

# Acute and potentially persistent effects of scuba diving on the blood transcriptome of experienced divers

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**Eftedal I, Ljubkovic M, Flatberg A, Jørgensen A, Brubakk AO, Dujic Z.** Acute and potentially persistent effects of scuba diving on the blood transcriptome of experienced divers. *Physiol Genomics* 45: 965–972, 2013. First published August 20, 2013; doi:10.1152/physiolgenomics.00164.2012.—During scuba diving, the circulatory system is stressed by an elevated partial pressure of oxygen while the diver is submerged and by decompression-induced gas bubbles on ascent to the surface. This diving-induced stress may trigger decompression illness, but the majority of dives are asymptomatic. In this study we have mapped divers' blood transcriptomes with the aim of identifying genes, biological pathways, and cell types perturbed by the physiological stress in asymptomatic scuba diving. Ten experienced divers abstained from diving for >2 wk before performing a 3-day series of daily dives to 18 m depth for 47 min while breathing compressed air. Blood for microarray analysis was collected before and immediately after the first and last dives, and 10 matched nondivers provided controls for pre-dive stationary transcriptomes. MetaCore GeneGo analysis of the pre-dive samples identified stationary upregulation of genes associated with apoptosis, inflammation, and innate immune responses in the divers, most significantly involving genes in the TNFR1 pathway of caspase-dependent apoptosis, HSP60/HSP70 signaling via TLR4, and NF- $\kappa$ B-mediated transcription. Diving caused pronounced shifts in transcription patterns characteristic of specific leukocytes, with downregulation of genes expressed by CD8<sup>+</sup> T lymphocytes and NK cells and upregulation of genes expressed by neutrophils, monocytes, and macrophages. Antioxidant genes were upregulated. Similar transient responses were observed after the first and last dive. The results indicate that sublethal oxidative stress elicits the myeloid innate immune system in scuba diving and that extensive diving may cause persistent change in pathways controlling apoptosis, inflammation, and innate immune responses.

acclimatization; apoptosis; diving; inflammation; innate immunity; oxidative stress

ACROSS THE GLOBE, MILLIONS of people are involved in diving for both professional and recreational purposes, enabled by the development of the self-contained underwater breathing apparatus (scuba) in the 1940s. Scuba allows divers to venture deeper and to spend more time under water than is possible during breath-hold diving, owing to the supply of breathing gas at a pressure equal to that of the surrounding water. The safety of scuba diving has been greatly advanced through development of better equipment and diving procedures. However, diving is

associated with the risk of decompression illness (DCI), a condition that comprises a plethora of symptoms ranging from minor pain to neurological manifestations, cardiac collapse, and death (49). Our knowledge of the processes that distinguish DCI development from normal physiological responses to the underwater hyperbaric environment is still incomplete.

Prior studies have shown that even in the absence of DCI, scuba diving is associated with acute reduction of vascular endothelial function (6). Scuba diving triggers proinflammatory development in blood, with increased expression of adhesion molecules, activation of coagulation, and elevated amounts of circulating micro-particles (22, 42, 45, 46). Such responses are triggered by oxidative stress and play important roles in upholding physiological homeostasis. However, when the oxidative stress becomes severe, redox homeostasis may be disrupted and the responses become maladaptive, possibly accelerating DCI development. The elevated partial pressure of oxygen (P<sub>O</sub><sub>2</sub>) at depth and the vascular gas bubbles formed during ascent to the surface (decompression) are major contributors to oxidative stress and proinflammatory development after diving. High P<sub>O</sub><sub>2</sub> accentuates oxidative stress and promotes inflammation via redox and oxygen-sensitive transcription factors (3, 14, 31, 41, 44). Inflammatory reactions to vascular gas bubbles have also been observed, and the severity of these reactions increases with increasingly stressful decompression (47, 51).

Whereas acute pathophysiological responses to diving in the circulatory system are documented, less is known about potential persistent changes over time in the circulatory system of healthy divers. It is an open question whether repetitive diving raises the risk of DCI via accumulated oxidative stress or provides protection through acclimatization (12, 37). There are indications that extensive asymptomatic diving alters the expression of inflammatory and antioxidant factors. In a study in which one or more daily scuba dives were performed over a period of 2 mo, increased blood levels of interleukin 8 (IL-8) and lipocalin, as well as a reduction in secretory leukocyte protease inhibitor, were observed at the end of the study (16). These results suggested a low-grade balanced activation of pro- and anti-inflammatory factors. Such activation may protect the circulatory homeostasis during diving; in line with this notion animal studies have indicated that repeated bouts of stressful hyperbaric exposure reduce the risk of morbidity after subsequent exposures (10, 34).

Transcriptomics is a useful tool for mapping of the biological basis of physiological responses. Using transcriptomics, we have previously shown that the rat aorta responds to simulated diving by activation of target genes for redox and oxygen-sensitive transcription factors such as hypoxia induc-

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ible factor 1- $\alpha$  and NF- $\kappa$ B (14). In human studies there are obvious limitations to the types of biological material that are available, but peripheral blood is accessible and suited for microarray-based transcriptome analysis. Blood interacts with all parts of the body, and previous studies have concluded that the blood transcriptome can be viewed as “an accessible window into the multiorgan transcriptome” (24, 33). Because diving activates the processes of inflammation and coagulation, blood transcriptomics is biologically relevant.

The aim of this study was to identify genes, biological pathways, and blood cell types that are perturbed by asymptomatic scuba diving. The study was performed in two steps: the first was to identify potentially persistent effects of extensive diving. For this, stationary blood transcriptomes from experienced divers were compared with those of matched nondivers. The second step was to identify acute effects of one single and several successive dives. For this, blood transcriptome changes were analyzed after one and three air scuba dives to a depth of 18 meters of seawater (msw). Circulating vascular bubbles, endothelial nitric oxide production, and vascular function after these dives have previously been published (32). To our knowledge this is the first report that describes the effects of diving on the expression of the human genome, and the results will enhance our understanding of the physiological responses to the underwater hyperbaric environment.

