subjects exposed to high hydrostatic pressure [102] and recent measurements taken in SCUBA and BH-divers at -40 m depth showed remarkable increases in the plasma concentrations of NO derivatives [103, 104]. Particularly, NO is the principal molecule involved in the regulation of vasoconstriction/vasodilatation mechanism, necessary to adapt the endothelium to the increased ambient pressure and the related regional modifications [102].

On the other hand the increase in pO_2 triggers the formation of ROS and RNS leading to oxidative stress, [62]. SCUBA and BH-divers can activate the endogenous antioxidant system to control vascular oxidative stress [64, 115]. This can explain the raise of serum CYST concentration: CYST is an important Cysteine source that is, with GLU and GLY, necessary for glutathione biosynthesis. GLY reduction may be due to the synthesis of glutathione: this is also confirmed by the increase of the glutathione peroxidase whose main biological role is to protect the organism from oxidative damage [63, 64, 297].

Finally, hypoxia occurs during the final part of BH-diving (ascent phase) [298]. SER is involved in the protection from hypoxia: mitochondrial serine catabolism protects from hypoxia maintaining mitochondrial redox balance and cell survival [250]. PRO decrease may be also related to the production of GLY, involved in the synthesis of antioxidants. The production of TAU precursors and TAU would underlie the tolerance to hypoxia: some authors registered a dose- dependent protective effect of TAU on the synaptic function of rat hippocampal slices exposed to a hypoxic insult [299, 300]. A similar protection mechanism may occur also in BH-diving despite the intermittent hypoxia. TAU decrease seems to be related to its antioxidant properties protecting tissues from highly toxic hypochlorite produced by inflammatory cells in the course of free radical processes [301] and other oxidative stress markers [302].

Our data seem to indicate a clear picture of the body adaption to hyperbaric exposure in BH-divers. From the interpretation of these results, it is clear that energetic metabolic request (for physical effort and for body adaptation to the extreme environment) is in large part supported by AA used as substrate for fuel metabolism. The reduction of several AA involved in energy support at T1 seems to be influenced by the characteristic of BH-diving, probably related to the "relax and comfort" training, diving experience and diving techniques adopted by expert BH-divers. Despite the absence of data related to serum AA changes in BH-diving, the major part of BH-Divers seems to perform their repetitive dives without an intensive muscle effort due to the correctness of the athletic gesture, the use of appropriate equipment and the adequate mental technique. Furthermore, it could be interesting to extend this test to other BH-diving specialties (static and dynamic apnea) in which the use of muscle is absent (static apnea) to use this model to understand better AA changes in BH-divers.

The short-term effect of serum AA profile changes found, represents the most important data in our results and may indicate a muscle activity more intense than that usually BH-diver perceived/referred.

Data related to the NO production and antioxidant synthesis could explain the well known BH-diving related vascular adaptation and the response to oxidative stress during diving deep phase, as observed by SCUBA and BH-diving underwater blood draw studies [103, 104]. Finally, there are interesting data related to the hypoxia [303, 304] stimulus that indirectly may confirm that the muscle apparatus works under strong exposure conditions notwithstanding the very short/low intensity of exercise, due to the intermittent hypoxia caused by repetitive diving. Obviously, the AA catabolism may be also explained in part by the increase of circulating AA besides cardiac and skeletal muscle work in the particular muscle activity conditions (increase of pressure, hypoxia in ascent phase, diving response) requiring several adaptation mechanisms including smooth muscle mediated massive vascular response.

Even if it is well known that water immersion affects fluid balance, causing a redistribution of blood volume and an increase in urine production which results in fluid loss (dehydration), our BH-divers did not show differences in blood volume, as calculated by the Dill and Costill formula, between pre and post diving,. This might be explained by the fact that all the BH-divers were expert instructors and/or high level athletes that commonly drink adequate amounts of water during the BH-diving training session.

