ASSOCIATION OF MICROPARTICLES AND NEUTROPHIL ACTIVATION WITH DECOMPRESSION SICKNESS

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ABSTRACT

Decompression sickness (DCS) is a systemic disorder assumed due to gas bubbles, but additional factors are likely to play a role. Circulating microparticles, vesicular structures with diameters of 0.1 to 1.0 µm, have been implicated but data in human divers has been lacking. We hypothesized that the number of blood-borne annexin V-positive microparticles (MPs) and neutrophil activation assessed as surface myeloperoxidase (MPO) staining would differ between SCUBA divers suffering from DCS versus 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 hours 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 versus 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 non-steroidal anti-inflammatory agents or emergency oxygen treatment, and 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 MPs production and neutrophil activation exhibit strong associations with DCS.
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 to 1.0 µm, are elevated in association with simulated as well as bona fide underwater diving (17-19, 23, 27, 28, 32). Maneuvers which decrease the incidence of DCS also diminish MPs 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 SCUBA (Self-contained underwater breathing apparatus) 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 co-authors. 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 prior to 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 minutes.

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) hours. 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 prior to arrival at hospital: 42 divers received supplemental O₂ and 6 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 clinic for a late follow-up medical evaluation prior to 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 prior to and at one or more times between 15 minutes and 144 hours 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 versus laboratory) and matched for time when samples were obtained post-diving, 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, Inc., Omaha, NE). Samples were sent by express mail to the first author’s laboratory where all analyses were performed following published techniques within 48 hours 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 weeks 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 (Becton Dickinson, San Jose, CA), the majority were performed with an 8-color, triple laser MACSQuant (Miltenyi Biotec Corp., Auburn, CA) using manufacturers’ acquisition software. MPs were stained with annexin V and analyzed exactly as previously described (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 non-staining 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 myeloperoxidase (MPO) assayed as previously described (27, 28). MPO% indicates the fraction of all CD66b-positive cells exhibiting positive staining for MPO, MPO-median the geometric median fluorescence value.

**Statistical analysis**: Results are summarized as median, 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, Point Richmond, 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 prior to developing DCS; only 18% developed DCS after a single dive. Most repetitive dives were performed on the same day, a minority over two 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 either ‘enriched air nitrox’ (EAN) comprised of 32% oxygen (O2)/68% nitrogen (N2), or Tri-mix gas which, depending on the dive depth and an individual diver’s preference, contained variable amounts of O2 (7-22%), helium (5-35%) and N2 (40-60%). Tri-mix divers in both groups used rebreather apparatus with fixed, user-selected O2 partial pressures that typically involved 1.0 to 1.3 ATA O2 at depth and up to 1.4 to 1.64 ATA O2 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 minutes to four hours after diving (Figure 1), but changes resolved within 24 hours. Obtaining blood at two hours post-diving 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 meters, 20-29.9 meters, etc.), all two hour-post diving values for neutrophil activation and MPs elevations were statistically significantly different from pre-dive values but none were significantly different from each other (data not shown).

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

Table 3 displays data from control divers and those with DCS. The first column contains pre-
dive data from the control group. The second column shows post-dive results from control
divers, but this data set differs slightly from the pre-dive 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 post-dive 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 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 better match the
data set for DCS divers, where blood samples were obtained at a median time of 24 (11.8,
55.0; range 0.5 – 144) hours after diving. By compiling the control diver data set in the
manner described, neutrophil and MPs changes could be assessed at a median time of 24 (7.4, 96) hours post-diving (range 0.5 – 144) hours.

Data from DCS diver samples obtained on presentation to hospital are identified as “acute” in the third column of Table 3. Values for neutrophil MPO staining and MPs expressing each of the protein sub-types were statistically significantly different from the post-dive 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 pre-dive values from the control group (column 1), but few were significantly different from post-dive 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 prior to onset of DCS. The only values exhibiting statistically significant differences were time when blood samples were obtained post-symptom 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) hours versus 26 (12, 72) hours (p=0.002) for those having performed repetitive dives. The CD142-positive MPs count for those performing a single dive was 18 (11, 50) MPs/µl versus 67 (29, 186) (p=0.012) MPs/µl 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 O2 prior to DCS diver presentation might influence various blood test results. However, using either NSAID or O2 use as the binary, dependent variable for multiple logistic regression analysis, we found no significant impact for these interventions on total MPs, any MPs sub-type or neutrophil activation.