## METHODS

**Ethical approval.** All procedures were approved by the Norwegian Regional Committee for Medical and Health Research Ethics and the Ethics Committee of the University of Split School of Medicine. They were conducted in compliance with the Declaration of Helsinki ethical principles for human experimentation. Participation was based on individual informed consent. All subjects held a valid medical certificate for diving and were provided with Divers Alert Network Europe divers insurance for 1 yr from the beginning of the study.

**Study and control groups.** The study group consisted of 10 certified and experienced male divers, with diving experience ranging from 4 to 25 yr, a mean age of  $40.3 \pm 2.6$  yr, height of  $1.8 \pm 0.1$  m, and a body weight of  $93.6 \pm 11.1$  kg. An equal sized group of male nondivers matched for age, weight, and general physical fitness were included as a control group for gene expression. All participants were healthy nonsmokers. At the start of the study, the divers had abstained from diving for a minimum of 2 wk. All participants had abstained from caffeine for  $>12$  h and from strenuous exercise for  $>48$  h before blood collection. Prior to each dive, the participants had a light breakfast consisting of low-fat food, but otherwise their diet was not controlled during the study. The number of individuals in the study groups is in compliance with recommended sample sizes for optimal strength in microarray gene expression analysis when two groups are compared (39).

**Diving.** The diving was performed at a site near Split in Croatia, with surface and bottom water temperatures of  $20 \pm 3^\circ\text{C}$  and  $16 \pm 1^\circ\text{C}$ , respectively. The site was reached by a 10 min boat ride from the on-shore diving facility. The participating divers followed the same routine, with single scuba dives while breathing compressed air on 3 consecutive days. The divers descended to a depth of 18 msw, stayed at that depth for 47 min while performing light physical exercise, and then ascended to the surface at a linear rate of 9 m per min. All dives were performed at the same time of day to minimize the effects of circadian variation on gene expression patterns.

**Blood samples.** Blood samples for analysis were taken from all the divers and matched nondiving controls. Venous blood (2.5 ml) was drawn into PAXgene tubes (PreAnalytix, Hombrechtikon, Switzerland) before dives and again immediately after the return to shore by

boat. All the samples were collected into a single batch of tubes by the same technician to minimize technical variation in the microarray data induced by sample handling. Samples were kept frozen during transport to the laboratory facility and were stored at  $-80^\circ\text{C}$  prior to RNA extraction.

**RNA preparation.** RNA from full blood was isolated using the PAXgene Blood RNA kit version 2 (PreAnalytix). The RNA concentration and quality were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA integrity number values of samples used for cRNA amplification ranged from 7.4 to 9.4.

**Microarray analysis.** The Illumina TotalPrep RNA amplification Kit (Ambion, Austin, TX) was used to amplify RNA for hybridization. The first-strand cDNA was synthesized by reverse transcription. Following second-strand cDNA replication and purification and in vitro transcription of cRNA, the gene expression profiles were measured using Illumina human HT-12 v4 Expression BeadChips (Illumina, San Diego, CA), which provides genome-wide expression analysis of  $>47,000$  transcripts. One prediving sample (diver no. 1) and one nondiving control sample had inadequate cDNA concentrations and were excluded from microarray analysis.

**Identification of differentially expressed transcripts, pathway enrichment analysis, and the predicted involvement of cell types.** The Illumina Genome Studio program, version 1.7.0, was used for background subtraction, and microarray data were exported into the R software for statistical computing (<http://www.r-project.org/>) using the lumi Bioconductor package version 1.1.0 (13). All signal intensity values  $<0$  were set to 0 prior to  $\log_2$  transformation. Intersample differences were normalized by quantile transformation. Probes with a detection  $P$  value outside the 0.01 threshold in more than half of the samples were excluded from further analysis. An exploratory analysis was then performed, with multilevel partial least squares (PLS) regression (48). The purpose of this analysis was to identify sensible choices for blocking structure in the subsequent differential testing and to obtain a global visualization of sample relations. The Rlimma package version 3.14.4 was used to evaluate the significance of differences in change of expression between the nondivers and experienced divers with a moderated  $t$ -statistic (43). We estimated the difference in gene expression caused by diving by a moderated paired  $t$ -test using each individual dive as blocking structure. In all tests, significance was decided by requiring a false discovery adjusted  $P$  value of  $<0.05$ . We generated the acute gene list by requiring probes to be significantly differentially expressed in diving and not present in the test between divers and nondivers. Conversely, a stationary gene list was generated from probes that were significant in the diver-versus-nondiver contrast and that were not differentially expressed after diving. Additional functional evaluation of gene lists was performed with MetaCore GeneGo software from Thomson Reuters, release 6.13 (New York, NY). Pathway enrichment analysis was performed on the gene lists and ranked by a hypergeometric distribution where the  $P$  value represents the probability of a particular pathway occurring by chance in the gene list compared with a background, which in this analysis was chosen to be all probes present on the Illumina HT12 chip. Analysis of tissue-specific coexpression patterns in the acute gene list was done using the TopFunn module of the TopGene Suite software (Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) (9). This analysis predicts the involvement of tissues and cell types based on the observed differential gene expression patterns.

**Database submission of microarray data.** The microarray data were submitted to the EMBL-EBI ArrayExpress repository (<http://www.ebi.ac.uk/arrayexpress/>) according to MIAME standards. The ArrayExpress accession code is E-MTAB-1179.

## RESULTS

Data for differentially expressed transcripts and their associated biological pathways and blood cell types are provided in the online data supplement.<sup>1</sup> Decompression-induced vascular bubbles, vascular function, and blood nitrite concentrations in these dives were previously reported by Marinovic et al. (32). In brief, both venous and arterial bubbles were observed after decompression. There was an insignificant trend toward reduced vascular function after each dive, but there were no differences in bubble loads or vascular function between successive dives. There were no changes in blood nitrite after diving. No symptoms of decompression illness were reported.