Our data seem to indicate that during a BH-diving training session, skeletal and cardiac muscles react to physical effort releasing stress-related substances, similar to those observed during intense sports activities (endurance). Although our data agree with those observed in other sports activities, the peculiar nature of BH-diving makes it difficult to understand if our results are related only to exercise induced muscle adaptation or whether acute hypoxia or a response to environmental changes (pressure) play a role to explain the observed changes, especially considering the additional difficulty in defining the role of hypoxia that recent observations showed occurring only in the final part of BH-diving (ascent phase) [305]. In this regard, it is intriguing to note that these muscle adaptations, comparable with those occurring in endurance sports, are

difficult to justify in BH-diving where significant muscle activity occurs only for a limited time, as body movements are effective but not explosive in order to optimize oxygen consumption, and the majority of a BH-diving training session is spent for surface recovery while the underwater time is shorter. Possibly, this BH-diving muscle response could be also explained, besides cardiac and skeletal muscle work, by the particular muscle activity conditions (increase of pressure, hypoxia, diving response) requiring several adaptations mechanisms including smooth muscle mediated massive vascular response.

It could also be possible that the effects on smooth muscle activity, negligible in normobaric conditions, play a more significant role in BH-diving due to the blood shift related continuous blood flow regulation.

As concerning AA changes, our results about changes in serum amino acid profile after repetitive breath-hold dives are an absolute novelty in this specific field, as far as we know, and could represent an interesting new approach in the study of BH-diving physiological adaptation and become very interesting to structure BH-diving much more specific protocols, than it was done in the first preliminary study, allowing to better select the numerous stimuli that a diver undergoes.

Finally, increased parasympathetic tone was present during diving. RR duration, SDNN; rMSSD, HF spectral power all increased during the dive above pre-dive levels. Conversely, HF power decreased (and the LF/HF increased) 30 min after the dive. Using FrD, a difference was detected between OC and CCR, which may be related to differences in partial pressure of oxygen breathed during the dive.

2.5 Paper White Blood Cells, Platelets, Red Blood Cells and Gas Bubbles in SCUBA Diving: Is There a Relationship?

2.5.1 Introduction

Type of dive (recreational or professional), depth, diving time and associated physical exercise can influence the hemodynamic response in divers and all these variables can originate physical and psychological stress inducing several body adaptations [102, 306]. Among the many parameters that show changes during and after diving, hematological parameters and particularly White Blood Cells (WBC) and platelets (PLT) changes are particularly interesting [307].

Some authors studied male divers after a single recreational dive at 30 m for 30 minutes [307], and they found a rapid increase of neutrophils, the most abundant type of granulocytes (GRAN), after surfacing and of lymphocytes (LYM) after 6 hours. On the other hand, monocyte (MONO) count decreased immediately after surfacing to increase again 6 hours after diving [307]. The neutrophil mobilization may be inducted by the stress associated to the dive that results in an increased release of cortisol and catecholamines [308-310] and also influenced by acute exercise, resulting in a rapid accumulation of neutrophils in muscles, related to both intensity and duration of exercise [311]. Neutrophils may be activated during exercise by several factors including muscle damage, growth hormone and IL-6 [312, 313]. Activated neutrophils could phagocytate cellular debris and release growth factors that recruit macrophages, which are involved in removing residual

cell fragments and in reconstructing muscle fiber [314]. Also, Nitric Oxide (NO), frequently studied in SCUBA diving for its role in the arterial flow regulation, exerts an important action on various neutrophil cells activities. NO plays a protective role in neutrophil-endothelial interaction by preventing neutrophil adhesion and endothelial cell damage by activated neutrophils [315]. Furthermore cold exposure during diving might influence WBC mobilization: some authors investigated haematological changes during a long swimming competition in cold water (6 C°) [316]. LYM increase H_2O_2 production after scuba diving, in normobaric condition, probably as a consequence of some mitochondrial changes [63]. After a hyperbaric exposure (e.g., scuba diving) LYM activate their antioxidant defenses in order to protect the cells against the induction of oxidative damage of macromolecules, especially DNA [317, 318]. Increased levels of reactive oxygen species (ROS) can activate the antioxidant machinery of LYM, and GPx is one of the first antioxidant systems activated to detoxify ROS [63]. According to these data, the increase of LYM observed by Perovic et al may be a consequence of the combination of physical activity, physiological stress, hyperbaria, hyperoxia and exposure to cold [307]. Physical exercise can induce a mild monocytosis often accompanied by neutrophil increase in the inflammatory response [319], especially in cold water [316]: it has been observed that MONO decreased immediately after diving [307]. MONO reduction may be due to a trans-endothelial migration caused by the alterations that can occur in the vascular/endothelial function observed after dive [163, 320].

