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Original Research

Inflammatory Biomarkers Comparison Between Breath-Hold and SCUBA Divers

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Abstract

Background: The pathogenesis of decompression sickness is multifactorial, involving bubbles and oxidative stress that lead to inflammatory responses. *Ex vivo* evidence indicates that oxidative stress occurs rapidly while at depth, prompting our hypothesis that biomarkers of inflammation would be similar between 12 subjects performing breath-hold (BH) dives lasting 1 to 2 minutes and 10 subjects performing Self-contained underwater breathing apparatus (SCUBA) (S) dives lasting 16 to 40 minutes. **Methods:** Consenting experienced diver research subjects traversed depths of 15 and 40 meters of seawater (msw). Laboratory measures were conducted on blood obtained prior to and following each dive. Vascular gas emboli were assessed by cardiac ultrasound. **Results:** Post-dive, there were significant elevations ($p < 0.05$, two-way within-subject ANOVA) in all divers for circulating levels of microparticles measured by flow cytometry, and post-diving microparticles (MPs) from S and BH divers exhibited equal propensity for activating human neutrophils. There also were significant elevations of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-10 and vascular cell adhesion molecule measured by enzyme-linked immunosorbent assays, and reactive oxygen species from electron paramagnetic resonance measurements. Mean fold-elevations for 15 msw BH dives were 1.51 ± 0.34 (standard deviation (SD)) and for S dives, 1.81 ± 0.67 (no significant difference, NS). For 40 msw dives, fold-elevation values for BH were 2.21 ± 0.81 and for S, 2.21 ± 0.86 (NS). For reactive oxygen species (ROS), TNF-α and IL-1β, some values were greater for S versus BH dives. Cardiac bubbles were rare for all divers with no significant difference between groups. **Conclusions:** We conclude that even among BH divers, oxidative stress and inflammatory responses to pressure occur rapidly. Some biomarkers are greater among S divers, which may reflect longer dive durations and higher systemic oxygenation.

Keywords: cytokines; extracellular vesicles; decompression sickness; oxidative stress

1. Introduction

The traditional view of decompression sickness (DCS) is that injuries occur due to bubbles formed in the body following gas supersaturation [1,2]. However, vascular gas bubbles are inconsistently present among DCS victims [1,2,3]. Gas bubbles are detected by ultrasound as venous gas emboli (VGE) [4]. The absence of VGE is indicative of decompression safety, but in those with DCS, high VGE numbers are found in only ~10% of divers and 5% of those exposed to altitude [3,5]. The inconsistent presence of bubbles in injured divers has led to efforts focused instead on assessing the relationship of inflammation to DCS onset [6,7,8,9,10,11].

High pressures of nitrogen and similar gases trigger singlet oxygen, leading to oxidative stress linked to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase activation at the cell membrane within minutes of exposure [12]. These events ultimately trigger assembly of the NOD-like receptor, pyrin-containing 3 (NLRP3) inflammasome. An associated component to this response is extracellular vesicle production, with microparticles (MPs, 0.1 to 1.0 µm) exhibiting a notable inflammatory potential [12,13,14]. Because these responses occur in a span of 30 minutes and begin while under pressure, we hypothesized that MPs and biomarkers of oxidative stress and inflammation would be closely matched between breath-hold (BH) and Self-contained underwater breathing apparatus (SCUBA) (S) divers despite differences in their durations of exposure [12,14]. This is an important pathophysiological issue because prevalence rates for neurological DCS causing cognitive and functional deficits are actually higher in commercial BH divers (~56%) versus 30% in compressed air divers [15,16,17].

There is growing evidence for oxidative stress and inflammatory responses to BH and S diving [18,19,20]. Among these responses, human (S) divers respond to high-pressure exposure/decompression with elevations of MPs [10,11,21]. BH divers also generate MPs [22], but no prior

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investigation has undertaken a direct comparison between BH and S divers exposed to the same pressures. MPs are linked to tissue injuries and functional deficits in a murine model and injuries identified in decompressed mice can be recapitulated by injecting decompression-induced MPs into naïve mice [23,24]. Tissue injuries are related to neutrophil activation and MPs content of interleukin (IL)-1β [12,13,23,24]. There is an association between MPs and IL-1β because the cytokine is packaged within a vesicle for secretion, as it cannot utilize the conventional secretory pathway [25,26]. There are also soluble inflammatory mediators that increase in divers, including tumor necrosis factor-alpha (TNF-α), IL-6, C-C Motif Chemokine Ligand (CCL)2 and CCL5 [10,27,28,29,30]. With these issues in mind, the goal of this study was to evaluate whether biomarkers of oxidative stress and inflammation were similar between BH and S divers. We hypothesized that physiological and biochemical stresses would supersede differences in dive duration, resulting in similar findings.

2. Methods and Materials

2.1 Study Subjects

All procedures were completed in accordance with the Declaration of Helsinki and approved by ethical committees from the University of Padova (No. HEC-DSB/03-18; addendum: November 2022) and the University of Maryland (HP-00109500). Subjects received a written explanation and an oral briefing of the methods and potential risks and gave their written informed consent. Subjects were healthy, trained SCUBA and breath-hold divers. Sample sizes were determined by logistical constraints and field feasibility rather than a priori statistical power calculations. No randomization or blinding was applied due to the experimental field setting. SCUBA divers and the breath-hold divers were different individuals, as each group specialized in a distinct diving discipline. On individual days, the number of breath-hold divers was not the same, as some unavailable and at times some unable to perform the dive. Therefore, the actual number of data points differed across the study time span.

Experiments were conducted in three stages between June 2024 and June 2025 at two open-seawater sites in Italy: The Gulf of Naples and the Tremiti Islands. Sites were selected based on diving safety, stable water temperature, availability of onshore infrastructure, access to a vessel suitable for laboratory setup, and the presence of a support boat for traffic control and emergency transport. Ambient air temperature ranged from 30 °C to 34 °C, with sea conditions from calm to slightly rough. Water temperature at target depths ranged from 17 °C to 25 °C.

Blood was collected by forearm venipuncture 30 min before diving and within 15 min after surfacing. One sample (5 mL) was collected in tubes using EDTA and lithium heparin as anticoagulant (Vacuette tube, Greiner Bio-One, Kremsmünster, Austria) and promptly centrifuged at 3000 ×g for 10 min and then frozen for later measurements of plasma superoxide and hydroxyl radical (reactive oxygen species (ROS)), IL-6, IL-10, TNF-α and vascular cell adhesion protein 1 (VCAM-1). Blood for MPs analysis (5 mL) was drawn into Cyto-Chex BCT test tubes that contain a proprietary preservative (Streck, Inc., Medimark Europe, Grenoble, France).