*Differential expression in the stationary blood transcriptomes of experienced divers.* Stationary blood transcriptomes from experienced divers were compared with those of nondiv-

ers to identify potentially persistent effects of prior diving. This revealed differential expression of 2,531 transcripts with 1,806 transcripts upregulated and 724 transcripts downregulated in the divers. A heat map of the most significant differentially expressed genes is shown in Fig. 1, and the top biological pathways associated with differential gene expression are shown in Table 1. Nineteen genes in the tumor necrosis factor receptor superfamily member 1A (TNFR1) apoptotic signaling pathway were differentially expressed in the divers (Fig. 2 and online data supplement), including those for the proinflammatory cytokine TNF- $\alpha$ , TNFR1-associated death domain protein (*TRADD*), and Fas-associated death domain protein (*FADD*), all of which were upregulated. In response to TNF- $\alpha$  signaling via TNFR1, TRADD recruits FADD into a complex that further activates the apoptotic caspase cascade and initiates transcription via activator protein 1 (AP-1) and NF- $\kappa$ B (4). Three initiator caspases (*CASP2*, 8, and 9) as well as *AP-1*, NF- $\kappa$ B genes *NF- $\kappa$ B2* and *RelB* and the NF- $\kappa$ B modulators

<sup>1</sup> The online version of this article contains supplemental material.

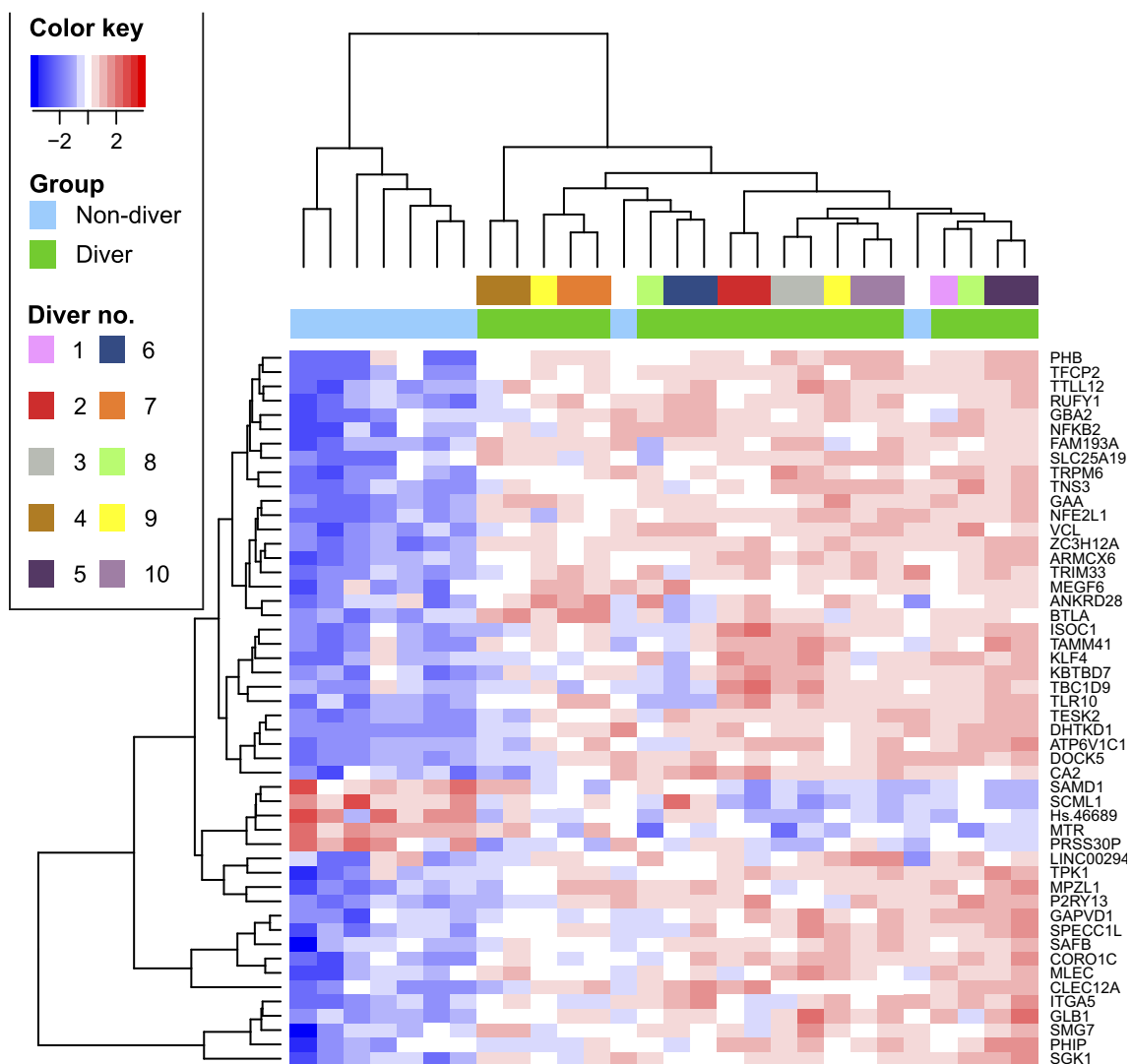


Fig. 1. Heat map visualization of mRNA levels for the top 50 differentially expressed genes in the stationary blood transcriptomes of divers ( $n = 10$ ) compared with those of nondivers ( $n = 9$ ). The columns show individual samples, whereas the rows show genes. Pre-dive data from the first and last study dive are included for nine divers (divers 2–10), whereas data from the last dive only is shown for diver 1. Data from both study dives cluster adjacently for most individual divers (7 out of 9), indicating that gene expression levels were similar prior to the 1st and last dive.