Red blood cells (RBC) are exposed to ROS and reactive nitrogen species (RNS) attack because of the high polyunsaturated free fatty acid content of their membranes and the high cellular concentrations of hemoglobin [321] but contain an elaborate endogenous antioxidant defense system that eliminates free radicals [322]. The oxidative membrane damage can be the reason of the RBC reduction observed in SCUBA divers by some researchers [5]: this may be due to increased pO_2 resulting from hyperbaric exposure during diving that could induce oxidative stress by raising ROS generation [188].

It's well-known that SCUBA diving can increase the number of vascular gas emboli (VGE), that may further exacerbate the endothelial homeostasis already impaired by ischaemia/reperfusion, physical contact or by an increased shear stress [163]. Several studies have focused on diving-related VGE formation [54, 176, 323] that plays a key role on the onset of decompression sickness (DCS). Notwithstanding the frequent presence of "silent" asymptomatic VGE in divers after diving, the link between circulating VGE and DCS is well accepted [324, 325].

PLT may have a key role in the onset of DCS [326]. Circulating bubbles can alter the clotting system both through activation of the coagulation cascade and the induction of PLT aggregation while circulating proteins adhere on bubble surface generating a thin layer that interacts with PLT [327]. PLT are one of the main sources of circulating microparticles (MP) [328]. During PLT activation, PLT-derived MP are generated in the bloodstream: some authors found a relationship between post-dive decrease of PLT-derived MP concentration reflecting PLT activation and bubble formation, probably due to their pro-coagulant activity that leads to the alteration of coagulation and thrombotic events in the pathogenesis of DCS [32].

The aim of this study was to investigate for possible relationship between hematological parameters and the predisposition to inert gas bubble formation after SCUBA diving.

2.5.2 Material and methods

2.5.2.1 Subjects and Diving Procedures

A total of 32 expert SCUBA divers (26 males and 6 females) were investigated during a single recreational dive in Y-40 "The Deep Joy" swimming pool (Montegrotto Terme, PD), 42 meters depth.

No subject reported previous episodes of DCS, historical or clinical evidence of arterial hypertension, cardiac, pulmonary or any other significant disease, none of them took prescription drugs, suffered any acute disease during the 15 days before the experiment, or reported assumption of anti-inflammatory drugs and exposure to high altitude in the 7 days before the experiment. All the divers received an explanation of the study's purposes, risks and benefits, were familiarized with the experimental protocol and read and signed a specific informed consent form before the experiment.

Divers were invitated to reach a bottom time of 7 minutes (descent and permanence at the bottom) so not to exceed the no-decompretion limit. After the dive, all the diving profile were downloaded into the Divers Alert Network (DAN) database using a common export fomat called Universal Dive Data Format (UDDF) and the Gradient Factor (GF) was calculated by a dedicated DAN Software using the Buhlmann ZHL16 C model and used to ascertain uniformity of hyperbaric exposure in addition to the analysis of compliance with the suggested diving profile.

The Maximum GF value is generally reached at the end of the dive. The GF measures the inert gas load in the diver's tissues, according to the selected decompression algorithm. This is a way to estimate inert gas supersaturation and to compare diving exposure in the different investigated subjects [54].

2.5.2.2 Material and Protocol

Venous blood samples were obtained from the antecubital vein of divers in heparin containing tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, United States). Blood samples were collected 30 min before the diving session and 30 min after surfacing (post diving).

All haematological analyses were performed within an hour after blood sampling on the same Abaxis Piccolo Xpress® chemistry analyzer (Union City, California, USA). Briefly, 100 µL of whole blood were transferred into the self-contained reagent disc. The disposable, single-use disc contains all the reagents and the diluent necessary to perform a complete multi-test chemistry panel. The following haematological parameters were measured: WBC, WBC differential blood count including, GRAN, LYM, and MONO, RBC, haemoglobin (HGB), mean corpuscular volume (MCV), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), PLT count, mean PLT volume (MPV) and plateletcrit (PCT).