Blood in Cyto-Chex tubes for MP analysis was stored at 4 °C until it was shipped to the University of Maryland for analysis, where analysis occurred within 4 days. Samples in EDTA tubes were aliquoted and stored in a portable liquid nitrogen dewar during transport to the Institute of Clinical Physiology laboratory (IFC-CNR Milan, Italy), then stored at –80 °C until assayed, and thawed only once before analysis. Subjects also provided urine samples that were collected at ~10 minutes prior to diving and within 10 minutes after each dive. Urine samples were collected via voluntary voiding in a sterile container provided to the subjects and frozen in liquid nitrogen prior to analysis.

2.2 VGE Assessment

VGE were assessed by two-dimensional echocardiography using a Versana Active Ultrasound Device (GE Healthcare, USA), equipped with a sector probe (1–4 MHz) using an apical 4-chambers view. Within 10 min after surfacing, divers were placed in the left-lateral decubitus position on board the vessel and scanning commenced. Bubble scores were graded according to the Eftedal–Brubakk scale [31].

2.3 SCUBA Diving Protocol

SCUBA divers used an open-circuit system and wore a buoyancy compensator device, fins, a face mask and a 5-millimeter neoprene wetsuit modified to allow arterial cannulation. Each diver carried a single 15-liter steel cylinder filled with compressed air. Divers reached target depths (15 or 40 m seawater (msw)) in pairs within ~2 min, after which fins were removed and all divers performed exercise on a submerged cycle ergometer (OKEO Srl, Genoa, Italy). Workload was set at 100 W with an additional estimated 50 W to overcome water resistance (total ~150 W), at 60 rpm for 10 min. Total bottom time was ~12 min.

Ascent rate was maintained at $\leq$3 m min^{-1} with a 3-minute safety stop at 5 msw. No decompression stops were required for the 15 msw dive. For the 40 msw dive, decompression followed dive computer guidance using a Bühlmann ZH-L16 algorithm (gradient factors 35/75), resulting in total dive times of up to 40 minutes. These conservative ascent parameters were chosen simply out of an abundance of caution.

2.4 Breath-Hold Diving Protocol

Breath-hold divers wore a 5-millimeter two-piece neoprene wetsuit modified for arterial cannulation and a standard face mask. Participants avoided exertion before dives

and followed a standardized warm-up consisting of surface apnea exercises and four preparatory dives (5–10 min intervals). Prior to the experimental dive, divers performed 5 min of controlled ventilation (8–9 breaths min^{-1}; inspiratory-to-expiratory ratio 1:2); lung packing was not permitted.

Experimental dives to 15, 25 or 40 msw were performed using a sled-assisted technique (head-down descent, head-up ascent). Safety measures included a professional breath-hold instructor, safety SCUBA divers at depth and at 10 m, onboard medical personnel with oxygen and emergency equipment, and a dedicated evacuation vessel. The duration of the 15, 25 and 40 msw index dives from leaving the water surface to return spanned, respectively, 1 minute 18 seconds ± 7.6 seconds and 1 minute 50 seconds ± 6.3 seconds and 2 minutes 16 seconds ± 3.4 seconds.

2.5 Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The antibodies and flow cytometry reagents used are as follows: annexin V-Fluorescein isothiocyanate (V-FITC) (BD, cat. no. 556419), Anti-β-actin (Sigma-Aldrich, St. Louis, Mo, cat. no. A2066), anti-CD66b (eBioscience, San Diego, CA, cat. no. 48-5931-82), anti-CD31 BV510 (BD cat. no. 563089), anti-CD41 PerCP Cy5.5 (BioLegend, San Diego, CA, cat. no. 133918), Anti-glial fibrillary acidic protein (GFAP) Brilliant Violet 421 (BioLegend, San Diego, CA cat. no. 644719), anti-transmembrane protein (TMEM) 119-APC (Abcam, cat. no. ab225494), anti-thrombospondin (TSP)-1 (Santa Cruz, cat. no. 393504 and 393504FITC), anti-CD18 (BioLegend, cal#366316), anti-myeloperoxidase (BioLegend, cat. no. 340655). Antibodies for flow cytometry were used specifically for that application as documented by the manufacturers and at the recommended concentrations. Positive staining in flow cytometry was determined following the fluorescence-minus-one control test.

2.6 Blood MP Acquisition and Processing

Blood-borne MPs were isolated and prepared for analysis by flow cytometry as previously described. Briefly, blood from Cyto-chex tubes was centrifuged for 5 min at 1500 g. EDTA was then added to the supernatant to achieve a concentration of 12.5 mM to prevent MPs aggregation. This suspension was centrifuged at 15,000 g for 30 minutes, and the supernatant was used for analysis. Separation of F-actin + MPs was achieved by incubating MPs suspensions with phalloidin-biotin conjugates followed by streptavidin conjugated to 1 µm diameter MagVigen® magnetic nanoparticles (Nvigen, Inc., Sunnyvale, CA, USA). This suspension was incubated for 12 hours before separation using a magnet. The washing and magnetic bead separation procedures followed the manufacturer's recommended protocols. Suspensions of MPs remaining after magnetic extraction were centrifuged at 100,000 g for 1 hour to pellet particles for analysis. A human-specific ELISA Kit (eBioscience, San Diego, CA, USA) was used to evaluate the concentration of IL-1β in MPs samples following the manufacturer's instructions.

2.7 MP Analysis

All reagents and solutions used for MP isolation and analysis were filtered with a 0.1 µm filter (EMD Millipore, Billerica, MA, USA). MPs were analyzed as described previously [11]. In brief, flow cytometry was performed with an 8-color, triple laser MACSQuant® Analyzer (Miltenyi Biotec Corp., Auburn, CA, USA) using MACSQuantify™ software version 2.13.3 for data analysis. MACSQuant was calibrated every other day using calibration beads (Miltenyi Biotec Corp., Auburn, CA, USA). Forward and side scatter were set at logarithmic gain. Photomultiplier tube voltage and triggers were optimized to detect sub-micron particles. Micro-beads of 3 different diameters, 0.3 µm (Sigma, Inc., St. Louis, MO, USA), 1.0 µm and 3.0 µm (Spherotech, Inc., Lake Forest, IL, USA), were used for the initial settings and before each experiment as an internal control. Samples were suspended in Annexin binding buffer solution (1:10 v/v in distilled water, BD Pharmingen, San Jose, CA, USA), with the previously listed antibodies. Examples of blood-borne and cervical particle analysis have been published previously [32]. All reagents and solutions used for MP analysis were sterile and filtered through a 0.1 µm filter. MPs were defined as annexin V-positive particles with diameters ranging from 0.3 to 1 µm. The concentration of MPs in the sample tubes was determined by MACSQuant® Analyzer according to the exact volume of solution from which MPs were analyzed.