Table 1. The most significant biological pathway changes in the divers' stationary blood transcriptomes

Pathway	Genes (n) in		P Value
	Data	Reference	
Apoptosis and survival: TNFR1 signaling pathway	19	43	7.413e-13
Immune response: CD28 signaling	20	56	2.127e-11
Translation: regulation of EIF4F activity	19	53	6.309e-11
Development: Flt3 signaling	17	44	1.640e-10
Immune response: HSP60 and HSP70/TLR signaling pathway	18	54	8.036e-10
Apoptosis and survival: anti-apoptotic action of gastrin	16	43	1.104e-9

The top 50 pathways determined by MetaCore GeneGo pathway analysis are shown in the online data supplement.

*IKK-γ* and *I-κB* were upregulated, as were the NF-κB target genes *IL-1β*, *IL-8*, and *CD86*. The expression of the receptor *TNFR1*, which transmits TNF-α signaling across the cellular membrane, was insignificantly increased in the divers prior to the study dives.

In the CD28 signaling pathway, genes for transcription factors AP-1 and nuclear factor of activated T cells (NFATC1 and NFATC2) were upregulated. These transcription factors function synergistically to balance immune reactions in activated lymphocytes (30). Genes for the small Rho GTPases RhoA, Rac1, and CDC42 were also upregulated. These GTPases are involved in a multitude of intracellular signaling pathways, explaining the involvement of regulation of EIF4F activity, Flt3 signaling, anti-apoptotic action of gastrin, and VEGF signaling in Table 1 (8). Most pertinently, small GTPases regulate and coordinate innate immune responses and induction of NF-κB-mediated transcription (5). Other upregulated genes included those for toll-like

receptor 4 (*TLR4*) and its associated lymphocyte antigen 96 (*MD-2*) (Fig. 2). The heat shock proteins HSP60 and HSP70 activate inflammatory signaling via TLR4 and small GTPases and participate in regulation of major histocompatibility complex MHC I and II function (25); the *HSP60*, *HSP70*, and *HLA-B* (MHC I), *HLA-DPB1*, and *HLA-DQAI* (MHC II) genes were all upregulated.

*Acute blood transcriptome changes after scuba diving.* Comparing the divers' pre- and postdive blood transcriptomes in the first and last study dives identified the genes, cell types, and biological pathways that were acutely affected by scuba diving. The numbers of differentially expressed transcripts were not the same in both dives; 395 transcripts were affected in the first and 1,537 in the last dive, but they gave similar results in for the most significantly associated pathways in the MetaCore GeneGo analysis. Altogether, there were 1,570 differentially expressed transcripts, of which 630 were upregulated and 940 were downregulated after diving. Data from both dives were merged for further analysis.

Analysis of gene coexpression after diving revealed a highly significant shift in the expression patterns associated with specific leukocyte subtypes, with decreased expression of genes characteristic of cytotoxic T lymphocytes (CD8+ T cells) and natural killer cells (NK cells) and increased expression of genes from cells of myeloid origin, most significantly neutrophils, macrophages, and classical monocytes (Table 2 and online data supplement).

The top three biological pathways associated with the acute gene expression changes after diving are shown in Table 3. The most significantly affected pathway was that of granzyme B apoptotic signaling. The initiating factors in this pathway, granzyme B (*GZMB*) and perforin (*PRF1*), were both downregulated after diving, and so were the genes for the pore-

Fig. 2. Acute and persistent effects of scuba diving on blood gene expression in the tumor necrosis factor receptor superfamily member 1A (TNFR1) and toll-like receptor 4 (TLR4)-mediated signaling pathways. Vertical arrows adjacent to protein symbols mark differential expression in the divers' pre-dive stationary transcriptomes. Stars mark acute changes in expression after scuba diving. The map is based on MetaCore GeneGo pathway analysis of microarray data.

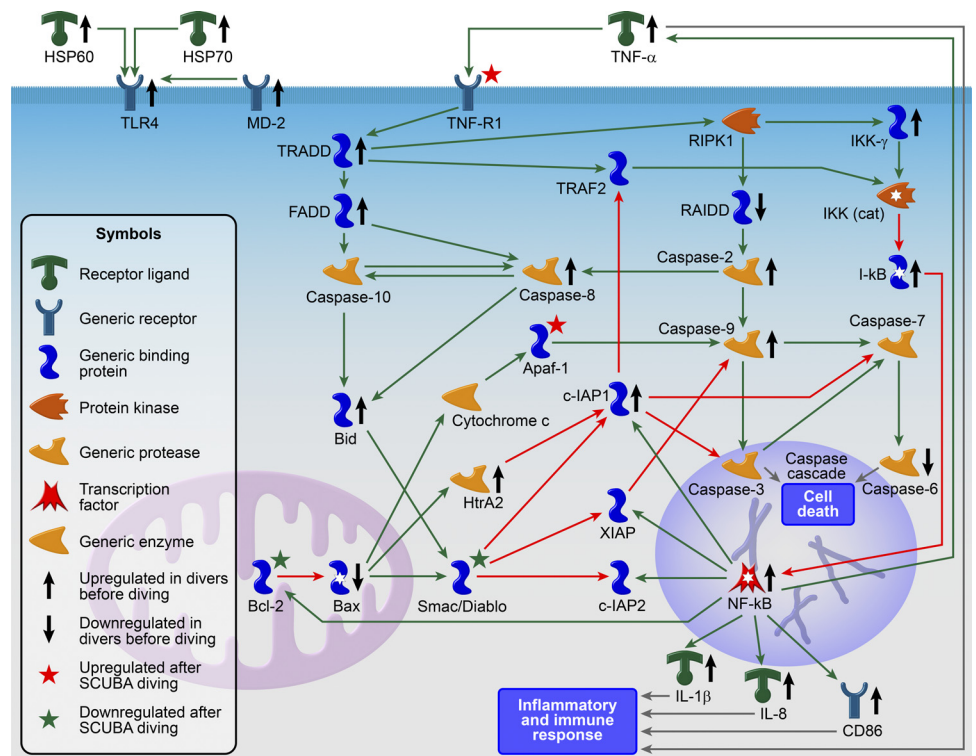


Table 2. Selected blood cell associations for genes that were down- and upregulated after scuba diving