Logistic regression of log transformed data found a positive association between both the magnitude of neutrophil activation and numbers of MPs in all sub-groups 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. Factoring in 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. 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 while diminishing the OR for MPO-median (last row in Table 4). Adding gender, body mass index or including multiple MPs sub-types 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 MPs subtypes, and strong correlations among the number of each of the  
MPs subtypes. No correlation was found between DCS and maximum diving depth,  
performing repetitive versus 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 Figure 2. We  
interpret 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  
O₂ and ballast, or what are viewed as inert gases such as nitrogen (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 versus MPs numbers and neutrophil  
activation can be discerned with inbred laboratory animals (29).
With regard to divers with DCS, neutrophil and MPs 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 MPs sub-types. Post-decompression MPs 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).

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

There appears to be persistent neutrophil activation and elevations in some MPs sub-types 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 on-going 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.

Persistent elevations of some MPs sub-types weeks after DCS could be related to rates of MPs 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 feed-back 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 pre-dive characteristics such that attributes of MPs or sensitivity for neutrophil activation place them at greater risk for DCS.

The relationship between MPs 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 MPs elevations and among MPs sub-types (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 auto-activation (14).
The dynamics between MPs and neutrophil activation may also be responsible for the trends with elevations of MPs sub-types in repetitive divers (Table 2), and elevations of CD142-positive MPs in repetitive DCS divers versus 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 between 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 MPs enlargement occurs post-decompression, MPs may be a source of decompression-induced vascular bubbles which have reported diameters of 24 to 160 μm (9, 10, 12).

Finally, the data provide some insight into perceived risks of DCS. There is on-going 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 which 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 over 42 years of age (3, 25). Another study found no age influence on DCS, however, there were no divers over age 50 in the series (13). We found age to significantly influence 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 MPs 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 when compared to 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 hospital at later times, the blood-borne changes described here might be useful as biomarkers to aid in diagnosing DCS (Figure 3). Further work will be needed, however, because values from the DCS and control groups exhibit some overlap. Some interventions that inhibit MPs elevations and tissue injuries in mice also diminish MPs 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

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FIGURE LEGENDS

Figure 1. Blood-borne annexin V-positive MPs and neutrophil myeloperoxidase (MPO) staining in blood from control divers before and after a single dive. Blood was obtained prior to a dive and from 15 minutes to 24 hours post-dive. As discussed in Methods, 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 brackets below the figure) differed at each time point. The number of MPs/µl of plasma is shown in the top figure. MPO% indicates the fraction of CD66b-positive cells exhibiting myeloperoxidase fluorescence above the fluorescence-minus-one threshold, MPO-median indicates the geometric median fluorescence value for MPO on CD66b-positive cells. The figure indicates median value as the horizontal line within grey boxes, the boxes display 25th and 75th percentile, error bars show 10th and 90th percentile, with outliers shown as single dots. *p<0.05 versus pre-dive sample.

Figure 2. MPs and neutrophil MPO staining in blood from control divers performing repetitive dives. Data show pre-dive values for the same parameters as described in Figure 1 within one hour before diving and at two hours post-diving. Dives were conducted at approximately 24 hour intervals, thus the pre-dive values for days two, three and six are values in blood about 22 hours after diving on days one, two and five. All post-dive values are significantly different (p<0.001) from pre-dive values on the same day. The (*) indicates
p<0.001 versus day one and day two post-dive values based on two-way repeated measures ANOVA of log-transformed data.

Figure 3. Differences between DCS and control/asymptomatic divers’ blood-borne annexin V-positive MPs and neutrophil myeloperoxidase (MPO) staining in blood at 24 or more hours post-diving. The first bar in each graph are pre-dive data for control divers (n=185), the second bar are data from DCS divers obtained 24 or more hours after the dive that incited DCS (n=59) and the third bar are data for control divers obtained 24 hours after their first dive (n=27). The figure illustrates the persistent elevations of blood-borne changes in DCS divers versus control/asymptomatic divers. Panel labels are the same as described for Table 3. The figure indicates median value as the horizontal line within grey 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 first and last control diver panels (p<0.05 ANOVA) and there are no significant differences between pre- and post-control diver values.
### Table 1. Characteristics of study population.

