J Appl Physiol 119: 427–434, 2015.
First published July 2, 2015; doi:10.1152/japplphysiol.00380.2015.

Association of microparticles and neutrophil activation with decompression sickness

Stephen R. Thom,[1] Michael Bennett,[2] Neil D. Banham,[3] Walter Chin,[4] Denise F. Blake,[5] Anders Rosen,[6] Neal W. Pollock,[7] Dennis Madden,[8] Otto Barak,[8] Alessandro Marroni,[9] Costantino Balestra,[9,10] Peter Germonpre,[11] Massimo Pieri,[9] Danilo Cialoni,[9] Phi-Nga Jeannie Le,[12] Christopher Logue,[12] David Lambert,[12] Kevin R. Hardy,[12] Douglas Sward,[1] Ming Yang,[1] Veena B. Bhopale,[1] and Zeljko Dujic[8]

[1]*Department of Emergency Medicine, University of Maryland School of Medicine, Baltimore, Maryland;* [2]*Department of Diving and Hyperbaric Medicine, Prince of Wales Hospital, New South Wales, Australia;* [3]*Department of Hyperbaric Medicine, Fiona Stanley Hospital, Perth, Western Australia;* [4]*Department of Hyperbaric Medicine, University of California Los Angeles Medical Center, Los Angeles, California;* [5]*Department of Emergency Medicine, The Townsville Hospital and College of Marine and Environmental Sciences, James Cook University, Townsville, Queensland, Australia;* [6]*Sahlgrenska University Hospital/Omrade2, Gothenburg, Sweden;* [7]*Divers Alert Network, Durham, North Carolina;* [8]*Department of Integrative Physiology, University of Split School of Medicine, Soltanska, Split, Croatia;* [9]*Divers Alert Network Europe Foundation and Diving Safety Laboratory, Roseto, Italy;* [10]*Environmental, Occupational, Ageing and Integrative Physiology Laboratory, Haute Ecole Paul-Henri Spaak, Brussels, Belgium;* [11]*Centre for Hyperbaric Oxygen Therapy, Military Hospital Brussels, Brussels, Belgium; and* [12]*Department of Emergency Medicine, Pereleman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

Submitted 12 May 2015; accepted in final form 30 June 2015

Thom SR, Bennett M, Banham ND, Chin W, Blake DF, Rosen A, Pollock NW, Madden D, Barak O, Marroni A, Balestra C, Germonpre P, Pieri M, Cialoni D, Le P-NJ, Logue C, Lambert D, Hardy KR, Sward D, Yang M, Bhopale VB, Dujic Z. Association of microparticles and neutrophil activation with decompression sickness. *J Appl Physiol* 119: 427–434, 2015. First published July 2, 2015; doi:10.1152/japplphysiol.00380.2015.—Decompression sickness (DCS) is a systemic disorder, assumed due to gas bubbles, but additional factors are likely to play a role. Circulating microparticles (MPs)—vesicular structures with diameters of 0.1-1.0 μm—have been implicated, but data in human divers have been lacking. We hypothesized that the number of blood-borne, Annexin V-positive MPs and neutrophil activation, assessed as surface MPO staining, would differ between self-contained underwater breathing-apparatus divers suffering from DCS vs. asymptomatic divers. Blood was analyzed from 280 divers who had been exposed to maximum depths from 7 to 105 meters; 185 were control/asymptomatic divers, and 90 were diagnosed with DCS. Elevations of MPs and neutrophil activation occurred in all divers but normalized within 24 h in those who were asymptomatic. MPs, bearing the following proteins: CD66b, CD41, CD31, CD142, CD235, and von Willebrand factor, were between 2.4- and 11.7-fold higher in blood from divers with DCS vs. asymptomatic divers, matched for time of sample acquisition, maximum diving depth, and breathing gas. Multiple logistic regression analysis documented significant associations ($P < 0.001$) between DCS and MPs and for neutrophil MPO staining. Effect estimates were not altered by gender, body mass index, use of nonsteroidal anti-inflammatory agents, or emergency oxygen treatment and were modestly influenced by divers' age, choice of breathing gas during diving, maximum diving depth, and whether repetitive diving had been performed. There were no significant associations between DCS and number of MPs without surface proteins listed above. We conclude that MP production and neutrophil activation exhibit strong associations with DCS.

Address for reprint requests and other correspondence: S. R. Thom, Dept. of Emergency Medicine, Univ. of Maryland School of Medicine, 655 W. Baltimore St., Bressler Research Bldg., Rm. 4-013, Baltimore, MD 21201 (e-mail: sthom@smail.umaryland.edu).

decompression sickness; myeloperoxidase; CD41; CD235; CD14; tissue factor; von Willebrand factor; platelet-endothelial cell-adhesion molecule

DECOMPRESSION SICKNESS (DCS) is a risk associated with compressed gas diving, tunneling, high-altitude aviation, and space exploration. Gas bubbles, long thought to be the inciting factor for DCS, are common and often asymptomatic; hence, additional pathophysiological factors have been sought to explain the development of the syndrome (7, 15, 16). There is now considerable evidence that microparticles (MPs), cell-derived membrane vesicles with diameters of 0.1–1.0 μm, are elevated in association with simulated as well as bona fide underwater diving (17–19, 23, 27, 28, 32). Maneuvers that decrease the incidence of DCS also diminish MP production (17, 18). Murine studies suggest that MPs play a role in high-pressure gas pathophysiology and possibly with gas-bubble nucleation (29, 30, 35, 36). In the mouse model, MPs have been shown to initiate a systemic inflammatory process that is related to neutrophil activation following decompression (29, 30, 35, 36). Injuries identified in decompressed animals can be recapitulated by injecting decompression-induced MPs into naïve mice (30, 35, 36).

There are, as yet, no data associating MPs with DCS in humans. The goal of this study was to examine MPs and neutrophil activation in blood obtained from self-contained underwater breathing-apparatus (SCUBA) divers. MPs were characterized, as is standard, by surface expression of antigenic markers from parent cells and based on Annexin V binding, because as MPs are formed, negatively charged phosphatidylserine residues become exposed. We hypothesized that differences would be identified between healthy, asymptomatic divers and those suffering from DCS. Blood samples were obtained from divers by a consortium of investigators around the globe who supervise diving and/or treat divers with DCS. As the study progressed, it became obvious that those with DCS exhibited marked differences

from asymptomatic divers. This prompted an examination of blood-borne changes following a variety of dives to different depths, using different breathing gases, and after multiple dive sessions.