2.8 MP Activation of Human Neutrophils

Activation of human neutrophils by divers' MPs was assessed following published methods [11,33]. In brief, 150,000 isolated neutrophils from a healthy donor suspended in 300 µL phosphate-buffered saline containing 1 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 5.5 mM glucose were incubated with 10,000 divers' MPs suspended for 30 minutes. Activation was assessed as production of new MPs and as increased expression of CD18 and myeloperoxidase on the neutrophil membrane. Parallel samples were co-incubated with 120 µmol phosphatidylserine as an antagonist to MPs-mediated cell activation [33].

2.9 ROS Production

Plasma samples were analyzed in an electron paramagnetic resonance (EPR) spectroscopy X-band instrument (E-Scan, 9.3 GHz Bruker Co., Billerica, MA, USA) following previously described methods [34]. The spin probe CMH (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethyl-pyrrolidine) was utilized to enable the acquisition of EPR spectra and the detection of ROS resonances. The CMH probe directly detects free radical adducts gen-

erated in real time, allowing for a comprehensive assessment of multiple oxygen radical species (e.g., superoxide, hydroxyl radical). Hence, the EPR signal intensity reflects the overall oxidative activity within the sample. The stable radical CP (3-carboxy2,2,5,5- tetramethyl1-1-pyrrolidinyloxy) was then adopted as an external reference to convert the ROS calculated data into absolute quantitative levels ($\mu mol \cdot min^{-1}$).

2.10 Quantification of Inflammatory Markers

IL-6, IL-10 and TNF-α plasma levels were determined by ultrasensitive ELISA immunoassays (Fine Test, Wuhan, China, Cat No.: EH0201; EH0173; EH0302), according to the manufacturer's instructions [34,35]. The assays are based on a double-antibody sandwich technique. The samples' concentrations were determined at 450 nm, and plasma levels of inflammatory markers in pg/mL were calculated. The coefficient of variation (CV) was indicated by the manufacturer for IL-6, IL-10 and TNF-α: inter-assay CV 4.62%, 5.26% and 5.85%, and intra-assay CV 5.35%, 5.05% and 5.99%, respectively.

2.11 VCAM-1 (Vascular Cell Adhesion Protein 1)

VCAM-1 plasma concentrations were determined by ultrasensitive ELISA immunoassays (Fine Test, Wuhan, China, Cat No.: EH0326) according to the manufacturer's instructions. The assays are based on a double-antibody sandwich technique with sample concentrations determined at 450 nm. The CV was indicated by the manufacturer for VCAM-1: inter-assay CV 5.92% and intra-assay CV 4.68%.

2.12 8-isoprostane (8-iso-PGF2α)

The concentration of 8-isoprostane, a marker of lipid peroxidation, was assessed in urine samples using an immunoassay (Cayman Chemical, Ann Arbor, MI, USA, Item No. 516351). Briefly, 50 µL urine samples were added to a 96-well plate pre-coated with mouse monoclonal antibody, followed by 50 µL of 8-iso PGF2α-tracer antiserum and incubated. After washing, 200 µL of Ellman's reagent was added and the results normalized using urine creatinine values. The samples were read at 412 nm and concentration determined using 8-iso-PGF2α. The CV indicated by the manufacturer was as follows: inter-assay CV 11.5%; intra-assay CV 8.9%.

2.13 Creatinine and Neopterin

Urinary neopterin and creatinine concentrations were measured in duplicate using isocratic high-pressure liquid chromatography (HPLC) following published methods [36]. The calibration curve was linear (1.25–10 mmol/L). The inter-assay and intra-assay variation coefficients were below 5%. Every urinary marker was standardized based on the amount of excreted creatinine, as the collection of the 24 h urine was not possible. In healthy human subjects in the absence of renal disease, the excretion rate of creatinine remains relatively constant [37].

2.14 Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SigmaStat version 12.5 (Jandel Scientific, San Jose, CA, USA). Data normality was assessed using the Shapiro–Wilk test, and for those few data sets with non-normal distribution, appropriate transforms were found. Data were analyzed by two-way within-subject ANOVA (factors: time: pressure, protocol). Biomarker changes with pressure were calculated as Δ% = ((post depth – pre depth) / pre depth) × 100. For all studies, we deemed a result to be statistically significant if $p < 0.05$. Post hoc multiple comparisons were assessed following the Holm–Sidak method.

3. Results

3.1 Breath-Hold (BH) and SCUBA (S) Divers Exhibit Elevations of Circulating Reactive Oxygen Species (ROS) and Inflammatory Proteins

BH and S divers performed 15 and 40 msw dives. BH divers also completed dives to 25 msw, so these values are included although there are no comparisons to S divers. Dive durations are summarized in Methods. The analyzed biomarkers (mean ± SD) in the two groups (BH and S) are shown in Fig. 1. In BH divers the ROS production rate exhibits an immediate increase, detectable as early as 15 msw (+16%); then ROS production rate, lipid peroxidation, systemic inflammation (IL-6 and IL-10; TNF-α; VCAM1, respectively) and immune activation (neopterin) significantly increased at 25 msw (+26, +107, +67, +154, +40 and +66%, respectively) and 40 msw (+44, +222, +78, +153, +73 and +150%, respectively) with respect to T0 (Fig. 1A–G). In S divers, the ROS production levels, lipid peroxidation, IL-10 and TNF-α significantly increased at 15 msw (+43, +78, +121, +152%, respectively) and at 40 msw (+48, +172, +173, +208%, respectively) with respect to T0 (Fig. 1a,b,d,e). While IL-6, VCAM-1 and neopterin significantly increased only at 40 msw (+77%, +82, and + 34%, respectively, Fig. 1c,f,g).