<table>
<thead>
<tr>
<th></th>
<th>DCS Group (90)</th>
<th>Control Subjects (185)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34 (27, 42)</td>
<td>40 (37, 44)</td>
</tr>
<tr>
<td></td>
<td>Range: 16 - 73</td>
<td>Range: 21 - 72</td>
</tr>
<tr>
<td>Dive depth (meters)</td>
<td>22 (16, 34)</td>
<td>18 (18, 33)</td>
</tr>
<tr>
<td># Female</td>
<td>23 (34%)</td>
<td>30 (20 %)</td>
</tr>
<tr>
<td>Compressed air</td>
<td>69 (76.7%)</td>
<td>130 (70.3%)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>25.8 (22.7, 28.3)</td>
<td>26.9 (24.1, 28.7)</td>
</tr>
<tr>
<td>EAN</td>
<td>19 (21.1%)</td>
<td>28 (15.1%)</td>
</tr>
<tr>
<td>Tri-mix</td>
<td>2 (2.2%)</td>
<td>27 (14.6%)*</td>
</tr>
</tbody>
</table>

Age, diving depth, gender distribution and body mass index between the divers with DCS and control subjects were not statistically significantly different. The last three rows indicate the breathing gas used by the divers. *p<0.001.
Table 2: MPs sub-types in blood from repetitive diver control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Day 1 Pre- (20)</th>
<th>Day 1 Post-</th>
<th>Day 2 Pre- (11)</th>
<th>Day 2 Post-</th>
<th>Day 3 Pre- (11)</th>
<th>Day 3 Post</th>
<th>Day 6 Pre- (8)</th>
<th>Day 6 Post-</th>
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<tr>
<td>CD66</td>
<td>3.6 (2.2, 8.3)</td>
<td>93 (35, 230)</td>
<td>8.1 (4.2, 14.1)</td>
<td>228 (39, 532)</td>
<td>5.6 (3.7, 11.3)</td>
<td>200 (115, 425)</td>
<td>5.1 (4.0, 12.3)</td>
<td>128 (55, 206)</td>
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<tr>
<td>CD41</td>
<td>6.6 (3.6, 9.6)</td>
<td>77.2 (31.8, 229)</td>
<td>7.0 (4.3, 11.5)</td>
<td>58.3 (39.5, 333)</td>
<td>6.2 (3.7, 10.5)</td>
<td>193.7 (108, 424)</td>
<td>8.0 (4.1, 15.7)</td>
<td>195.9 (114, 390)</td>
</tr>
<tr>
<td>CD31</td>
<td>6.3 (2.8, 10.0)</td>
<td>103 (37, 233)</td>
<td>9.0 (3.5, 18.5)</td>
<td>233 (40, 365)</td>
<td>7.7 (5.5, 12.1)</td>
<td>220 (176, 290)</td>
<td>6.5 (4.7, 12.0)</td>
<td>226 (138, 357)</td>
</tr>
<tr>
<td>CD142</td>
<td>0.3 (0.06, 2.9)</td>
<td>19 (9, 39)</td>
<td>5.4 (2.1, 5.5)</td>
<td>22 (10, 40)</td>
<td>0.7 (0.5, 0.9)</td>
<td>86 (43, 127)</td>
<td>0.9 (0.5, 1.3)</td>
<td>50 (41, 189)</td>
</tr>
<tr>
<td>CD14</td>
<td>4.9 (3.9, 6.8)</td>
<td>11 (6, 27)</td>
<td>5.9 (3.3, 6.3)</td>
<td>17 (7, 72)</td>
<td>7.7 (6.2, 9.2)</td>
<td>122 (56, 157)</td>
<td>7.5 (6.3, 11.6)</td>
<td>79 (59, 299)</td>
</tr>
<tr>
<td>CD235</td>
<td>5.4 (3.7, 6.3)</td>
<td>15 (9, 72)</td>
<td>5.0 (4.1, 5.8)</td>
<td>12 (8, 34)</td>
<td>8.7 (5.7, 9.1)</td>
<td>98 (51, 315)</td>
<td>8.4 (6.4, 10.3)</td>
<td>134 (81, 307)</td>
</tr>
<tr>
<td>vWF</td>
<td>5.5 (3.6, 6.3)</td>
<td>21 (11, 73)</td>
<td>5.6 (4.8, 6.4)</td>
<td>10 (7.3, 217)</td>
<td>5.2 (3.2, 6.6)</td>
<td>80 (44, 118)</td>
<td>6.0 (4.7, 10.9)</td>
<td>52 (38, 184)</td>
</tr>
</tbody>
</table>