EXPERIMENTAL PROCEDURES

Subjects. All procedures were completed in accordance with the Declaration of Helsinki and approved by ethical committees of all organizations involved with this investigation. Participants provided informed, written consent. Divers with DCS symptoms were approached by clinical teams when they presented to hospitals for evaluation and treatment. A comparison group of divers, who were not suffering DCS, was developed by soliciting cooperation from sport SCUBA divers. These were experienced, certified divers, monitored by one or more of the coauthors. The dive profiles, frequency of diving, and choice of breathing gases were selected by the divers and were independent of the study protocol. Activities were planned for other purposes, often as recreation, and the research component was solely a willingness to undergo phlebotomy before and at a range of times after diving. Under supervision, these divers swam continuously while at depth at a pace assumed similar to that which a normal diver would follow—activity that for most represents a sustained, moderate work rate. Diving profiles were chosen to be within accepted standards, so there would be no decompression requirement. Total dive times ranged from 17 to 178 min.

Divers evaluated for DCS. The signs and symptoms reported covered the gamut that is typically seen and well described in other publications (22). Pain, as one of the primary complaints, was reported by 69 (72.6%), sensory abnormalities by 52 (54.7%), weakness by 45 (47.4%), and central nervous symptoms by 15 (15.8%). The median interval of time from termination of the last dive to onset of DCS signs and/or symptoms was 0.55 (25th and 75th percentile: 0.08, 4.25) h. There were no obvious violations of decompression algorithms based on divers' reports and—where possible—confirmed by interrogation of dive computers in 72 (80%) cases. Violations of dive tables and/or alerts from dive computers were identified in 18 (20%) cases, typically due to diver decisions or equipment failure that resulted in an uncontrolled ascent from depth. Some divers had first aid/emergency interventions before arrival at hospital: 42 divers received supplemental O_2, and six received nonsteroidal anti-inflammatory agents (NSAID).

There is no definitive test by which to establish a diagnosis of DCS. We defined DCS as having occurred when a diver presented with complaints consistent with DCS, such that a clinical decision was made to initiate therapeutic recompression, and where recompression was associated with an improvement in signs or symptoms. Participation in the study involved obtaining blood at the time of the initial evaluation and after recompression treatment. Divers were also asked to provide a sample when returning to the clinic for a late follow-up medical evaluation before any return to diving.

Control divers. Divers participating in this project used their own equipment. Venous blood was collected from an antecubital arm vein by a trained phlebotomist before and at one or more times between 15 min and 144 h after diving. Phlebotomy was often carried out at a remote beach site, but where feasible, it was done at shore-based laboratory facilities. When results were compared among the field sites or according to the location where phlebotomy was done (remote beach site vs. laboratory) and matched for time when samples were obtained postdiving, there were no statistically significant differences.

Materials and standard laboratory procedures. Blood (~5 ml) was drawn into Cyto-Chex BCT test tubes that contain a proprietary preservative (Streck, Omaha, NE). Samples were sent by express mail to the first author's laboratory, where all analyses were performed following published techniques within 48 h after arrival, from 4 to 9 days after collection (27). Prior work has shown that MPs and neutrophil characteristics remain unchanged when samples are processed within 3 wk from time of acquisition (27). All supplies, reagents, and manufacturer sources have been described in previous publications (17, 18, 27, 28).

Flow cytometry. Early studies were performed with a 10-color FACSCanto (BD Biosciences, San Jose, CA); the majority was performed with an eight-color, triple-laser MACSQuant (Miltenyi Biotec, Auburn, CA) using manufacturers' acquisition software. MPs were stained with Annexin V and analyzed exactly as described previously (27, 28). Surface markers were evaluated with use of the Fluorescence Minus One Control test (31). This analysis provides a way to define the boundary between positive and negative particles in an unbiased manner by defining the maximum fluorescence expected for a given subset after outlining the area in a two-dimensional scatter diagram when a fluorophore-tagged antibody is omitted from the stain set. This analysis allows a simple decision as to where to place the upper boundary for nonstaining particles in a fluorescence channel. We define MPs as Annexin V-positive particles with diameters up to 1 μm. Neutrophils in whole blood were identified by CD66b staining and surface expression of MPO assayed as described previously (27, 28). MPO% indicates the fraction of all CD66b-positive cells exhibiting positive staining for MPO and MPO-median, the geometric median fluorescence value.

Statistical analysis. Results are summarized as median (with 25th and 75th percentiles). Log transformations were used for logistic regression analysis and to carry out two-way ANOVA. Correlations were evaluated by the Spearman rank order test. We used SigmaStat software (Systat Software, San Jose, CA) for the statistical analysis. Statistical significance level was set as $P < 0.05$.

RESULTS

Blood samples were obtained from 280 divers. Table 1 displays characteristics of the study population. Among 95 divers who presented to hospitals with signs and/or symptoms thought to be due to DCS, complaints improved with recompression therapy in 90. They had performed a median of two (1.5, 3.0) dives before developing DCS; only 18% developed DCS after a single dive. Most repetitive dives were performed on the same day—a minority over 2 or more consecutive days. There was no statistically significant correlation between DCS and the maximum depth of the most recent dive before presentation.

There were 185 divers in the control group. Age, gender distribution, body mass index, and median of the maximum depth of diving were not statistically, significantly different from the DCS group (Table 1). The majority of divers in both groups used compressed air as breathing gas; the rest used

Table 1. *Characteristics of study population*

	DCS Group (90)	Control Subjects (185)
Age (yr)	34 (27, 42) Range: 16–73	40 (37, 44) Range: 21–72
Dive depth (m)	22 (16, 34)	18 (18, 33)
No. female	23 (34%)	30 (20%)
Body mass index	25.8 (22.7, 28.3)	26.9 (24.1, 28.7)
Compressed air (%)	69 (76.7)	130 (70.3)
EAN (%)	19 (21.1)	28 (15.1)
Tri-mix (%)	2 (2.2)	27 (14.6)*

Age, diving depth, gender distribution, and body mass index between the divers with decompression sickness (DCS) and control subjects were not statistically, significantly different. The last 3 rows indicate the breathing gas used by the divers. EAN, enriched air nitrox (comprised of 32% O_2/68% N_2); Tri-mix contained variable amounts of O_2 (7–22%), helium (5–35%), and N_2 (40–60%). *$P < 0.001$.

either enriched air nitrox (EAN), comprised of 32% O_2/68% N_2, or Tri-mix gas, which depending on the dive depth and an individual diver's preference, contained variable amounts of O_2 (7–22%), helium (5–35%), and N_2 (40–60%). Tri-mix divers in both groups used a rebreather apparatus, with fixed, user-selected O_2 partial pressures that typically involved 1.0–1.3 atmospheres absolute (ATA) O_2 at depth and up to 1.4–1.64 ATA O_2 during the final stages of decompression. A higher percentage of divers used Tri-mix in the control group than in the DCS group.

Analysis of blood from control divers demonstrated that neutrophil activation assessed as MPO on the cell surface, and elevations of MPs did occur from 15 min to 4 h after diving (Fig. 1), but changes resolved within 24 h. The procurement of blood at 2 h postdiving was a frequent part of the research protocol. When results from control divers were separated by decades of maximum diving depth (e.g., 10–19.9, 20–29.9 m, etc.), all 2-h postdiving values for neutrophil activation and MP elevations were statistically, significantly different from predive values, but none was significantly different from the other (data not shown).