Cell Types	Genes (n) in		P Value
	Data	Reference	
<i>Downregulation</i>			
Activated T cells, CD8+ CD45.1+	65	387	3.514e-18
NK cells, NK1.1+ TCRb- Ly49H+	61	370	1.747e-16
NK cells, NK1.1+ CD3- Ly49C/I+	62	385	2.913e-16
<i>Upregulation</i>			
Neutrophils, CD11b+ Ly6-G+	54	418	5.072e-20
Macrophages, CD45+ F4/80+ CD11b+	38	402	6.142e-9
Classical monocytes HMCII-, CD115+ B220- CD43+ Ly6C+	36	408	1.737e-7

The 20 most significant cell type associations and the differentially expressed genes in their signature overlaps are shown in the online data supplement.

forming protein granulysin (*GNLY*) and granzymes A and H (*GZMA* and *GZMH*) (Fig. 3). The median expression levels for these genes were similar before the first and third dives, indicating that the change was transient with transcription returning to pre-dive levels between dives. Postdive expression levels were also similar, indicating that the divers responded equally in both dives. Granzymes, granulysin, and perforin constitute the cytotoxic entity of CD8+ T and NK cells. Among the genes that were most downregulated after diving were a number of CD8+ T and NK cell receptors: GPR56, CD160, killer cell immunoglobulin-like receptors (KIR2DL1, 2DL2, 2DL4, and KIR3DL1), and killer cell lectin-like receptors (KLRC3, D1, and F1) (2, 27, 40).

In the granulocyte development pathway, there was upregulation of the genes for granulocyte colony stimulating receptor CSF3R and for the transcription factors C/EBP-alpha, PU.1, RAR-α, STAT3, and STAT5, which orchestrate the development of neutrophils and monocytes during hematopoietic stem cell differentiation (18, 21). Conversely, lymphocyte growth and activity are stimulated by transcriptional activation via calmodulin and catalytic calcineurin in the T cell receptor signaling pathway (11), and both calmodulin and calcineurin were downregulated after diving.

As shown in Fig. 2, more genes in the TNFR1 apoptotic pathway were differentially expressed after diving, in addition to those already affected in the divers' stationary transcriptomes. The *TNFRSF1A* gene was upregulated. Its product

Table 3. The most significant biological pathway associations for the divers' blood transcriptome changes after scuba diving

Pathway	Genes (n) in		P Value
	Data	Reference	
Apoptosis and survival: granzyme B signaling	11	32	3.110e-8
Development: transcription regulation of granulocyte development	9	32	3.901e-6
Immune response: T cell receptor signaling pathway	11	53	8.393e-6

The top 50 pathways determined by MetaCore GeneGo pathway analysis are shown in the online data supplement.

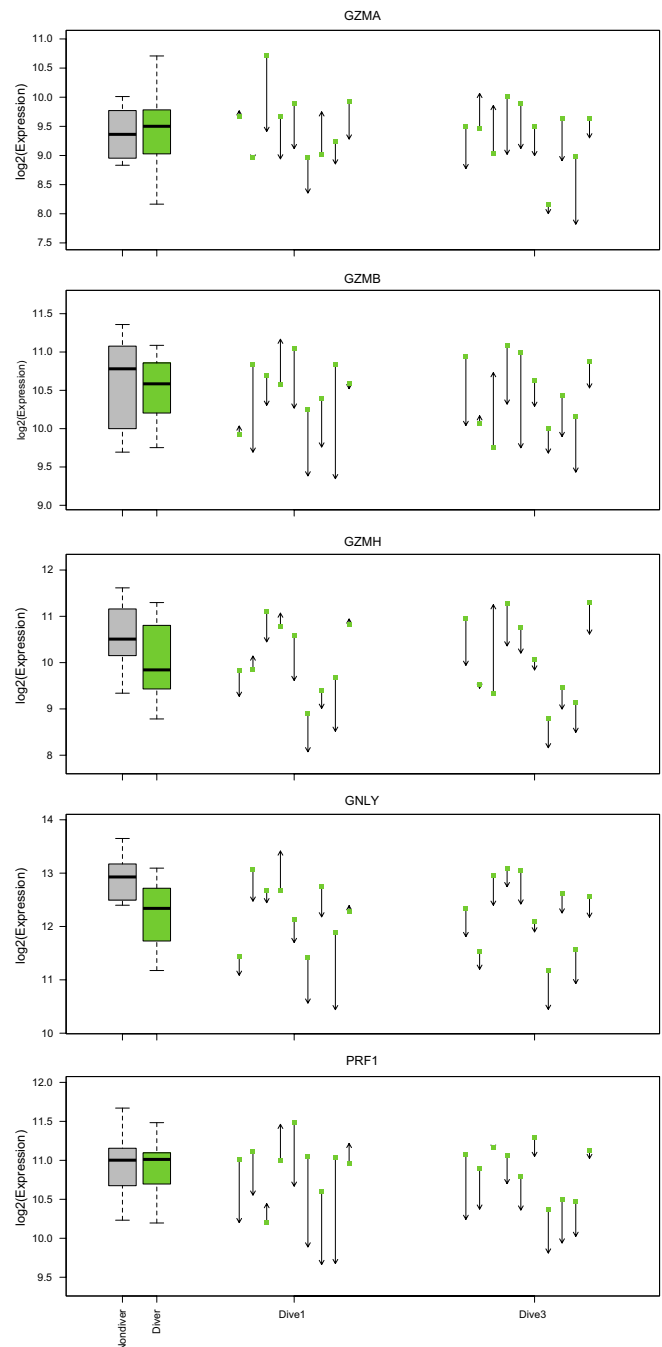


Fig. 3. Acute effects of diving on the expression of granzyme A (*GZMA*), B (*GZMB*), and H (*GZMH*), granulysin (*GNLY*), and perforin (*PRF1*). Nondiver and diver pre-dive stationary expression is shown as box plots; band, bars, and whiskers indicate median values, lower and upper quartiles, and min/max values, respectively. The arrows illustrate gene expression changes in the 1st and last study dive: the square ends are pre-dive expression levels, whereas arrowheads indicate the direction and magnitude of change after diving. Due to exclusion of 1 pre-dive sample (diver 1), only 9 arrows are shown for the 1st dive. The order of individual divers is the same in all panels.