Data are median, 25th and 75th percentiles for repetitive control subject divers, (n) in the pre-dive columns indicates the number of individual diver samples in each pre/post-dive set. All post-dive values are statistically significantly greater than pre-dive values (p<0.001).

Although a trend appears suggesting that post-dive values for days three and six are greater than the post-dive values for day one and/or day two, the differences are not statistically significant based on two-way ANOVA on log-transformed data. All rows indicate the number/µl 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 (von Willebrand factor).
Table 3. MPs and neutrophil activation data on blood samples.

<table>
<thead>
<tr>
<th></th>
<th>Pre-dive Control subjects (185)</th>
<th>Post-dive Control Subjects (n=162)</th>
<th>DCS Divers-Acute (n=90)</th>
<th>DCS Divers-follow-up (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MPs/µl</td>
<td>1448 (946, 2165)</td>
<td>2391 (1258, 5123)*</td>
<td>2716 (945, 6920)*</td>
<td>2047 (779, 3682)</td>
</tr>
<tr>
<td>MPO %</td>
<td>2.6 (1.9, 3.5)</td>
<td>5.8 (2.7, 12.2)</td>
<td>11.5 (4.3, 24.4)*</td>
<td>10.3 (4.6, 18.3)*</td>
</tr>
<tr>
<td>MPO-median</td>
<td>12.2 (11.4, 14.0)</td>
<td>15.3 (13.5, 21.6)*</td>
<td>16.4 (13.8, 19.9)*</td>
<td>14.9 (12.8, 22.3)</td>
</tr>
<tr>
<td>MPs-CD66b/µl</td>
<td>4.8 (1.8, 38.2)</td>
<td>31.1 (15.9, 44.3)*</td>
<td>84.0 (22.8, 148.9)*†</td>
<td>55.7 (18.8, 128.9)*</td>
</tr>
<tr>
<td>MPs-CD41/µl</td>
<td>9.6 (4.5, 33.7)</td>
<td>45.0 (18.4, 87.5)*</td>
<td>110.4 (52.2, 400.9)*†</td>
<td>88.2 (19.5, 337.4)*</td>
</tr>
<tr>
<td>MPs-CD31/µl</td>
<td>13.1 (5.3, 64.2)</td>
<td>37.9 (21.0, 242.8)*</td>
<td>186.9 (70.3, 606.3)*†</td>
<td>137.6 (53.5, 298.3)* †</td>
</tr>
<tr>
<td>MPs-CD142/µl</td>
<td>1.4 (0.3, 16.9)</td>
<td>16.4 (2.1, 132.3)*</td>
<td>66.7 (24.5, 194.6)*†Δ</td>
<td>19.4 (8.1, 46.9)*</td>
</tr>
<tr>
<td>MPs-CD235/µl</td>
<td>6.4 (3.9, 15.8)</td>
<td>32.5 (9.0, 126.7)*</td>
<td>385.9 (56.6, 692.6)*†Δ</td>
<td>73.4 (34.1, 248.6)* †</td>
</tr>
<tr>
<td>MPs-vWF/µl</td>
<td>6.5 (4.0, 18.0)</td>
<td>38.2 (6.6, 148.3)*</td>
<td>248.9 (36.0, 558.0)*†Δ</td>
<td>58.1 (4.1, 272.0)*</td>
</tr>
<tr>
<td>MPs-CD14/µl</td>
<td>7.7 (4.1, 18.0)</td>
<td>25.5 (6.2, 81.8)*</td>
<td>271.2 (101.4, 765.0)*†</td>
<td>206.6 (26.5, 309.1)* †</td>
</tr>
</tbody>
</table>