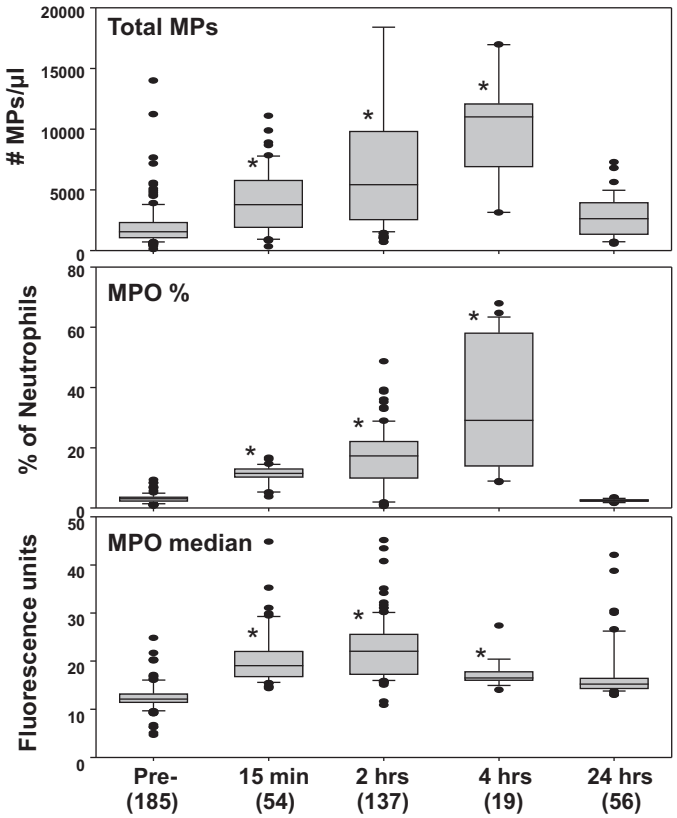

Fig. 1. Blood-borne Annexin V-positive microparticles (MPs) and neutrophil MPO staining in blood from control divers before and after a single dive. Blood was obtained before a dive and from 15 min to 24 h postdive. As discussed in EXPERIMENTAL PROCEDURES, participants performed dives based on their own choices, and involvement in this project entailed their willingness to undergo phlebotomy at intervals. Therefore, the number of samples (shown in parentheses below the figure) differed at each time point. The number of MPs/microliter of plasma is shown in the *top*. MPO % indicates the fraction of CD66b-positive cells exhibiting MPO fluorescence above the fluorescence-minus-1 threshold (*middle*); MPO median indicates the geometric median fluorescence value for MPO on CD66b-positive cells (*bottom*). The figure indicates median value as the horizontal line within gray boxes. The boxes display 25th and 75th percentile; error bars show 10th and 90th percentile, with outliers shown as single dots. $*P < 0.05$ vs. predive sample.

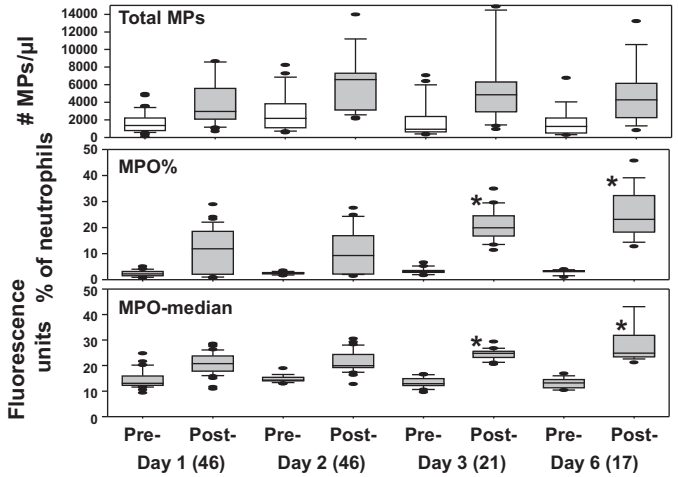

Fig. 2. MPs and neutrophil MPO staining in blood from control divers performing repetitive dives. Data show predive values for the same parameters, as described in Fig. 1, within 1 h before diving and at 2 h postdiving. Dives were conducted at ~24-h intervals; thus the predive values for days 2, 3, and 6 are values in blood ~22 h after diving on days 1, 2, and 5. All postdive values are significantly different ($P < 0.001$) from predive values on the same day. $*P < 0.001$ vs. days 1 and 2 postdive values based on 2-way repeated-measures ANOVA of log-transformed data.

Some individuals in the control group dove only once, and blood was obtained at one or more times, from 0.25 to 96 h later. Others performed one or two dives/day for up to 6 days. Figure 2 displays the pattern of MPs and neutrophil MPO staining when control divers conducted repetitive dives. Predive values on all days were not statistically, significantly different from each other. Statistically, significant elevations were found in all 2-h postdive samples, but only postdive neutrophil MPO on days 3 and 6 were greater than postdive values on days 1 and 2 (Fig. 2). The pattern of elevations with each day of diving and normalization of values before diving the next day were also observed within MP subgroups (Table 2). Most of the repetitive divers performed just one dive/day, but data were not significantly different among 22 divers, who performed two dives in 1 day, and blood obtained 24 h after the second dive (data not shown).

Table 3 displays data from control divers and those with DCS. The first column contains predive data from the control group. The second column shows postdive results from control divers, but this data set differs slightly from the predive set. As was outlined in Table 1, the control subjects used Tri-mix gas ~15% of the time (27 divers). Because it is unknown how this usage may influence neutrophil activation and MPs, data from only the first four Tri-mix divers enrolled in the study were used so that the proportions matched those for divers with DCS (a post hoc analysis demonstrated that data for these four were not statistically, significantly different from the 23 other control Tri-mix divers). Hence, data represent results from 162 divers; 130 (80.2%) used compressed air, 28 (17.3%) used EAN, and four (2.5%) used Tri-mix.