TNFR1 transmits TNF-α signals to the caspase/Bcl-2-dependent apoptotic pathway; in this pathway *APAF-1* was upregulated and *BCL-2* and *DIABLO* were downregulated. Also, genes coding for the antioxidant enzymes mitochondrial superoxide dismutase, glutathione peroxidase 4, and thioredoxin (*SOD2*, *GPX4*, *TXN1*) were upregulated after diving.

## DISCUSSION

This study identifies differences in the pre-dive stationary blood transcriptomes of experienced divers compared with nondivers and shows that asymptomatic scuba diving causes further acute changes. The dive profile used is common in recreational diving; thus the results may be relevant to a large number of people. However, when interpreting the results one must consider that the measurements are limited to the transcriptional level and the potential involvement of biological pathways and cell types is predicted from the microarray data.

*Persistent changes in pathways of apoptosis, inflammation, and immune responses in the blood transcriptomes of experienced divers.* Genes associated with apoptosis, inflammation, and innate immune responses were differentially expressed in the divers' stationary blood transcriptomes (Table 1). Most of the significant associations were caused by upregulated transcription, as observed for the cytokines and heat shock proteins TNF- $\alpha$ , IL-1 $\beta$ , IL-8, HSP60, and HSP70. Increased serum levels of IL-8 has previously been reported after extensive scuba diving (16). The intracellular signaling pathways activated by TNF- $\alpha$  and HSP60/HSP70 via TNFR1 and toll-like receptors converge into NF- $\kappa$ B-mediated transcription, which is essential in cellular stress responses (23). De novo transcription is not required: NF- $\kappa$ B is rapidly activated when its inhibitor I- $\kappa$ B is degraded by IKK. However, in the divers there was stationary upregulation of NF- $\kappa$ B subunits NF- $\kappa$ B2 and RelB, as well as of I- $\kappa$ B, the IKK-subunit IKK- $\gamma$ , and the small GTPases RhoA, Rac1, and CDC42. The mRNAs for central factors that modulate transcription of NF- $\kappa$ B target genes were thus present in higher amounts in the divers' blood, which could enhance the cellular capacity for responses to NF- $\kappa$ B-activating signals. Similarly, the differential expression of initiator caspases and other genes in the TNFR1 signaling pathways could facilitate rapid induction of apoptosis. Thus, the most significantly affected genes and pathways in the divers' stationary transcriptomes indicate a cellular state of sustained alertness toward exogenous stress. This may be a persistent effect of extensive prior diving. However, we have no associated physiological markers that distinguish the divers from the nondiving controls, and it is unknown whether these changes affect the risk of DCI in later diving.

*Blood transcriptome changes after scuba diving indicate sublethal oxidative stress, with suppression of lymphocyte activity and activation of the myeloid innate immune system.* A pronounced shift in transcription indicates that the activity of CD8+ T cells and NK cells fell, whereas the activity of neutrophils, monocytes, and macrophages increased as a result of scuba diving (Table 2). The most pronounced effect was the downregulation of genes involved in granule-dependent lymphocyte cytotoxicity (*GZMA*, *GZMB*, *GZMH*, *GZML*, and *PRFI*). In these processes, proteolytic granzymes and granzysin delivered from cytotoxic cells via granule exocytosis cause activation of caspase-dependent apoptosis in stressed or pathogenic target cells (17, 28). Together with the observed downregulation of CD8+ T and NK cell receptors, our results suggest that diving impeded lymphocyte function. T lymphocytes and NK cells are central players in both innate and adaptive immunity (50), but they may lose their activity and enter apoptosis in response to reactive oxygen species (ROS) (1, 35). In saturation divers, increased NK cell cytotoxicity has

been observed after 2 days of exposure to heliox at 240 msw with a PO<sub>2</sub> of 35–70 kPa (26). However, a single diver who developed high-pressure nervous syndrome primarily contributed this increase. Recent work has demonstrated that lymphocytes may not be essential in balanced physiological responses to oxidative stress; healthy cells exposed to low-level ROS selectively attract classical monocytes but not NK cells or other lymphocytes (20). Upon attraction and activation, monocytes differentiate into macrophages and dendritic cells, which in turn mediate innate immune responses. Neutrophils are also activated by oxidative stress (29). They are by far the most abundant type of human leukocytes, and their function is to instigate rapid innate responses of host defense against pathogens or exogenous stress. It is interesting to note that in our study, calmodulin and calcineurin, which activate lymphocyte transcription factors, were downregulated after diving, whereas transcription factors controlling neutrophil and monocyte/macrophage development were upregulated.