2.5.2.3 Ecocardiography protocol

The ecocardiography protocol is descibed above.

2.5.3 Results

A total of 32 experienced SCUBA divers, 26 males and 6 females, mean age 45.1 ± 12,4; mean height 173.4 cm ± 7.9; mean weight 78.5 kg ± 14.2, and BMI 26.0 ± 3.8 were studied during a single dive in the Y-40 "The Deep Joy" swimming pool. The dive profile implied a mean depth of 41.5 ± 0.5 meters, a mean diving time of 50.4 ± 7.7 minutes, mean temperature of 32.7 ± 1.2 °C and a mean GF of 0.83 ± 0.03.

A point-by-point analysis of diving profiles confirmed that all divers respected the diving planning and also the calculated GF at the end of dives confirmed a similar hyperbaric exposure (0.83 +/- 0.03).

The echocardiography protocol allowed to allocate 9 B and 19 NB divers while 4 subjects showed intermediate conditions (grade two) and were excluded from the protocol. As reported in Figure 29, we found a statistically significant higher number of total WBC in NB as compared to the B divers in the pre diving sample (p=0.001). These data are confirmed by two different elements of white series, GRAN p=0.03, LYM p=0.0003 while no statistical differences were found in MONO p=0.06.

These data were also confirmed in the post diving blood sample analysis (Figure 30) for both GRAN and LYM (GRAN p=0.02, LYM p=0.001 respectively) (Table 2).

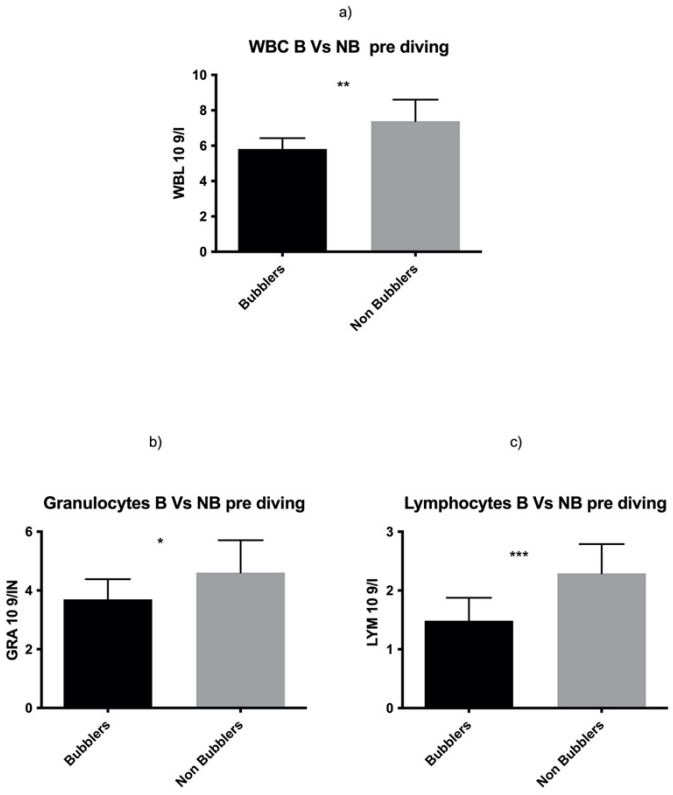

Figure 29. (a) WBC, (b) GRA (c) LYM in NB compared to B divers in the pre diving sample show a higher count in NB.