The postdive data in the second column of Table 3 were generated using only one blood sample from each control diver. The sample chosen was always the last obtained from

Table 2. *MP subtypes in blood from repetitive diver control subjects*

	Day 1 Pre (20)	Day 1 Post	Day 2 Pre (11)	Day 2 Post	Day 3 Pre (11)	Day 3 Post	Day 6 Pre (8)	Day 6 Post
CD66b	3.6 (2.2, 8.3)	93 (35, 230)	8.1 (4.2, 14.1)	228 (39, 532)	5.6 (3.7, 11.3)	200 (115, 425)	5.1 (4.0, 12.3)	128 (55, 206)
CD41	6.6 (3.6, 9.6)	77.2 (31.8, 229)	7.0 (4.3, 11.5)	58.3 (39.5, 333)	6.2 (3.7, 10.5)	193.7 (108, 424)	8.0 (4.1, 15.7)	195.9 (114, 390)
CD31	6.3 (2.8, 10.0)	103 (37, 233)	9.0 (3.5, 18.5)	233 (40, 365)	7.7 (5.5, 12.1)	220 (176, 290)	6.5 (4.7, 12.0)	226 (138, 357)
CD142	0.3 (0.06, 2.9)	19 (9, 39)	5.4 (2.1, 5.5)	22 (10, 40)	0.7 (0.5, 0.9)	86 (43, 127)	0.9 (0.5, 1.3)	50 (41, 189)
CD14	4.9 (3.9, 6.8)	11 (6, 27)	5.9 (3.3, 6.3)	17 (7, 72)	7.7 (6.2, 9.2)	122 (56, 157)	7.5 (6.3, 11.6)	79 (59, 299)
CD235	5.4 (3.7, 6.3)	15 (9, 72)	5.0 (4.1, 5.8)	12 (8, 34)	8.7 (5.7, 9.1)	98 (51, 315)	8.4 (6.4, 10.3)	134 (81, 307)
vWF	5.5 (3.6, 6.3)	21 (11, 73)	5.6 (4.8, 6.4)	10 (7.3, 217)	5.2 (3.2, 6.6)	80 (44, 118)	6.0 (4.7, 10.9)	52 (38, 184)

Data are median (with 25th and 75th percentiles) for repetitive control subject divers; (*n*) in the predive columns (Pre) indicates the number of individual diver samples in each pre/postdive set. All postdive values (Post) are statistically, significantly greater than predive values ($P < 0.001$). Although a trend appears, suggesting that postdive values for days 3 and 6 are greater than the postdive values for day 1 and/or day 2, the differences are not statistically significant based on 2-way ANOVA on log-transformed data. All rows indicate the number/microliters plasma for microparticles (MPs), manifesting the following surface markers: CD66b (neutrophil specific), CD41 (platelet specific), CD31 (platelet-endothelial cell-adhesion molecule), CD142 (tissue factor), CD14 (leukocyte common antigen), CD235 (erythrocyte specific), von Willebrand factor (vWF).

those who did a single dive ($n = 65$). For repetitive divers ($n = 97$), the sample chosen for analysis was obtained just before diving on the last day. This approach was taken to match the data set better for DCS divers, where blood samples were obtained at a median time of 24 (11.8, 55.0; range 0.5–144) h after diving. By compiling the control diver data set in the manner described, neutrophil and MP changes could be assessed at a median time of 24 (7.4, 96) h postdiving (range 0.5–144).

Data from DCS diver samples obtained on presentation to the hospital are identified as "acute" in the third column of Table 3. Values for neutrophil MPO staining and MPs expressing each of the protein subtypes were statistically, significantly different from the postdive control values. Among the 90 divers with DCS, 35 returned to clinics for follow-up evaluations and provided blood samples at a median time of 28 (13.5, 35) days after treatment (last column in Table 3). Divers were all asymptomatic at this time. All values in this group were statistically, significantly different from predive values from the control group (column 1), but few were significantly different from postdive control group values (column 2). Leukocyte and platelet counts were not significantly different among samples from control subjects, the acute samples obtained from DCS divers, and the late follow-up samples (data not shown).

We also compared DCS diver data sets between those who performed a single dive and those who had conducted repetitive dives before onset of DCS. The only values exhibiting statistically significant differences were time when blood samples were obtained postsymptom onset and the number of CD142-positive MPs. All other blood analyses listed in Table 3 were not significantly different. The median time of blood sample acquisition for those performing a single dive was 12 (4, 48) h vs. 26 (12, 72) h ($P = 0.002$) for those having performed repetitive dives. The CD142-positive MP count for those performing a single dive was 18 (11, 50) MPs/microliter vs. 67 (29, 186; $P = 0.012$) MPs/microliter for those having performed repetitive dives. Interestingly, a trend for elevations in CD142 MPs with repetitive dives in the control group also appeared in Table 2.

It was feasible that ingestion of NSAID medication or emergency use of supplemental O_2 before DCS diver presentation might influence various blood-test results. However, with the use of either NSAID or O_2 as the binary dependent variable for multiple logistic regression analysis, we found no significant impact for these interventions on total MPs, any MP subtype, or neutrophil activation.

Table 3. *MPs and neutrophil activation data on blood samples*

	Predive Control Subjects ($n = 185$)	Postdive Control Subjects ($n = 162$)	DCS Divers, Acute ($n = 90$)	DCS Divers, Follow-up ($n = 35$)
Total MPs/μl	1,448 (946, 2,165)	2,391 (1,258, 5,123)*	2,716 (945, 6,920)*	2,047 (779, 3,682)
MPO%	2.6 (1.9, 3.5)	5.8 (2.7, 12.2)	11.5 (4.3, 24.4)*	10.3 (4.6, 18.3)*
MPO-median	12.2 (11.4, 14.0)	15.3 (13.5, 21.6)*	16.4 (13.8, 19.9)*	14.9 (12.8, 22.3)
MPs-CD66b/μl	4.8 (1.8, 38.2)	31.1 (15.9, 44.3)*	84.0 (22.8, 148.9)*†	55.7 (18.8, 128.9)*
MPs-CD41/μl	9.6 (4.5, 33.7)	45.0 (18.4, 87.5)*	110.4 (52.2, 400.9)*†	88.2 (19.5, 337.4)*
MPs-CD31/μl	13.1 (5.3, 64.2)	37.9 (21.0, 242.8)*	186.9 (70.3, 606.3)*†	137.6 (53.5, 298.3)*†
MPs-CD142/μl	1.4 (0.3, 16.9)	16.4 (2.1, 132.3)*	66.7 (24.5, 194.6)*†‡	19.4 (8.1, 46.9)*
MPs-CD14/μl	7.7 (4.1, 18.0)	25.5 (6.2, 81.8)*	271.2 (101.4, 765.0)*†	206.6 (26.5, 309.1)*†
MPs-CD235/μl	6.4 (3.9, 15.8)	32.5 (9.0, 126.7)*	385.9 (56.6, 692.6)*†‡	73.4 (34.1, 248.6)*†
MPs-vWF/μl	6.5 (4.0, 18.0)	38.2 (6.6, 148.3)*	248.9 (36.0, 558.0)*†‡	58.1 (4.1, 272.0)*

Data are predive values for control subjects ($n = 185$, 1st column); postdive control subjects ($n = 162$, 2nd column) included only 4 divers who used Tri-mix gas, as described in the text, and the samples analyzed were obtained at the longest time after diving to match the time when samples were obtained in the acute DCS group (column 3). Column 4 displays data from divers with DCS, who returned for follow-up evaluations after treatment. Data are median (25th and 75th percentiles). *$P < 0.001$ vs. predive control subject values;. †$P < 0.05$ vs. postdive control subject values;. ‡$P < 0.05$ vs. late follow-up DCS values. Rows are labeled as follows: MPO% indicates the fraction of CD66b-positive cells exhibiting MPO fluorescence above the fluorescence-minus-1 threshold (see EXPERIMENTAL PROCEDURES); MPO-median indicates the geometric median fluorescence value for MPO on CD66b-positive cells. All other rows indicate the number/microliter plasma for MPs manifesting the following surface markers: CD66b (neutrophil specific), CD41 (platelet specific), CD31 (platelet-endothelial-cell adhesion molecule), CD142 (tissue factor), CD14 (leukocyte common antigen), CD235 (erythrocyte specific), vWF.