To ease comparisons, Table 1 displays mean (±SD) values between BH and S divers adjacent to each other. In this format, it is somewhat easier to summarize the findings by considering fold-elevations. ROS assessed by EPR increased 1.16-fold after 15 msw BH ($p < 0.001$) and by 1.40-fold ($p < 0.001$) after S dives, with a significant difference ($p = 0.008$) between 15 msw BH and S values. After the 40 msw dives, the fold elevation for BH was 1.40 ($p < 0.001$) and for S 1.49 ($p < 0.001$), with no significant difference between BH and S values. Values for TNF-α increased after 15 msw BH by 1.73-fold ($p < 0.001$) and by 2.93-fold ($p < 0.001$) for S dives. BH and S values were significantly different ($p < 0.001$). After 40 msw dives, the BH value increased by 3.25-fold ($p < 0.001$) and 3.76-fold for S (p

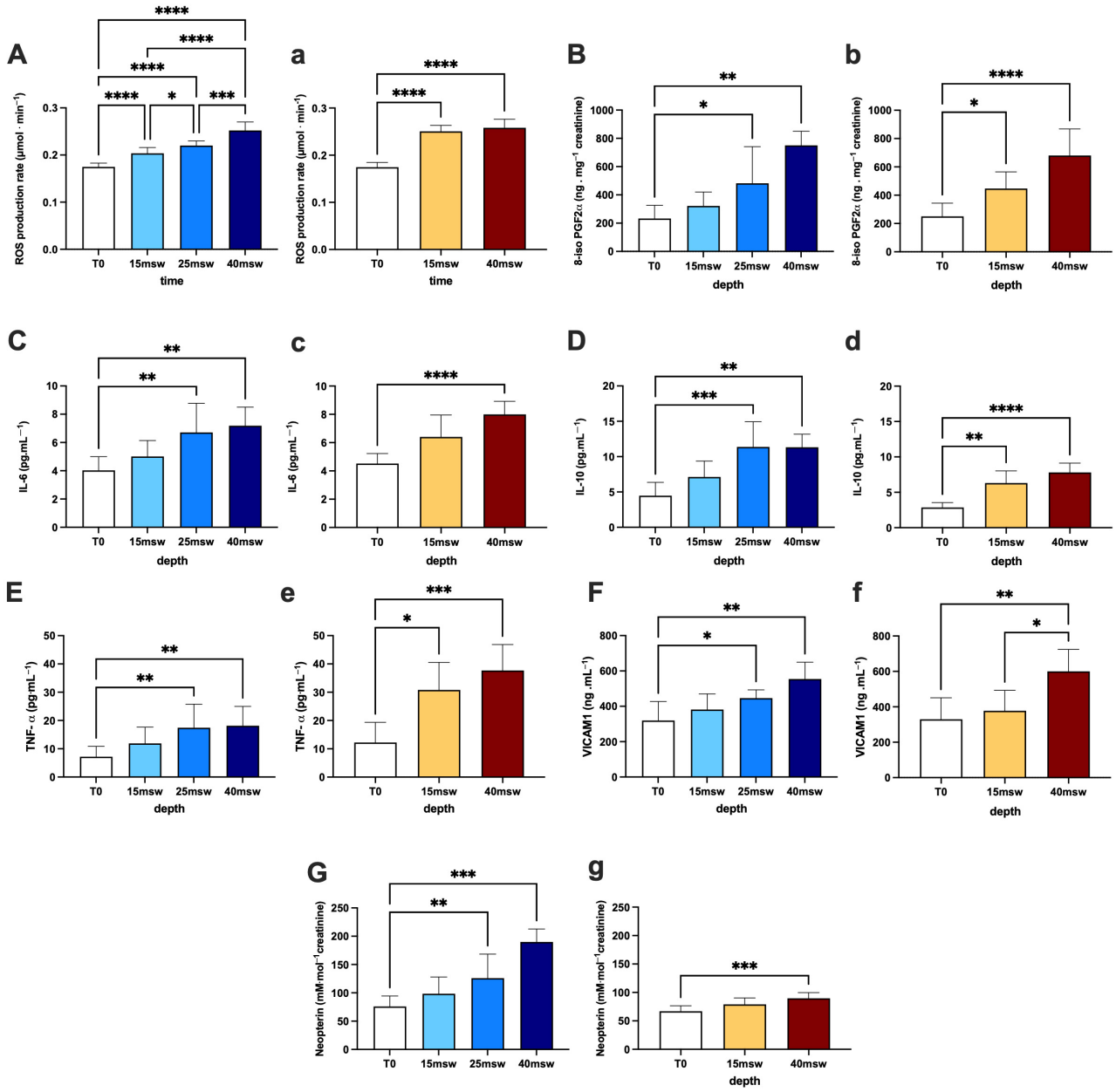

Fig. 1. Data obtained by BH (blue tone) and SCUBA (orange/red). Results are expressed as mean ± SD. Histograms of: (A,a) ROS production rate (µmol·min^{-1}) detected by EPR technique, (B,b) lipid peroxidation (8-iso-PGF2-α pg·mg^{-1} creatinine); pro- and anti-inflammatory cytokines; (C,c) IL-6 (pg·mL^{-1}), (D,d) IL-10 (pg·mL^{-1}), (E,e) tumor necrosis factor-alpha TNF-α (pg·mL^{-1}); (F,f) vascular cell adhesion molecule-1 VCAM-1/CD106 (ng·mL^{-1}) and (G,g) neopterin concentration (µM·mol^{-1} creatinine). Significant differences symbols: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.00001$. BH, breath-hold; SCUBA, self contained underwater breathing apparatus; SD, standard deviation; ROS, reactive oxygen species; EPR, electron paramagnetic resonance; IL, interleukin; VCAM-1, vascular cell adhesion protein 1.

< 0.001), and BH and S values were significantly different ($p < 0.001$). For IL-6, IL-10 and VCAM, elevations were not significantly different between BH and S dives, but for each group the values were significantly increased over pre-dive control ($p < 0.001$). Thus, for IL-6 after 15 msw, the elevation was 1.23-fold for BH and 1.43-fold for S, and after 40 msw, 2.00-fold for BH and 1.79-fold for S. For VCAM after 15 msw, the elevation was 1.31-fold for BH and 1.36-fold for S, and after 40 msw, 2.40-fold for BH and 1.96-fold for S. Values for the anti-inflammatory cytokine IL-10 were generally higher among BH versus S divers, although values were not always statistically significant. Values between ambient pressure (control) samples differed significantly, suggesting an inherent difference between the two groups. After the 15 msw dive, the fold elevation was 2.04 for BH and 2.36 for S, and after the 40 msw

Table 1. Plasma and urinary biomarkers.