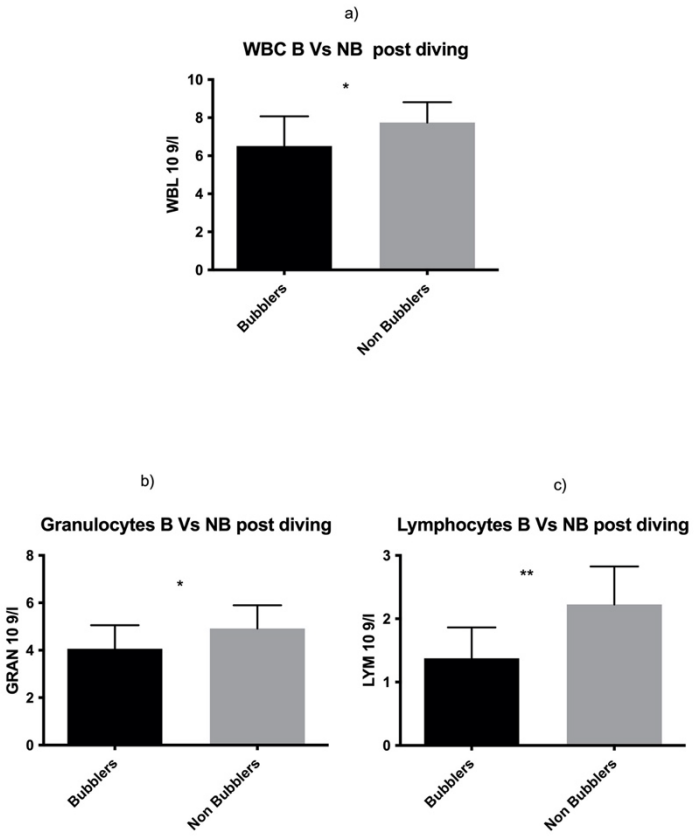

Figure 30. (a) WBC, (b) GRA (c) LYM in NB compared to B divers in the Post diving sample show a higher count in NB.

We found statistically significant increase in GRAN ($p<0.001$) in post diving value with respect to basal value while LYM and MONO didn't show any statistical difference ($p>0.05$) (Table 2).

Table 1. – WBC and WBC-related parameters.

White series	B	NB	Results
WBC pre (10^9)	5.81 ± 0.62	7.39 ± 1.21	P=0.001
WBC post (10^9)	6.51 ± 1.56	7.74 ± 1.07	P=0.021
GRANs pre (10^9)	3.70 ± 0.69	4.61 ± 1.01	P=0.031
GRAN post (10^9)	4.07 ± 0.99	4.92 ± 0.98	P=0.041
LYM pre (10^9)	1.49 ± 0.39	2.29 ± 0.50	P=0.0003
LYM post (10^9)	1.38 ± 0.49	2.23 ± 0.60	P=0.001
MONO pre (10^9)	0.39 ± 0.10	0.49 ± 0.12	N.S.[1]
MONO post (10^9)	0.42 ± 0.13	0.42 ± 0.11	N.S.
	Pre	**Post**	
WBC (10^9)	6.82 ± 1.23	7.32 ± 1.34	P=0.0050
GRAN (10^9)	4.19 ± 1.06	4.60 ± 1.00	P=0.001

LYM (10^9)	2.09 ± 0.62	2.00 ± 0.71	N.S.
MONO (10^9)	0.45 ± 0.13	0.43 ± 0.11	N.S.

[1] not statistically significant

We did not find any post diving vs pre diving statistical difference between NB and B for RBC, HCT, MCH and MCHC, while HGB and MCV showed a statistically significant increase after diving (HGB p=0.026, MCV p=0.019). (Table 3).

Table 2. RBC and RBC related parameters.