Diving caused differential expression in the Bcl-2 apoptotic pathway, including downregulation of *BCL-2*, in agreement with previous reports showing the involvement of the Bcl-2 protein family members in induction of apoptosis by hyperoxia and ROS (7, 19). Upregulation of genes for antioxidant enzymes superoxide dismutase, glutathione peroxidase, and thioredoxin (*SOD2*, *GPX4*, and *TXN1*) was also observed. As part of the antioxidant defense system, these enzymes act in concert to catalyze the conversion of ROS into oxygen and water (36, 38). Taken together, our observations suggest that the acute transcriptome changes after asymptomatic scuba diving were caused by augmented oxidative stress. Both elevated PO<sub>2</sub> (59 kPa in the bottom phase) and vascular bubbles [median venous bubble grade of 3 on the Eftedal/Brubakk scale (15)] could have led to increased exogenous ROS production and activation of innate immunity, and we cannot distinguish between the impacts of hyperoxia and bubbles in the present study.

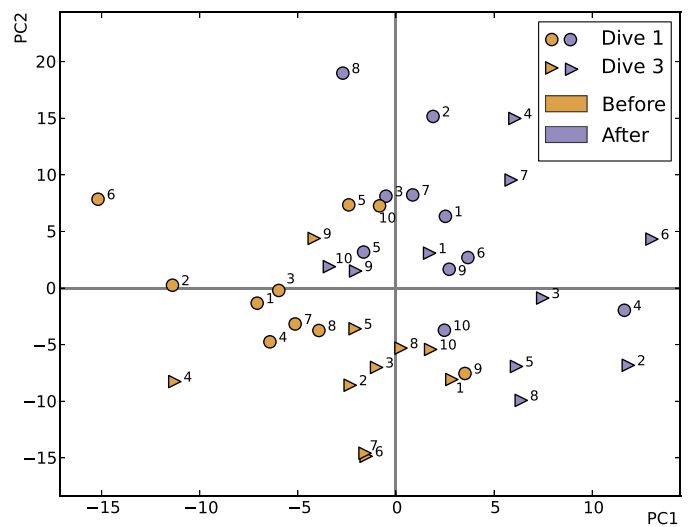


Fig. 4. Multilevel partial least square (PLS) cores of within diver variation in the 2 first principal components of the microarray data, showing the separation in measurements before and after diving in the 1st and last study dive. Numbers indicate data from individual divers. A distinct common direction is seen for the data obtained before and after diving for both dive series.



*Similar transcriptome responses after one and three successive scuba dives.* The two series of dives (the first and last in the study) affected similar biological pathways. As shown in Fig. 3, genes involved in initiation of granule-mediated apoptosis were equally affected in both dives, with expression levels returning to pre-dive values in the interval between the dives. Also, the heat map in Fig. 1 shows that the top 50 differentially expressed genes in the divers' stationary transcriptomes had similar expression levels before the first and last study dive for most divers. Multilevel PLS dimension reduction of the microarray dataset (Fig. 4) shows a dominant effect of the act of diving (before and after) over the contributions of the particular dive (*dive 1* and *dive 3*). We conclude that both dives produced comparable transient responses, consistent with the observations of Marinovic et al. (32) that vascular bubble grades, vascular function, and blood nitrite were similar in the first and third dives. However, three dives may be too few to induce significant acclimatization. Also, these analyses were conducted on blood from experienced divers whose transcriptomes were already different from those of the nondiving controls. Whether the responses of naïve divers would be the same is unknown.

### Limitations

The study design and the analyses are both potential sources of errors. To identify the acute effects of diving we compared pre- and postdive data from the same group, so the results are unlikely to be affected by unacknowledged variance. However, to identify potentially persistent effects of diving we compared transcriptomes from different diving and nondiving individuals. The matching of divers and nondivers was carefully arranged, and the microarray data were checked prior to analysis (see METHODS), but we cannot rule out the possibility that the dietary status or other unacknowledged differences may have confounded the outcome of the analyses. However, in light of the highly significant association of the divers' stationary transcriptomes with signaling pathways activated by exogenous stress, we consider it likely that the results reflect genuine biology. As leukocytes are the nucleated components of blood, they are the primary source of RNA in this study. We therefore believe that the results reflect the effects of diving on leukocyte function. This is supported by the observation that the most significant pathways were associated with defined leukocyte subsets. We note that platelets, microparticles, and immature erythrocytes also contain RNA, and because platelets and microparticles are activated by scuba diving it is possible that they have contributed to the results (42, 45).

### Conclusions

In conclusion, asymptomatic scuba diving significantly affects the blood transcriptome. The acute effects identified in this study are characteristic of cellular responses to sublethal oxidative stress and indicate that scuba diving elicits the myeloid innate immune system by activating neutrophils, monocytes, and macrophages while suppressing lymphocyte activity. Persistent effects of diving were implied from stationary gene expression changes in pathways of apoptosis, inflammation, and innate immune responses in experienced divers. These persistent effects are novel findings, possibly indicating

acclimatization to augmented oxidative stress. However, their potential influence on DCI risk remains to be determined.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

### AUTHOR CONTRIBUTIONS

Author contributions: I.E., M.L., A.O.B., and Z.D. conception and design of research; I.E. and M.L. performed experiments; I.E. and A.F. analyzed data; I.E., A.F., A.J., and Z.D. interpreted results of experiments; I.E. prepared figures; I.E. and A.J. drafted manuscript; I.E., M.L., A.J., A.O.B., and Z.D. edited and revised manuscript; I.E., M.L., A.F., A.J., A.O.B., and Z.D. approved final version of manuscript.