		Ambient	15 msw	25 msw	40 msw
ROS	BH	0.18 ± 0.01 (12)	0.20 ± 0.01 (9)*	0.22 ± 0.01 (8)*#	0.25 ± 0.02 (4)*#!
	SCUBA	0.18 ± 0.01 (10)	0.25 ± 0.01 (7)* †		0.26 ± 0.02 (8)**
TNF-α	BH	7.2 ± 3.7 (12)	11.9 ± 5.7 (10)*	17.4 ± 8.4 (8)*	18.2 ± 6.8 (4)**
	SCUBA	12.2 ± 7.1 (10)	30.8 ± 9.8 (8)* †		37.7 ± 9.2 (8)* †#
IL-6	BH	4.0 ± 0.99 (12)	5.0 ± 1.1 (9)*	6.7 ± 2.1 (8)*#	7.2 ± 1.3 (4)*#
	SCUBA	4.5 ± 0.7 (10)	6.4 ± 1.6 (8)*		8.0 ± 0.9 (8) *
IL-10	BH	4.5 ± 1.9 (12)	7.1 ± 2.3 (9)*	11.4 ± 3.6 (8)*#	11.3 ± 1.9 (4) *#
	SCUBA	2.9 ± 0.7 (10) †	6.3 ± 1.7 (8)*		7.8 ± 1.3 (8) *†#
VCAM	BH	320 ± 107 (12)	382 ± 89 (9)*	446 ± 47 (8)*	554 ± 95 (4)*#
	SCUBA	330 ± 121 (10)	378 ± 115 (8)*		601 ± 124 (8)*
8-isoprostane	BH	233 ± 93 (12)	322 ± 98 (9)	482 ± 259 (8)*	750 ± 100 (4)*#!
	SCUBA	250 ± 93 (10)	447 ± 117 (8)*		680 ± 188 (8)*
neopterin	BH	76.0 ± 18.5 (12)	98.3 ± 29.8 (12)*	125.9 ± 52.6 (8)*#	189.9 ± 22.9 (4)*#!
	SCUBA	67.1 ± 9.4 (10)	79.1 ± 11.2 (8)**		89.7 ± 10.0 (8)* †
creatinine	BH	0.40 ± 0.15 (12)	1.05 ± 0.49 (12)*	1.54 ± 0.74 (8)*#	1.98 ± 0.57 (4)*#
	SCUBA	0.30 ± 0.20 (8)	0.75 ± 0.23 (8)*		1.34 ± 0.38 (8)*

Biomarkers were analyzed as described in Methods.

Units of measure were ROS (μmol·min^{-1}), TNF-α (pg·mL^{-1}), IL-6 (pg·mL^{-1}), IL-10 (pg·mL^{-1}), VCAM-1 (ng·mL^{-1}), 8-isoprostane (pg·mg^{-1} creatinine), Neopterin (μmol·mol^{-1} creatinine), and creatinine (g·L^{-1}). Data are mean ± SD; subject numbers are indicated within (brackets); *$p < 0.001$ vs ambient pressure, **$p < 0.01$ vs ambient pressure, #$p < 0.02$ vs 15 msw, !$p < 0.05$ vs 25 msw, and †$p < 0.01$ vs BH at the same pressure. msw, meters of seawater.

dive, 3.25-fold for BH and 2.88-fold for S. The 40 msw BH and S diver values were significantly different ($p < 0.01$). Urinary neopterin and 8-isoprostane were also assayed and results are shown in Table 1. Elevations for BH and S dives were identified.

3.2 Breath-Hold (BH) and SCUBA (S) Divers Exhibit Comparable Elevations of MPs

Elevations of blood-borne MPs in BH and S divers are shown in Table 2 as total MPs number and various subgroups exhibiting specific surface proteins. Those expressing filamentous (F-) actin and thrombospondin-1 have been shown to play major roles in inflammatory responses in the murine DCS model [13,23,24,38,39,40]. Elevations occurred for both BH and S divers with no statistically significant differences between the BH and S groups. Dives to 15 msw resulted in an MPs fold-elevation of 1.56 for BH and 1.42 for S. Dives to 40 msw resulted in MPs fold-elevations of 1.23 for BH and 1.21 for S. There were some significant differences within the BH and S groups for deeper dives. Within the BH group, most often elevations were higher after the 25 msw depth than after the 40 msw dives. Similarly, for S divers, the values after the 15 msw dive were often slightly greater than after 40 msw dives. These differences are outlined further in Discussion.

Neutrophil activation is mediated by MPs generated in association with pressure exposure [12]. This response is prominent in human divers, and it is a required event for pathogenesis of DCS in the murine model [23,24,38,39]. We compared the ability of MPs isolated from S and BH divers to activate fresh neutrophils from a healthy human donor. This was a delayed study using fixed, stored MPs available from four S and BH divers. Activation was assessed as the number of new MPs generated by the neutrophils and as increases in neutrophil expression of CD18, a component of β_2 integrins, and myeloperoxidase (MPO), an index of cell degranulation. Results shown in Fig. 2 demonstrate an equivalent stimulatory ability of MPs from S and BH divers after 15 and 40 msw dives (Fig. 2A). Note that the number of MPs generated by neutrophils stimulated with post-40 msw MPs was significantly greater than the production caused by post-15 msw MPs. In this regard, it is notable that there were no significant differences in the number of post-dive F-actin MPs between 15 and 40 msw dives (Table 2). This is an important issue because in some prior studies, only F-actin-expressing MPs were found to cause neutrophil activation [33]. The F-actin shell renders MPs membrane phosphatidylserine (PS) immunogenic, so that when samples are co-incubated with 5 μmol PS or more, neutrophil activation was entirely inhibited [33]. In the current studies, however, incubation up to 120 μmol PS caused at most 50% inhibition (see black bars in Fig. 2). As shown in Fig. 2B,C, neutrophil activation could also be demonstrated by increased neutrophil CD18 and MPO expression. The elevations were statistically significant on cells incubated with post-40 msw MPs ($p < 0.001$) but not with post-15 msw MPs.

Table 2. Circulating microparticle counts.

MPs/μL		Ambient	15 msw	25 msw	40 msw
Total MPs	Breath-hold	947 ± 88 (12)	1525 ± 461 (9)*	1389 ± 170 (5)*	1183 ± 176 (4)**
	SCUBA	946 ± 88 (8)	1706 ± 576 (8)*		1321 ± 320 (8)**
GFAP	Breath-hold	214 ± 82 (12)	312 ± 181 (9)*	424 ± 53 (5)*#!	225 ± 158 (4)*
	SCUBA	269 ± 51 (8)	445 ± 130 (8)*		335 ± 59 (8)*
CD146	Breath-hold	156 ± 59 (12)	229 ± 130 (9)*	304 ± 43 (5)*#!	170 ± 120 (4)*#
	SCUBA	209 ± 44 (8)	287 ± 92 (8)*		256 ± 62 (8)*#
TSP	Breath-hold	65 ± 17 (12)	111 ± 48 (9)*	122 ± 25 (5)*	78 ± 46 (4)**#
	SCUBA	79 ± 16 (8)	148 ± 88 (8)*		105 ± 26 (8)**
CD66	Breath-hold	75 ± 26 (12)	129 ± 61 (9)*	157 ± 27 (5)*#!	83 ± 53 (4)**#
	SCUBA	88 ± 20 (8)	131 ± 69 (8)*		111 ± 30 (8)**#
F-actin	Breath-hold	126 ± 44 (12)	203 ± 93 (9)*	247 ± 38 (5)*#!	142 ± 90 (4)**#
	SCUBA	149 ± 38 (8)	201 ± 101 (8)*		183 ± 52 (8)**
TMEM	Breath-hold	208 ± 63 (12)	324 ± 141 (9)*	405 ± 40 (5)*#!	232 ± 121 (4)**#
	SCUBA	257 ± 38 (8)	436 ± 233 (8)*		318 ± 65 (8)**#
TSP+GFAP	Breath-hold	11 ± 6 (12)	19 ± 15 (9)**	10 ± 2 (5)	20 ± 11 (4)**
	SCUBA	16 ± 13 (8)	70 ± 78 (8)**		25 ± 22 (8)
CD41	Breath-hold	56 ± 5 (12)	101 ± 12 (9)*	90 ± 16 (5)*#!	95 ± 31 (4)*
	SCUBA	67 ± 8 (8)	106 ± 11 (8)*		92 ± 7 (8)*