Table 4. *Association of MPO on neutrophils and MPs with DCS*

Adjustment	MPO%	MPO-Median	CD66b	CD41	CD31	CD142	CD14	CD235	vWF
Unadjusted	1.6 (1.2, 2.0)	6.9 (2.1, 23.1)	1.4 (1.2, 1.8)	1.5 (1.2, 1.8)	1.7 (1.4, 2.2)	1.4 (1.2, 1.7)	2.0 (1.4, 2.7)	1.6 (1.3, 2.2)	1.7 (1.3, 2.3)
	$P < 0.001$	$P = 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Time	1.6 (1.2, 2.1)	7.2 (2.1, 24.8)	1.7 (1.3, 2.1)	1.7 (1.4, 2.2)	2.1 (1.6, 2.8)	1.9 (1.5, 2.4)	2.2 (1.5, 3.1)	2.2 (1.5, 3.2)	2.1 (1.5, 3.0)
	$P < 0.001$	$P = 0.002$	$P < 0.001$	<0.001	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Age	1.6 (1.2, 2.1)	8.6 (2.4, 31.5)	1.4 (1.1, 1.7)	1.4 (1.2, 1.6)	1.6 (1.3, 2.1)	1.4 (1.2, 1.7)	2.0 (1.4, 2.9)	1.6 (1.2, 2.2)	1.7 (1.2, 2.4)
	$P < 0.001$	$P = 0.001$	$P = 0.005$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.003$	$P = 0.001$
Time, depth, gas, repeat dive, age, gender	1.6 (1.2, 2.1)	7.1 (1.8, 27.8)	1.5 (1.2, 2.0)	1.6 (1.2, 2.1)	2.2 (1.6, 3.1)	2.0 (1.5, 2.6)	3.4 (1.8, 6.4)	2.4 (1.4, 4.1)	2.5 (1.5, 4.2)
	$P = 0.001$	$P = 0.005$	$P = 0.003$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.001$	<0.001

Logistic regression was performed on log-transformed data to assess the odds ratios (ORs) for DCS using values of MPO on the neutrophil surface and MP subtypes. Blood samples used for this analysis were the pretreatment samples from 90 divers with DCS and 190 blood samples from divers who were deemed not to have DCS. This included the latest blood sample obtained postdiving from each of the control group ($n = 185$), as described in RESULTS, and also the 5 divers who presented to hospitals with signs/symptoms thought due to DCS but who did not improve with recompression treatment. Unadjusted ORs are shown, as well as ORs adjusted by including the length of time from the end of the last self-contained underwater breathing-apparatus dive to when blood was collected for analysis (identified as Time in table), diver's age, and including time, maximum diving depth, breathing gas (compressed air, EAN, or Tri-mix), whether repetitive diving was involved, diver's age, and diver's gender. Data are ORs, 95% confidence limits, and *P* values. Columns are labeled as was described for rows in the legend of Table 3.

Logistic regression of log-transformed data found a positive association between both the magnitude of neutrophil activation and numbers of MPs in all subgroups and DCS, with odds ratios (ORs) from 1.4 to 6.9 (Table 4). These ORs increased when adjusted for the time when the blood samples were obtained after diving. The factoring in of diving characteristics, such as maximum depth, breathing gas, and whether repetitive diving was performed, along with time of sample acquisition, had little impact on ORs (data not shown). The OR for MPO-median, adjusted for a diver's age, increased the value to 8.6 (95% confidence limits: 2.4, 31.5; $P = 0.001$), and by including time of sample acquisition and age, the OR was 9.0 (2.4, 33.3; $P = 0.001$). A diver's age did not modify the effect estimate of any other variable, and gender and body mass index did not modify the effect estimate of any variable. Adjustment of ORs, by including factors related to dive characteristics and also diver age, increased ORs for some variables modestly, whereas it diminished the OR for MPO-median (Table 4, bottom row). The addition of gender, body mass index, or inclusion of multiple MP subtypes in this multiple variable analysis did not alter the ORs. There was no significant association between DCS and total number of Annexin V-positive MPs.

Consistent with the regression analysis, DCS exhibited a significant correlation with all variables (Table 5). There were weak, statistically significant correlations between MPO on neutrophils and most MP subtypes and strong correlations among the number of each of the MP subtypes. No correlation was found between DCS and maximum diving depth, performing repetitive vs. a single dive, or gender. Age was negatively correlated with DCS (-0.25; $P < 0.0001$).

DISCUSSION

Our results provide a number of insights regarding human responses to SCUBA diving. Elevations of MPs and neutrophil MPO surface staining occur predictably, but there are no statistically significant differences in the responses based on depth of diving. It does appear, however, that repetitive diving augments neutrophil activation, as shown in Fig. 2. We inter-

Table 5. *Spearman correlation analysis*

	MPO%	MPO-Median	CD66b	CD41	CD31	CD142	CD14	CD235	vWF
DCS	0.38	0.19	0.28	0.29	0.41	0.33	0.46	0.43	0.42
	<0.0001	<0.01	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MPO%		0.41	0.26	NS	0.30	0.25	0.54	0.32	0.53
		<0.0001	<0.0001		<0.0001	<0.01	<0.0001	<0.0001	<0.0001
MPO-median			0.23	0.14	0.21	0.26	0.44	0.35	0.59
			<0.01	<0.0001	0.01	<0.01	<0.0001	<0.0001	<0.0001
CD66b				0.70	0.69	0.78	0.76	0.61	0.74
				<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CD41					0.80	0.73	0.78	0.71	0.80
					<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CD31						0.72	0.70	0.88	0.87
						<0.0001	<0.0001	<0.0001	<0.0001
CD142							0.73	0.66	0.78
							<0.0001	<0.0001	<0.0001
CD14								0.74	0.82
								<0.0001	<0.0001
CD235									0.79
									<0.0001

Correlation coefficients and *P* values are shown for analyses conducted using the same data set as described for Table 4. Values are more highly correlated with coefficients approaching 1.0.

pret elevations of MPs as a response to high gas-pressure exposures. The mechanism has been discerned for neutrophils as an oxidative stress response due to an interaction between O_2 and ballast or what is viewed as inert gases, such as N_2 (26); studies with other vascular cells are underway. Similar MPs and neutrophil activation responses occur in the murine model, but a dose response between gas pressure vs. MP numbers and neutrophil activation can be discerned with inbred laboratory animals (29).