Data are pre-dive values for control subjects (n=185, first column), post-dive control subjects (n=162, second column) included only four 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), (*) indicates p<0.001 versus pre-dive control subject values; †indicates p<0.05 vs post-dive control subject values, Δ=p<0.05 versus late follow-up DCS values. Rows are labeled as follows: MPO% indicates the fraction of CD66b-positive cells exhibiting myeloperoxidase fluorescence above the fluorescence-minus-one threshold (see Methods), MPO-median indicates the geometric median fluorescence value for MPO on
CD66b-positive cells. All other rows indicate the number/µl 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 (von Willebrand factor).
Table 4. Association of MPO on neutrophils and MPs with DCS.

<table>
<thead>
<tr>
<th>Adjustment</th>
<th>MPO%</th>
<th>MPO-median</th>
<th>CD66</th>
<th>CD41</th>
<th>CD31</th>
<th>CD142</th>
<th>CD14</th>
<th>CD235</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>1.6</td>
<td>(1.2, 2.0)</td>
<td>6.9</td>
<td>1.4</td>
<td>1.5</td>
<td>1.7</td>
<td>1.4</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>1.6</td>
<td>(1.2, 2.1)</td>
<td>7.2</td>
<td>1.7</td>
<td>1.7</td>
<td>2.1</td>
<td>1.9</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.6</td>
<td>(1.2, 2.1)</td>
<td>8.6</td>
<td>1.4</td>
<td>1.4</td>
<td>1.6</td>
<td>1.4</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time, depth, gas, repeat dive, age</td>
<td>1.6</td>
<td>(1.2, 2.1)</td>
<td>7.1</td>
<td>1.5</td>
<td>1.6</td>
<td>2.2</td>
<td>2.0</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.005</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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 MPs sub-types. Blood samples used for this analysis were the pre-treatment samples from 90 divers with DCS, and 190 blood samples from divers who were deemed to not have DCS. This included the latest blood sample obtained post-diving from each of the control group (n=185), as described in the Results text, and also the five 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 SCUBA 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 Trimix), if 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 caption of Table 3.
Table 5. Spearman Correlation Analysis.

<table>
<thead>
<tr>
<th></th>
<th>MPO%</th>
<th>MPO-median</th>
<th>CD66b</th>
<th>CD41</th>
<th>CD31</th>
<th>CD142</th>
<th>CD14</th>
<th>CD235</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCS</td>
<td>0.38</td>
<td>&lt;0.0001</td>
<td>0.19</td>
<td>&lt;0.01</td>
<td>0.28</td>
<td>&lt;0.0001</td>
<td>0.29</td>
<td>&lt;0.0001</td>
<td>0.41</td>
</tr>
<tr>
<td>MPO%</td>
<td>0.41</td>
<td>&lt;0.0001</td>
<td>0.26</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td></td>
<td>0.30</td>
<td>&lt;0.0001</td>
<td>0.25</td>
</tr>
<tr>
<td>MPO-median</td>
<td>0.23</td>
<td>&lt;0.01</td>
<td>0.14</td>
<td>&lt;0.0001</td>
<td>0.21</td>
<td>0.01</td>
<td>0.26</td>
<td>&lt;0.0001</td>
<td>0.44</td>
</tr>
<tr>
<td>CD66b</td>
<td></td>
<td></td>
<td>0.70</td>
<td>&lt;0.0001</td>
<td>0.69</td>
<td>0.78</td>
<td>0.76</td>
<td>&lt;0.0001</td>
<td>0.61</td>
</tr>
<tr>
<td>CD41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.80</td>
<td>&lt;0.0001</td>
<td>0.73</td>
<td>&lt;0.0001</td>
<td>0.78</td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.72</td>
<td>&lt;0.0001</td>
<td>0.70</td>
</tr>
<tr>
<td>CD142</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.73</td>
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<td>CD14</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD235</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

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.