The MPs were identified based on size (0.3 to 1 μm diameter) and surface expression of annexin V. Proteins probed on the surface of the MPs include those from astrocytes (glial fibrillary acidic protein, GFAP), endothelium (CD146), neutrophils (CD66), microglia (transmembrane protein 119, TMEM) and those expressing thrombospondin-1 (TSP) or filamentous actin (F-actin). Data are mean ± SD; subject numbers are indicated within (brackets); *$p < 0.001$ vs ambient pressure, **$p < 0.01$ vs ambient pressure, #$p < 0.02$ vs 15 msw, !$p < 0.05$ between 25 and 40 msw. There are no statistically significant differences between BH and S. MPs, microparticles.

As discussed in the Introduction, IL-1β plays a prominent role in tissue injuries in the murine model and the cytokine is found solely within F-actin-expressing MPs [24,41]. The presence of IL-1β in the total number of MPs from SCUBA divers has been reported [11] but its presence in F-actin-positive versus F-actin-negative MPs has not, and no studies have previously examined this relationship in BH divers. Unfortunately, MPs samples were inadequate to assay 15 or 25 msw dives. For samples from 40 msw dives, there was no IL-1β detectable in F-actin-negative MPs for BH or S divers. Table 3 exhibits values for IL-1β in MPs following BH and S, with significantly higher IL-1β in S versus BH divers.

Table 3. IL-1β content (pg/1000 MPs) in F-actin-expressing MPs from S and BH divers.

IL-1β (pg/1000 MPs)	Ambient	40 msw
BH (4)	3.73 ± 0.17	7.32 ± 0.51*
S (4)	3.70 ± 0.36	9.05 ± 0.69*†

Data are mean ± SD (n = 4 for all groups), *$p < 0.001$ vs ambient pressure, †$p < 0.001$ vs BH at same pressure.

3.3 Bubble Score

Among all divers, post-dive cardiac bubbles were detected in three BH and two S divers. One S diver had a bubble score of 1 after a 15 msw dive and one had a score of 2 after a 40 msw dive. One BH diver had a score of 3 after a 25 msw dive, and after a 40 msw dive, one had a score of 1 and one a score of 3.

4. Discussion

Because exposures to elevations of ambient pressure rapidly cause increases in intracellular ROS and oxidative stress responses, we hypothesized that inflammatory responses would be similar between BH and S divers despite considerable differences in the duration of pressure exposures [12,14,42]. Fig. 3 illustrates the organization of our study and elements of the investigation. The goal was to compare oxy-inflammatory responses after the divers had been exposed to the same depths. Results support our hypothesis, as shown at the bottom of the figure. BH and S divers generated virtually the same magnitude of MPs and many oxidative stress and inflammatory responses were comparable. However, some differed and these will be highlighted below.

It is notable that although ROS increased in response to all dives, values were significantly different between S and BH 15 msw dives. We speculate that the higher values