With regard to divers with DCS, neutrophil and MP responses are markedly greater than among asymptomatic divers. There are statistically significant associations between these variables and DCS. Diving depth, breathing gas, and participation in repetitive diving had no meaningful impact on the associations between DCS and neutrophil activation or MP subtypes. Postdecompression MP elevations and neutrophil activation are clearly linked to injuries to the vasculature and brain in the murine model, but obviously, results from this project do not identify the pathophysiological relationship between these blood-borne changes and clinical findings in humans (30, 35, 36).

Postdive values for control divers in the second column of Table 3, obtained at a median time of 24 h postdiving, are significantly different from the predive values. This may appear to contradict findings in Fig. 1, where resolution of dive-induced changes occur within 24 h. It is important to note, however, that 65 (40.1%) of the samples in the Table 3 analysis were obtained from divers 0.5–2 h after diving. It was our belief that the inclusion of these early postdive data provided a more balanced comparison for findings to the DCS diver group, as many of the injured presented to a hospital within a few hours of diving.

There appears to be persistent neutrophil activation and elevations in some MP subtypes long after divers suffered DCS (last column in Table 3). These divers had been instructed not to participate in SCUBA diving until presenting for follow-up evaluations. Assuming that most were compliant with instructions, the findings could suggest that ongoing or long-term changes occur after DCS. It is important to note, however, that the magnitude of neutrophil activation related to diving is much less than that in response to chemicals thought similar to some pathological stimuli (27). These results are not evidence for a fulminant systemic inflammatory response syndrome. Indeed, the late follow-up divers did not express physical complaints.

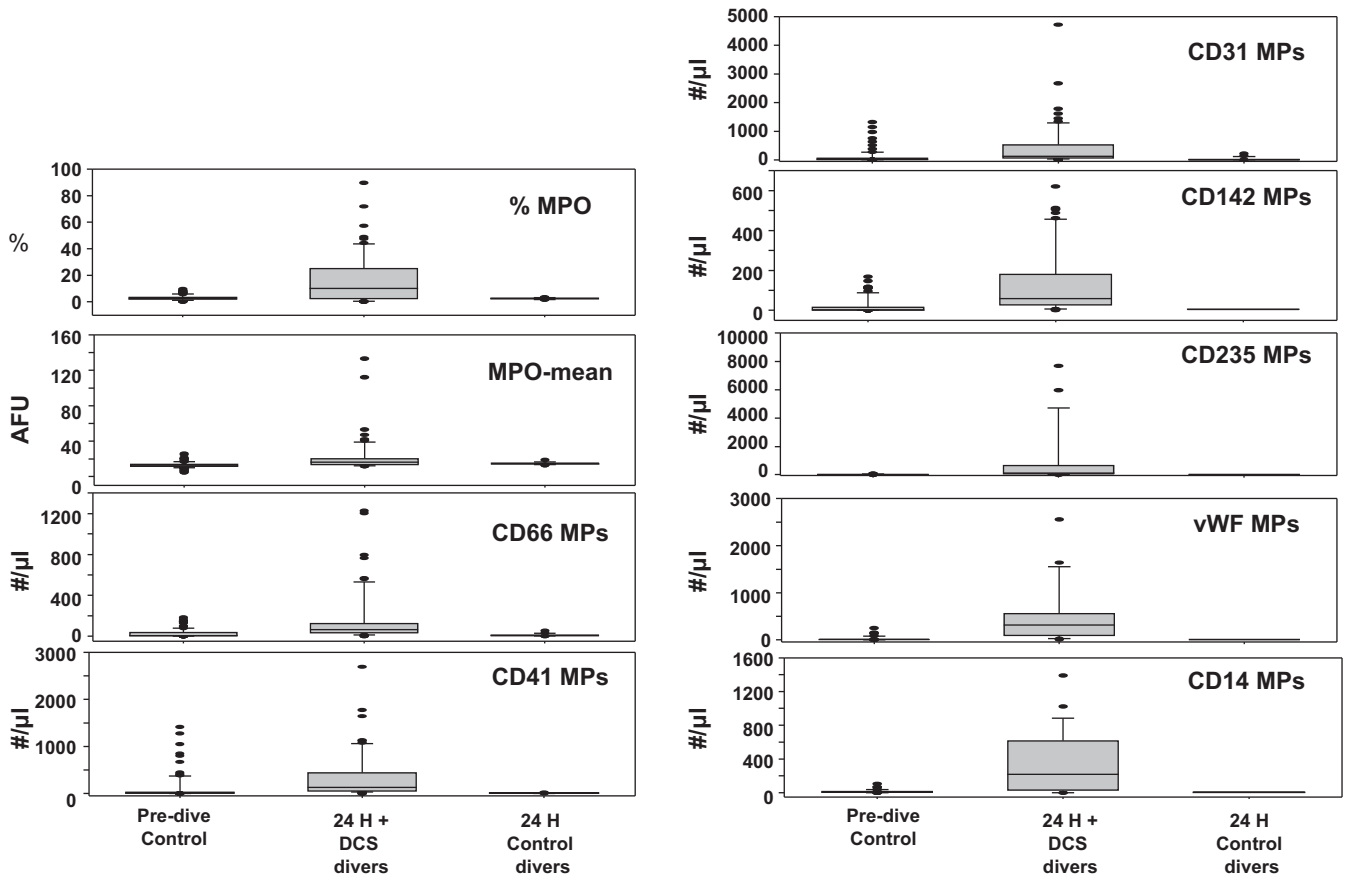

Fig. 3. Differences between decompression sickness (DCS) and control/asymptomatic divers' blood-borne Annexin V-positive MPs and neutrophil MPO staining in blood at 24 or more h postdiving. The 1st bar in each graph represents predive data for control divers ($n = 185$), the 2nd bar represents data from DCS divers obtained 24 or more h after the dive that incited DCS ($n = 59$), and the 3rd bar represents data for control divers obtained 24 h after their 1st dive ($n = 27$). The figure illustrates the persistent elevations of blood-borne changes in DCS divers vs. control/asymptomatic divers. Panel labels are the same as described for Table 3 (AFU, arbitrary fluorescence units; CD66, CD66b; vWF, von Willebrand factor). The figure indicates median value as the horizontal line within gray boxes. The boxes display 25th and 75th percentile; error bars show 10th and 90th percentile, with outliers shown as single dots. For all measurements, the data for DCS divers are statistically, significantly different from the 1st and last control diver panels ($P < 0.05$ ANOVA), and there are no significant differences between pre- and postcontrol diver values.

Persistent elevations of some MP subtypes weeks after DCS could be related to rates of MP clearance. Surface phosphatidylserine on MPs constitutes a recognition signal that enables phagocytosis (1). In the mouse model, there are marked differences in clearance rates among MPs, but data in humans are lacking (36). The results may be interpreted as a feedback loop, whereby persistent elevations of MPs are causing neutrophil activation, a phenomenon shown to occur in the murine model (30, 35, 36). An alternative possibility, however, is that the elevations found late after treatment actually represent these individuals' baseline or predive characteristics, such that attributes of MPs or sensitivity for neutrophil activation place them at greater risk for DCS.