RBC	B	NB	Results
RBC pre (10^{12})	5.13 ± 0.79	3.92 ± 0.95	N.S.[1]
RBC post (10^{12})	5.26 ± 0.56	5.20 ± 0.65	N.S.
HGB pre (g/L)	15.94 ± 1.60	15.02 ± 2.45	N.S.
HGB post (g/L)	15.74 ± 1.02	14.66 ± 1.05	N.S.
MCV pre (fL)	86.36 ± 3.85	85.49 ± 13.36	N.S.
MCV post (fL)	86.97 ± 3.90	83.78 ± 9.72	N.S.
HCT pre (%)	45.09 ± 2.82	42.88 ± 5.81	N.S.
HCT post (%)	45.48 ± 3.13	43.03 ± 3.75	N.S.
MCH pre (pg)	30.56 ± 1.90	28.83 ± 4.61	N.S.
MCH post (pg)	30.12 ± 2.34	28.47 ± 4.20	N.S.
MCHC pre (g/L)	35.14 ± 2.02	33.99 ± 2.20	N.S.
MCHC post (g/L)	34.64 ± 1.50	33.38 ± 3.22	N.S.
	Pre	**Post**	
RBC(10^{12})	5.12 ± 0.88	5.22 ± 0.61	N.S.
HGB (g/L)	15.32 ± 2.23	15.01 ±1.84	P=0.026
MCV (fL)	85.77 ± 11.11	84.77 ± 8.35	P=0.019
HCT (%)	43.59 ± 5.10	43.81 ±3.69	N.S.
MCH (pg)	29.38 ± 3.99	29.00 ± 3.74	N.S.
MCHC pre (g/L)	34.36 ± 2.17	33.79 ± 2.82	N.S.

[1] not statistically significant

Finally, PLT and MPV showed a statistically significant increase post diving as compared to pre diving (PLT p=0.01; MPV p=0.0003). Regarding PLT related parameters, we did not find any statistically significant difference in unit per microliter of blood, in mean MPV and PCT, in NB Vs B both in pre diving and post diving values (Table 4).

Table 3. Platelets and platelets related parameters.

Platelet	B	NB	Results
PLT pre (10^9)	183.20 ± 56.3	196.80 ± 70.9	N.S.[1]
PLT post (10^9)	198.8 ± 55.4	218.2 ± 67.6	N.S.
MPV pre (fL)	9.56 ± 0.92	9.29 ± 1.03	N.S.
MPV pre (fL)	9.20 ± 1.26	8.67 ± 1.17	N.S.
PCT pre (%)	0.17 ± 0.06	1.01 ± 3.63	N.S.
PCT post (%)	0.18 ± 0.05	0.19 ± 0.05	N.S.
	Pre	**Post**	
PLT (10^9)	192.5 ± 65.89	212.0 ± 63.56	P=0.01
MPV (fL)	12.56 ± 16.97	8.85 ± 1.20	P = 0.0003
PCT (%)	0.74 ± 2.99	0.18 ± 0.05	N.S.

[1] not statistically significant

2.5.4 Discussion

This study aimed to investigate a possible relation between the evidence of inert gas bubbles and hematological parameters after a SCUBA dive. As a secondary scope, we investigated changes, in the same hematological parameters, between pre and post dive samples. SCUBA diving exposes the human body to a combination of several factors including hyperbaria, hyperoxia, breathing resistance, physical effort and inert gas bubble generation [73]. Bubbles can occur in blood circulation, activating vascular endothelium and inducing inflammation with the activation of leukocytes associated to the production of cytokines [326, 329]. After a dive at -42 m for 40 minutes, we observed an increase in WBC, (GRAN and LYM) according to the data obtained by other authors [63, 115, 307] even if the protocol was different as time points. Neutrophil mobilization is exacerbated by the production of stress biomarkers such as catecholamines and cortisol [308, 330]. All the investigated divers performed similar diving profile and dived into an approximate GF interval, ranging from 0.80 to 90, usually associated to the peak of bubble formation [54], guaranteeing a suitable diving exposure for our purpose.

The most interesting result obtained during our protocol emerged when divers were divided according to their circulating bubble production, into B and NB. In the NB group, we found a higher amount of WBC (GRAN