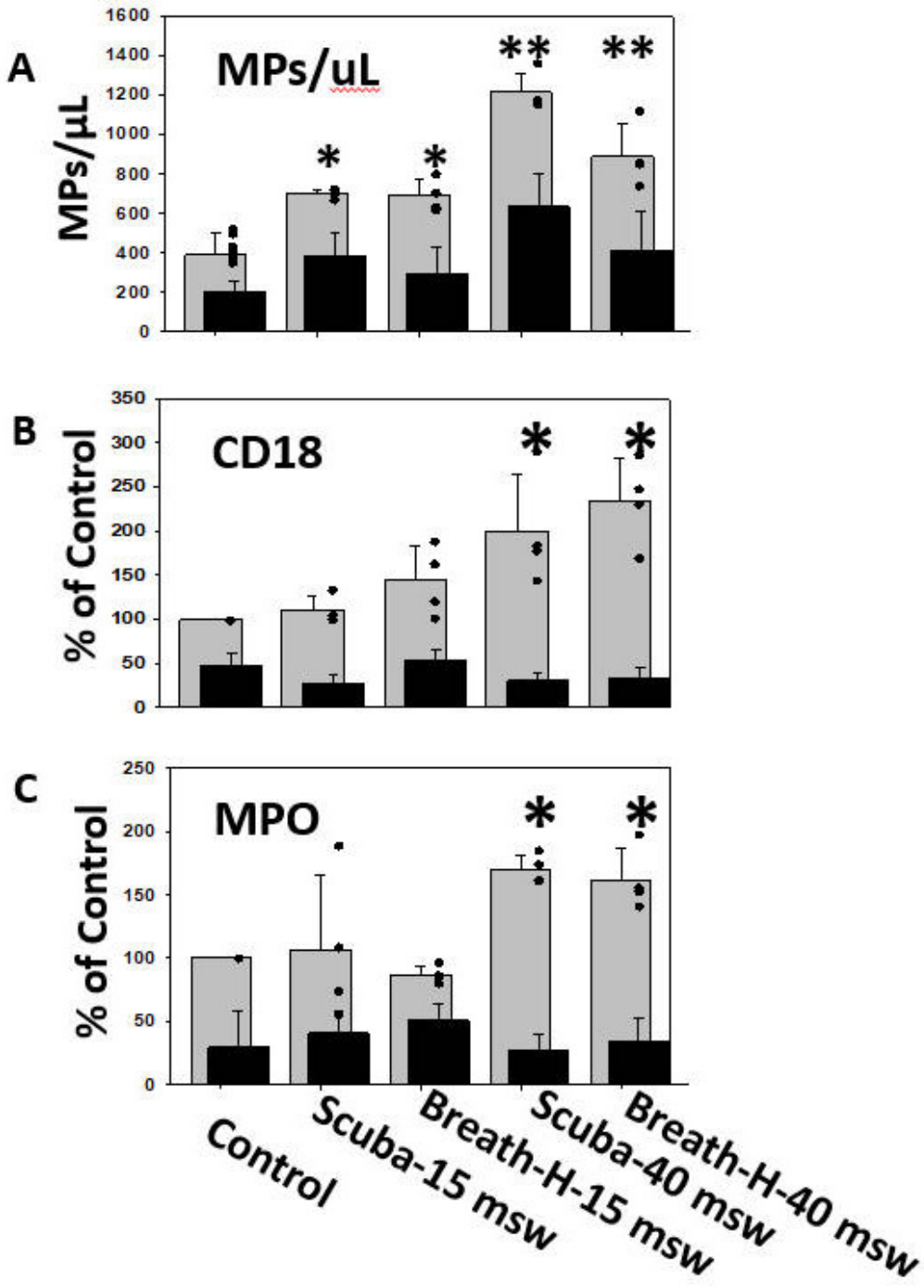

Fig. 2. MPs-mediated neutrophil activation. Microparticles from four SCUBA and four breath-hold divers were incubated for 30 minutes with fresh, isolated neutrophils from a healthy donor. Neutrophil activation was assessed as production of new MPs (number/µL) (A) and as an increase in expression over baseline, control (cells incubated without MPs) of cell-surface CD18 (B) and myeloperoxidase (MPO) (C). Illustrations depicted with grey bars show neutrophil responses; individual data points are also shown, and black bars show responses when cells + MPs are co-incubated with 120 µmol phosphatidylserine. Significant difference symbols: *$p < 0.01$ versus control, **$p < 0.001$ versus control and 15 msw dive values.

in S divers are related to the longer dive duration and greater oxygenation. However, values were the same among 40 msw BH and S divers and equaled the value seen in 15 msw S divers. ROS have a fleeting half-life, and while higher rates of production are expected at 40 msw, equal measurements may reflect a threshold for detection due to competing/quenching reactions hastened in both groups at 40 msw.

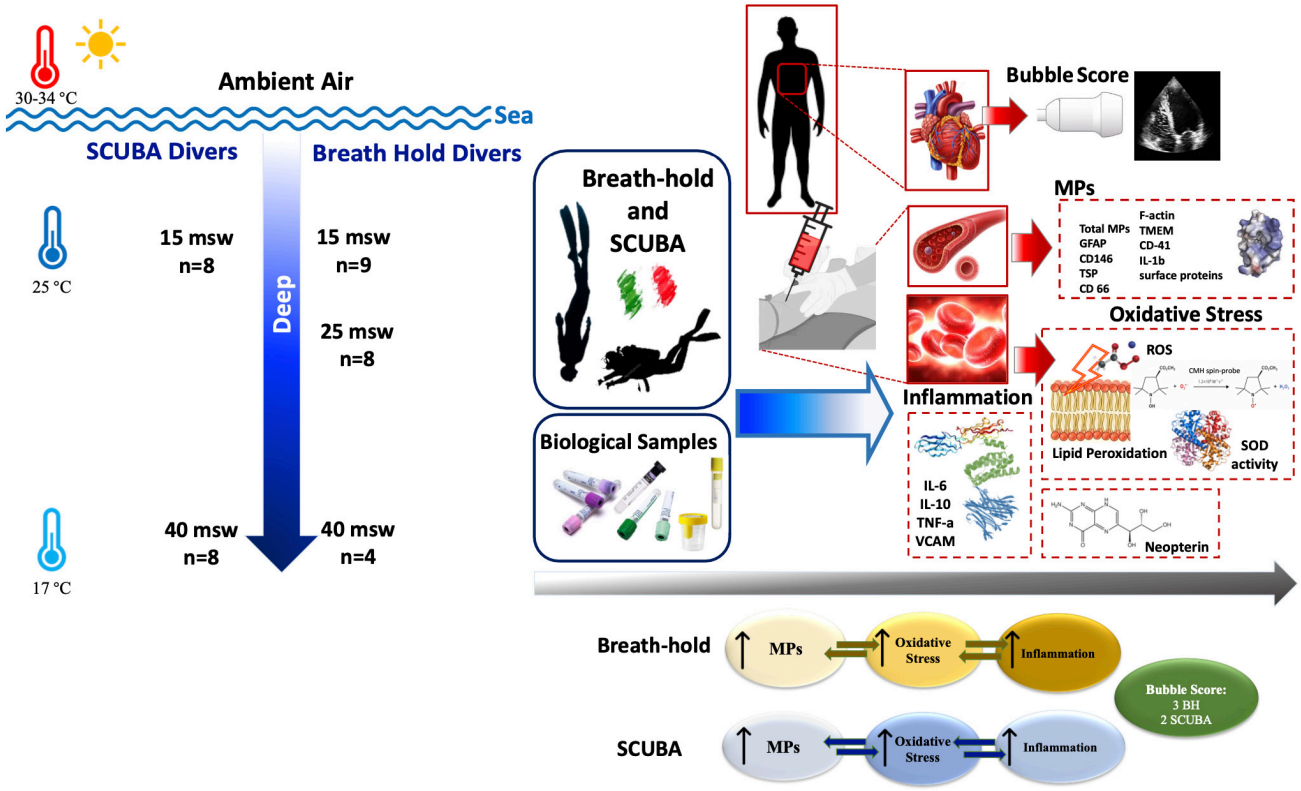

Fig. 3. Synopsis of the study. The illustration outlines the organization and findings on breath-hold and SCUBA dives at depths of 15, 25 and 40 msw, and the overall conceptual framework of the study. Divers performed dives to the same depths. After returning to the sea surface, VGE were analyzed, blood and urine samples were obtained. Laboratory testing was performed to assess MPs, biomarkers of oxidative stress and inflammation. The majority of the results support the study hypothesis that biomarkers of inflammation and oxidative stress would be comparable between BH and S divers despite differences in dive durations. VGE, venous gas emboli.

Among the cytokine assays, IL-1β and TNF-α stand out as exhibiting differences between S and BH divers. TNF-α and IL-1β promptly increase in response to oxidative stress. They both mediate innate immune responses and induce changes in gene expression via similar transcription factors. We speculate that the markedly higher values for TNF-α in S versus BH divers may also reflect the longer duration at pressure and thus extended oxidative stress. IL-1β was detected in F-actin-positive MPs and the value after the 40 msw S dives was greater than in BH divers. We believe this is due to response times for biochemical responses. Elevations of ROS occur within 5 minutes of high-pressure exposure, whereas NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome activation with concomitant MPs production may not reach a maximum for 30 minutes or more [12]. Elevations among IL-6, IL-10 and VCAM were greater after 40 msw than after 15 msw dives, but values did not differ between BH and S divers. Even if all elevations can be ascribed to oxidative stress, it is likely too much to expect that all downstream responses would follow a straightforward dose-response.