The relationship between MP elevations and neutrophil activation is complex, and each can lead to the other, as well as the development of vascular injuries (30, 35, 36). Human divers exhibit vascular dysfunction, assessed as a decrease in conduit artery endothelial function. Measured as flow-mediated dilatation, it occurs after a single dive and can persist for several days (2, 21). Correlations between neutrophil activation and MP elevations and among MP subtypes (Table 5) are consistent with murine studies (29, 30, 35, 36). Additionally, neutrophil activation results in some MPO adhering to the cell surface, and MPO on the neutrophil surface can cause autoactivation (14).

The dynamics between MPs and neutrophil activation may also be responsible for the trends with elevations of MP subtypes in repetitive divers (Table 2) and elevations of CD142-positive MPs in repetitive DCS divers vs. those diving only once. Intravascular expression of CD142 (tissue factor) is a primary mechanism of inflammation-induced coagulation activation, and it is the most important initiator of thrombin formation (4). In this regard, it is of interest that reductions of plasma fibrinogen occur with repetitive diving, and on rare occasions, coagulopathy occurs with DCS (6).

Of course, these results do not resolve the role for bubbles in DCS. The relationship among intravascular bubbles, MPs, and neutrophil activation is influenced by variables, such as diver exertion, as well as breathing gases and possibly diet or dietary supplements (28, 33, 34). There is evidence supporting the presence of a gas phase in some MPs (36). These could serve as bubble nucleation sites, and given that MP enlargement occurs postdecompression, MPs may be a source of decompression-induced vascular bubbles, which have reported diameters of 24–160 μm (9, 10, 12).

Finally, the data provide some insight into perceived risks of DCS. There is ongoing debate whether women have greater risk, possibly linked to menstrual changes (24). We found no statistically significant association between gender and DCS nor did gender influence the effect estimates of the various blood-borne measurements (Table 4). In some but not all studies, obesity appears to be one of the factors that increases the risk of DCS (5, 11); however, we did not find body mass index to be associated with DCS. Surprisingly, age was negatively correlated with DCS. There has not been much effort focused on investigating the association between age and DCS. One study reported that age was a contributing factor to intravascular bubble formation, and another found an increased incidence of altitude-induced DCS in those >42 yr of age (3, 25). Another study found no age influence on DCS; however, there were no divers >50 yr old in the series (13). We found age to influence significantly the effect estimate of MPO-median value on DCS. This is an interesting issue, as age is generally viewed as having a negative influence on neutrophil functions, such as priming and degranulation (8, 20).

In conclusion, whereas neutrophil activation and MP elevations are a common response to diving, individuals who develop DCS exhibit more exuberant responses than do the control/asymptomatic divers that were studied. Increased levels of MPs and activated neutrophils are associated with the development of DCS symptoms compared with divers who have not experienced DCS symptoms while conducting dives with similar profiles. Time of blood sample acquisition post-diving greatly impacts measurements. At least among those divers who present to the hospital at later times, the blood-borne changes described here might be useful as biomarkers to aid in diagnosing DCS (Fig. 3). Further work will be needed, however, because values from the DCS and control groups exhibit some overlap. Some interventions that inhibit MP elevations and tissue injuries in mice also diminish MP elevations and neutrophil activation in human divers (33, 34). This offers an opportunity to examine whether similar interventions could improve the safety of provocative diving. It remains unclear, however, whether there are pre-existing differences within the population that contribute to development of DCS.

ACKNOWLEDGMENTS

The authors thank Jelena Pavic, Jenna M. Wiley, and Stefanie D. Martina for field-testing assistance and Marina Bogush and Tatyana N. Milovanova at the University of Pennsylvania for technical assistance in the laboratory.

GRANTS

Support for this work was provided by grants from the U.S. Office of Naval Research, Divers Alert Network, Unity through Knowledge Fund (Grant No. 33/08), and Croatian Ministry of Science, Education and Sports (Grant No. 216-2160133-0130).

DISCLOSURES

Funding sponsors played no role in the study design. No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.R.T. and Z.D. conception and design of research; S.R.T., M.Y., and V.B.B. performed experiments; S.R.T., M.B., N.D.B., W.C., D.F.B., A.R., N.W.P., D.M., O.B., A.M., C.B., P.G., M.P., D.C., P-N.J.L., C.L., D.L., K.R.H., D.S., M.Y., V.B.B., and Z.D. analyzed data; S.R.T. and Z.D. interpreted results of experiments; S.R.T. prepared figures; S.R.T. drafted manuscript; S.R.T., M.B., N.D.B., W.C., D.F.B., A.R., N.W.P., D.M., O.B., A.M., C.B., P.G., M.P., D.C., P-N.J.L., C.L., D.L., K.R.H., D.S., M.Y., V.B.B., and Z.D. edited and revised manuscript; S.R.T., M.B., N.D.B., W.C., D.F.B., A.R., N.W.P., D.M., O.B., A.M., C.B., P.G., M.P., D.C., P-N.J.L., C.L., D.L., K.R.H., D.S., M.Y., V.B.B., and Z.D. approved final version of manuscript.

REFERENCES

1. **Bevers EM, Williamson PL.** Phospholipid scramblase: an update. *FEBS Lett* 584: 2724–2730, 2010.
2. **Brubakk AO, Duplancic D, Valic Z, Palada I, Obad A, Bakovic D, Wisloff U, Dujic Z.** A single air dive reduces arterial endothelial function. *J Physiol* 566: 901–906, 2005.
3. **Carturan D, Boussuges A, Vanuxem P, Bar-hen A, Burnet H, Gardette B.** Ascent rate, age, maximal oxygen uptake, adiposity, and circulating venous bubbles in diving. *J Appl Physiol* 93: 1349–1356, 2002.
4. **Chu AJ.** Tissue factor mediates inflammation. *Arch Biochem Biophys* 440: 123–132, 2005.
5. **Dembert ML, Jekel JF, Mooney LW.** Health risk factors for the development of decompression sickness among U.S. Navy divers. *Undersea Biomed Res* 11: 395–406, 1984.