and LYM) as compared to the B group, already in pre-diving blood sample (therefore not diving related), confirmed in the post dive blood analyses. This aspect could be very important to explain one of the possible causes of inter-subject differences in bubble formation in divers performing the same dive profile [54]. Despite the absence of an investigation related to anti-inflammatory mediator changes, these data may be explained considering that SCUBA-related acute inflammation is modulated by circulating WBC and leads to the stimulation of toll like receptors (TLRs) and, consequently, the activation of NF-κB pathway [331]. Even if the role of anti-inflammatory mediators were not directly investigated in this protocol, it is known that some authors evaluated the inflammatory status measuring IL-6, IL-8, MIP-1β and other pro-inflammatory molecules [332], including chemokines in recreational divers (Spisni, Marabotti et al. 2017). Some circulating chemokines, namely CCL2 and CCL5, increased after diving: CC chemokines are stored in and released from PLT and activated LYM [333]. Circulating chemokine CCL5 is known to contribute to endothelial activation and the interaction between endothelial cells and MONO [334]. CCL5 secretion facilitates endothelial progenitor cell recruitment and increases NO production in endothelial cells [335], protecting vascular endothelium from endothelial dysfunction. Also, it is well known that SCUBA diving induces cytokine response [336] and some authors observed the increase of circulating IL-6 IL-8, IL-10, and IL-1b cytokines with anti-inflammatory role [188]. This cytokine cascade seems to be involved in mediating the health beneficial effects of exercise and to play an important role in the modulation of low-grade inflammation induced by exercise oxidative stress [337]. All these mediators are produced by the immune system LYM and GRAN and may explain our results suggesting a protective role of WBC toward the production of bubbles through a WBC mediated anti-inflammatory response in absence of DCS symptom onset. According to our findings and secondary scope, Spisni et al found an increase in WBC in divers without any episode of DCS [39]. Bubbles in the vascular system can obstruct blood flow activating inflammatory pathways [44]: the WBC increase against inflammation, caused by the bubbles, raises the WBC availability and this could be an additional protective factor.

Increased pO_2 due to hyperbaric conditions can raise the activity of LYM antioxidant enzymes that may be in part responsible for the reduced levels of H_2O_2 during SCUBA diving [306]. Also, we can't exclude an active role of neutrophils to remove inert gas (nitrogen) bubbles from the blood by phagocytosis or simple inclusion by diffusion.

We did not find significant changes in RBC and their related factors after diving Vs pre diving and also in their number per cubic ml in NB Vs B. Despite RBC vulnerability to ROS/RNS attack during hyperbaric hyperoxia, endogenous antioxidant defenses were activated to protect RBC from free radical damage. The unchanged RBC value may reflect an unchanged marker of cellular damage in erythrocytes indicating that the cellular antioxidant response is enough to avoid the ROS-induced damage [338].

The HGB and MCV reduction may be explained by the haemolysis that might occur during hyperbaric exposure [339, 340].

Circulating bubbles can have inflammatory effects [341] and PLT activation plays a key role in the response to inflammation [342], because their receptors participate in neutrophil extracellular bacteria trapping in septic blood that induces PLT to adhere to neutrophils [343] and also knowing that bubbles cause PLT adhesion in animals [344, 345] and humans reducing free circulating PLT [346]. On the other hand, PLT changes during SCUBA diving are controversial: some authors found a decrease of PLT count related to bubble formation [347, 348] that may be due to individual physiology, dive profile and diver training/experience. PLT activation by bubbles is a slow process that involves several changes of plasma proteins at the bubble interface. After dive, lungs eject many inert gas bubbles minimizing PLT activation related inflammation and thrombosis. Before this process is complete, many bubbles can disappear and this delay probably may contribute to the latency period of DCS [349]. Some authors observed that breathing hyperbaric or normobaric O_2 before a dive reduced PLT activation as consequence of O_2 increase in fat tissue, reducing decompression-induced air bubble formation and alleviating decompression induced PLT activation [350]. On the other hand, increased pO_2, breathing nitrox mixtures, can reduce the level of decompression induced PLT activation [351]. The small numbers of subject investigated doesn't permit to give firm conclusion about the relationship between PLT count and bubble formation.

We did not find relationship between PLT count, PLT-related parameters and bubble formation. On the other hand, we found a statistically significant post dive increase in PLT count and MPV.

However, no differences were found in PLT levels and their associated parameters between B and NB.

The main limitation of this study is that only one blood sample, carried out 30 minutes after the surfacing, is not enough to evaluate the WBC kinetics that could be a relevant information in future studies in this field. Another limitation is the absence of investigation about cytokine and inflammatory marker trends.

Finally, the imbalance between the number of male and female divers and the reduced sample size in this study represent a limitation according to our point of view.

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