Circulating MPs increased similarly in response to S and BH dives (Table 2). Further, post-dive MPs from S and BH divers were equally effective at triggering activation of healthy human neutrophils (Fig. 2). This has pathophysiological implications for DCS development because neutrophil activation occurs in response to diving and it is a required event for progression of tissue damage in the murine DCS model [23,24]. Interestingly, we found that neutrophil activation by post-diving MPs could be inhibited by up to 50% when suspensions were co-incubated with PS. Prior work with MPs from diabetic patients has demonstrated that the F-actin shell on MPs renders membrane surface phosphatidylserine immunogenic [33]. As such, co-incubating MPs with PS could completely inhibit neutrophil activation [33]. Thus, our current investigation indicates there are additional factors causing neutrophil activation by divers' MPs besides PS. This issue is also supported by our finding that post-40 msw MPs triggered significantly more MPs to be generated by neutrophils than post-15 msw MPs. The number of F-actin-positive MPs was the same in 15 and 40 msw dives. Further investigation will be needed to ascertain the MPs' characteristics responsible for neutrophil activation.

Paradoxically, the numbers of circulating MPs in S divers were often fewer after the 40 versus 15 msw dives. This contrasts with the regular pattern of elevations seen in BH divers between 15, 25 and 40 msw. For all divers, blood

and urine samples were obtained within 15 minutes of surfacing, so sampling delays post-diving cannot be the basis for differences. We believe the MPs pattern in S divers may be due to a combination of the oxy-inflammation driving production and reduction by clearance from the circulation. Particle production is rapid and proportional to gas partial pressures, but clearance kinetics are largely unknown. In the murine model, MPs can adhere to the vascular lining via the CD36 receptor and more generally, MPs are thought to be cleared from the circulation via the reticulo-endothelial system [43].

There were elevations of urinary neopterin and 8-isoprostane in divers, again supporting the presence of inflammation and oxidative stress. These urinary measures have been validated across diverse settings as a non-invasive biomarker reflecting systemic inflammation and immune status [44]. However, contrary to the ROS, IL-1β and TNF-α data discussed above, the urinary data do not exhibit a consistent pattern where values were greater in S versus BH divers. Aromatic pteridines (neopterin) are produced by monocytes and macrophages upon stimulation by interferon-γ and rarely by other cytokines. 8-isoprostane is a relatively stable, non-enzymatic peroxidation product from arachidonic acid and a biomarker of oxidative stress. Multiple pathways with different rate-limiting steps are involved in generating urinary products, and perhaps it is the combination that leads to the more uniform pattern of results between BH and S divers.

Although VGE were scant in our subjects, this does not negate the possible role for insoluble gas with inflammatory responses, as current technology cannot detect micron-sized bubbles. No divers in the study sustained DCS, and more generally the relationship between inflammatory responses and DCS remains obscure. Inflammation in the murine model is reflected by elevations in IL-1β and MPs and linked to tissue damage because when purified MPs are isolated from decompressed mice and injected into naïve mice, the same injuries occur as in decompressed mice [13,23,24]. These studies include an important additional finding, however. Injected MPs obtained from decompressed mice are larger than MPs in mice kept at ambient pressure [39]. Because MPs enlargement is reversed by hydrostatic pressure, the data indicate that the inflammatory MPs contain a gas phase. Importantly, no injuries occur when size-reversed MPs (via hydrostatic recompression) are injected into mice [39]. Recently, similar enlargement and hydrostatic pressure reversal of MPs was shown in samples from human SCUBA divers [45]. This suggests that during decompression some insoluble gas diffuses into MPs, which may help to explain the difference in propensity for DCS between S and BH divers. In conjunction with the current results, the data suggest that while MPs are a manifestation of an inflammatory process, progression to overt DCS may require MP enlargement and/or concomitant formation of bubbles to exacerbate tissue injuries. BH divers do not incur as much gas supersaturation into tissues as S divers. DCS in BH divers is most typically seen among professional fishermen performing multiple dives in rapid succession and thus building up insoluble gas within tissues [15].

5. Study Limitations

The magnitude of hypoxia sustained by BH divers can be variable, and we did not directly measure oxygenation status between the BH and S groups. Most BH divers will experience hyperoxia at depth concurrent with lung compression, but some sustain hypoxia that is ascribed to vascular shunting [46]. We also did not perform formal assessments of whether BH divers had patent foramen ovale or physiological shunts. We recognize that a limitation in this work is an imprecise matching of physical effort between BH and S divers. To approximate BH diver exertion, S divers performed moderate exercise while at depth. Dive depths were matched between BH and S divers, but we cannot quantify the effort BH divers expend to return to the sea surface, as energy expenditure may exceed that due simply to swimming [47]. There also is the challenge of relatively small sample numbers. The choice of sample size was based on logistical considerations, and there also was no randomization undertaken. It is reassuring, however, that the variability within the data sets was relatively low. In future studies, larger data sets and further efforts to match the two groups may provide further insight into (patho)physiological responses to diving.

6. Conclusions

We conclude that oxidative stress and inflammatory responses to pressure are often comparable between BH and S divers. However, some values such as intra-MPs IL-1β and total particle numbers are greater in S divers due to factors such as longer dive durations, greater hyperoxia and ROS production, as well as compensatory mechanisms such as MPs clearance rates.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

GB conceived and designed research, SRT and GB acquired funding, administered the project and supervised research activities; SRT, SMS, AV, ARB, AKA, and GB developed methodology and conducted the investigation; SRT, SMS, AV, TAG, MP, DW, ZI, ARB, AKA, and GB acquired data; SRT, SMS, AV, and GB conducted formal analysis; SMS, TAG, MP, and GB participated in subject accrual and management; SRT, SMS, AV, TAG, MP, DW, ZI, ARB, AKA, and GB edited, revised and approved final

version of manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All work was approved by ethical committees from the University of Padova (No. HEC-DSB/03-18; addendum: November 2022) and University of Maryland (HP-00109500). The study was carried out in accordance with the guidelines of the Declaration of Helsinki. Subjects received a written explanation and an oral briefing of the methods and potential risks and gave their written informed consent.

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Conflicts of Interest

The authors declare no conflicts of interest.

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