6. **Eckenhoff RG, Hughes JS.** Hematologic and hemostatic changes with repetative air diving. *Aviat Space Environ Med* 55: 592–597, 1984.
7. **Eftedal OS, Lydersen S, Brubakk AO.** The relationship between venous gas bubbles and adverse effects of decompression after air dives. *Undersea Hyperb Med* 34: 99–105, 2007.
8. **Fulop T, Larbi A, Douziech N, Fortin C, Guerard KP, Lesur O, Khalil A, Dupuis G.** Signal transduction and functional changes in neutrophils with aging. *Aging Cell* 3: 217–226, 2004.
9. **Gersh I, Hawkinson GE, Rathburn EN.** Tissue and vascular bubbles after decompression from high pressure atmospheres—correlation of specific gravity with morphological changes. *J Cell Comp Physiol* 24: 35–70, 1944.
10. **Grulke DC, Marsh PL, Hills BA.** Experimental air embolism: measurement of microbubbles using the coulter counter. *Br J Exp Pathol* 54: 684–691, 1973.
11. **Hagberg M, Ornhagen H.** Incidence and risk factors for symptoms of decompression sickness among male and female dive masters and instructors—a retrospective cohort study. *Undersea Hyperb Med* 30: 93–102, 2003.
12. **Hills BA, Butler BD.** Size distribution of intravascular air emboli produced by decompression. *Undersea Biomed Res* 8: 163–170, 1981.
13. **Hoiberg A.** Consequences of U.S. Navy diving mishaps: decompression sickness. *Undersea Biomed Res* 13: 383–394, 1986.
14. **Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, Brummer J, Rudolph V, Munzel T, Heitzer T, Meinertz T, Baldus S.** Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc Natl Acad Sci USA* 102: 431–436, 2005.
15. **Ljubkovic M, Dujic Z, Mollerlokken A, Bakovic S, Obad A, Breskovic T, Brubakk AO.** Venous and arterial bubbles at rest after no-decompression air dives. *Med Sci Sports Exerc* 43: 990–995, 2011.
16. **Ljubkovic M, Marinovic J, Obad A, Breskovic T, Gaustad SE, Dujic Z.** High incidence of venous and arterial gas emboli at rest after trimix diving without protocol violations. *J Appl Physiol* 109: 1670–1674, 2010.
17. **Madden D, Thom SR, Milovanova TN, Yang M, Bhopale VM, Ljubkovic M, Dujic Z.** Exercise before SCUBA diving ameliorates decompression-induced neutrophil activation. *Med Sci Sports Exerc* 46: 1928–1935, 2014.
18. **Madden D, Thom SR, Yang M, Bhopale VM, Milovanova TN, Ljubkovic M, Dujic Z.** High intensity cycling before SCUBA diving reduces post-decompression microparticle production and neutrophil activation. *Eur J Appl Physiol* 114: 1955–1961, 2014.
19. **Madden LA, Chrismas BC, Mellor D, Vince RV, Midgley AW, McNaughton LR, Atkins SL, Laden G.** Endothelial function and stress response after simulated dives to 18 msw breathing air or oxygen. *Aviat Space Environ Med* 81: 41–51, 2010.
20. **McLaughlin B, O'Malley K, Cotter TG.** Age-related differences in granulocyte chemotaxis and degranulation. *Clin Sci (Lond)* 70: 59–62, 1986.
21. **Obad A, Palada I, Valic Z, Ivancev V, Bakovic D, Wisloff U, Brubakk AO, Dujic Z.** The effects of acute oral antioxidants on diving-induced alterations in human cardiovascular function. *J Physiol* 578: 859–870, 2007.
22. **Ozyigit T, Egi SM, Denoble P, Balestra C, Aydin S, Vann RD, Marroni A.** Decompression illness medically reported by hyperbaric treatment facilities: cluster analysis of 1929 cases. *Aviat Space Environ Med* 81: 3–7, 2010.
23. **Pontier JM, Gempp E, Ignatescu M.** Blood platelet-derived microparticles release and bubble formation after an open-sea dive. *Appl Physiol Nutr Metab* 37: 1–5, 2012.
24. **St Leger Dowse M, Gunby A, Phil D, Moncad R, Fife C, Morsman J, Bryson P.** Problems associated with scuba diving are not evenly distributed across a menstrual cycle. *J Obstet Gynaecol* 26: 216–221, 2006.
25. **Sulaiman ZM, Pilmanis AA, O'Connor RB.** Relationship between age and susceptibility to altitude decompression sickness. *Aviat Space Environ Med* 68: 695–698, 1997.
26. **Thom SR, Bhopale VM, Yang M.** Neutrophils generate microparticles during exposure to inert gases due to cytoskeletal oxidative stress. *J Biol Chem* 289: 18831–18845, 2014.
27. **Thom SR, Milovanova TN, Bogush M, Bhopale VM, Yang M, Bushmann K, Pollock NW, Ljubkovic M, Denoble P, Dujic Z.** Microparticle production, neutrophil activation and intravascular bubbles following open-water SCUBA diving. *J Appl Physiol* 112: 1268–1278, 2012.
28. **Thom SR, Milovanova TN, Bogush M, Yang M, Bhopale VM, Pollock NW, Ljubkovic M, Denoble P, Madden D, Lozo M, Dujic Z.** Bubbles, microparticles and neutrophil activation: changes with exercise level and breathing gas during open-water SCUBA diving. *J Appl Physiol* 114: 1396–1405, 2013.
29. **Thom SR, Yang M, Bhopale VM, Huang S, Milovanova TN.** Microparticles initiate decompression-induced neutrophil activation and subsequent vascular injuries. *J Appl Physiol* 110: 340–351, 2011.
30. **Thom SR, Yang M, Bhopale VM, Milovanova TN, Bogush M, Buerk DG.** Intra-microparticle nitrogen dioxide is a bubble nucleation site leading to decompression-induced neutrophil activation and vascular injury. *J Appl Physiol* 114: 550–558, 2013.
31. **Tung JW, Parks DR, Moore WA, Herzenberg LA, Herzenberg LA.** New approaches to fluorescence compensation and visualization of FACS data. *Clin Immunol* 110: 277–283, 2004.
32. **Vince RV, McNaughton LR, Taylor L, Midgley AW, Laden G, Madden LA.** Release of VCAM-1 associated endothelial microparticles following simulated SCUBA dives. *Eur J Appl Physiol* 105: 507–513, 2009.
33. **Yang M, Barak OF, Dujic Z, Madden D, Bhopale VM, Bhullar J, Thom SR.** Ascorbic acid diminishes microparticle elevations and neutrophil activation following SCUBA diving. *Am J Physiol Regul Integr Comp Physiol*. First published June 17, 2015; doi: 10.1152/ajpregu.00155.2015.
34. **Yang M, Bhopale VM, Thom SR.** Ascorbic acid abrogates microparticle generation and vascular injuries associated with high-pressure exposure. *J Appl Physiol (1985)* 119: 77–82, 2015.
35. **Yang M, Kosterin P, Salzberg BM, Milovanova TN, Bhopale VM, Thom SR.** Microparticles generated by decompression stress cause central nervous system injury manifested as neurohypophiseal terminal action potential broadening. *J Appl Physiol* 115: 1481–1486, 2013.
36. **Yang M, Milovanova TN, Bogush M, Uzan G, Bhopale VM, Thom SR.** Microparticle enlargement and altered surface proteins after air decompression are associated with inflammatory vascular injuries. *J Appl Physiol* 112: 204–211, 2012.