### REFERENCES

- Allan IM, Lunec J, Salmon M, Bacon PA. Reactive oxygen species selectively deplete normal T lymphocytes via a hydroxyl radical dependent mechanism. *Scand J Immunol* 26: 47–53, 1987.
- Anumanthan A, Bensussan A, Boumsell L, Christ AD, Blumberg RS, Voss SD, Patel AT, Robertson MJ, Nadler LM, Freeman GJ. Cloning of BY55, a novel Ig superfamily member expressed on NK Cells, CTL, and intestinal intraepithelial lymphocytes. *J Immunol* 161: 2780–2790, 1998.
- Barak M, Katz Y. Microbubbles: pathophysiology and clinical implications. *Chest* 128: 2918–2932, 2005.
- Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11: 372–377, 2001.
- Bokoch GM. Regulation of innate immunity by Rho GTPases. *Trends Cell Biol* 15: 163–171, 2005.
- Brubakk AO, Duplancic D, Valic Z, Palada I, Obad A, Bakovic D, Wisloff U, Djucic Z. A single air dive reduces arterial endothelial function in man. *J Physiol* 566: 901–906, 2005.
- Budinger GRS, Tso M, McClintock DS, Dean DA, Sznajder JI, Chandel NS. Hyperoxia-induced apoptosis does not require mitochondrial reactive oxygen species and is regulated by Bcl-2 proteins. *J Biol Chem* 277: 15654–15660, 2002.
- Bustelo XR, Sauzeau V, Berenjano IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* 29: 356–370, 2007.
- Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucl Acids Res* 37: W305–W311, 2009.
- Chen Y, Montcalm-Smith E, Schlaerth C, Auken C, McCarron RM. Acclimation to decompression: stress and cytokine gene expression in rat lungs. *J Appl Physiol* 111: 1007–1013, 2011.
- Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 357: 695–697, 1992.
- Doolette DJ. Health outcome following multi-day occupational air diving. *Undersea Hyperb Med* 30: 127–134, 2003.
- Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 24: 1547–1548, 2008.
- Eftedal I, Jørgensen A, Røsbjerg R, Flatberg A, Brubakk AO. Early genetic responses in rat vascular tissue after simulated diving. *Physiol Genomics* 44: 1201–1207, 2012.

15. Eftedal O, Brubakk AO. Agreement between trained and untrained observers in grading intravascular bubble signals in ultrasonic images. *Undersea Hyperb Med* 24: 293–299, 1997.
16. Ersson A, Walles M, Ohlsson K, Ekholm A. Chronic hyperbaric exposure activates proinflammatory mediators in humans. *J Appl Physiol* 92: 2375–2380, 2002.
17. Fellows E, Gil-Parrado S, Jenne DE, Kurschus FC. Natural killer cell-derived human granzyme H induces an alternative, caspase-independent cell-death program. *Blood* 110: 544–552, 2007.
18. Friedman AD. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* 21: 3377–3390, 2002.
19. Ganguly BJ, Tomomura N, Benson RM, Osborne BA, Granowitz EV. Hyperbaric oxygen enhances apoptosis in hematopoietic cells. *Apoptosis* 7: 499–510, 2002.
20. Geiger-Maor A, Levi I, Even-Ram S, Smith Y, Bowdish DM, Nussbaum G, Rachmilewitz J. Cells exposed to sublethal oxidative stress selectively attract monocytes/macrophages via scavenger receptors and MyD88-mediated signaling. *J Immunol* 188: 1234–1244, 2012.
21. Giron-Michel J, Caignard A, Fogli M, Brouty-Boye D, Briard D, van Dijk M, Meazza R, Ferrini S, Lebousse-Kerdiles C, Clay D, Bompais H, Chouaib S, Peault B, Azzarone B. Differential STAT3, STAT5, and NF-kappaB activation in human hematopoietic progenitors by endogenous interleukin-15: implications in the expression of functional molecules. *Blood* 102: 109–117, 2003.
22. Glavas D, Markotic A, Valic Z, Kovacic N, Palada I, Martinic R, Breskovic T, Bakovic D, Brubakk AO, Dujic Z. Expression of endothelial selectin ligands on human leukocytes following dive. *Exp Biol Med (Maywood)* 233: 1181–1188, 2008.
23. Hoffmann A, Baltimore D. Circuitry of nuclear factor  $\kappa$ B signaling. *Immunol Rev* 210: 171–186, 2006.
24. Kohane IS, Valtchinov VI. Quantifying the white blood cell transcriptome as an accessible window to the multiorgan transcriptome. *Bioinformatics* 28: 538–545, 2012.
25. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 8: 279–289, 2008.
26. Krog J, Tonnesen EK, Jepsen CF, Parner E, Segadal K, Hope A, Ulvik RJ, Hokland ME. Natural killer cells as biomarkers of hyperbaric stress during a dry heliox saturation dive. *Aviat Space Environ Med* 81: 467–474, 2010.
27. Lanier LL. NK cell receptors. *Ann Rev Immunol* 16: 359–393, 1998.
28. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* 3: 361–370, 2003.
29. Lorne E, Zmijewski JW, Zhao X, Liu G, Tsuruta Y, Park YJ, Dupont H, Abraham E. Role of extracellular superoxide in neutrophil activation: interactions between xanthine oxidase and TLR4 induce proinflammatory cytokine production. *Am J Physiol Cell Physiol* 294: C985–C993, 2008.
30. Macian F, Lopez-Rodriguez C, Rao A. Partners in transcription: NFAT and AP-1. *Oncogene* 20: 2476–2489, 2001.
31. Madden LA, Laden G. Gas bubbles may not be the underlying cause of decompression illness - the at-depth endothelial dysfunction hypothesis. *Med Hypotheses* 72: 389–392, 2009.
32. Marinovic J, Ljubkovic M, Breskovic T, Gunjaca G, Obad A, Modun D, Bilopavlovic N, Tsikas D, Dujic Z. Effects of successive air and nitrox dives on human vascular function. *Eur J Appl Physiol* 112: 2131–2137, 2012.
33. Mohr S, Liew CC. The peripheral-blood transcriptome: new insights into disease and risk assessment. *Trends Mol Med* 13: 422–432, 2007.
34. Montcalm-Smith EA, McCarron RM, Porter WR, Lillo RS, Thomas JT, Auker CR. Acclimation to decompression sickness in rats. *J Appl Physiol* 108: 596–603, 2010.
35. Nakamura K, Matsunaga K. Susceptibility of natural killer (NK) cells to reactive oxygen species (ROS) and their restoration by the mimics of superoxide dismutase (SOD). *Cancer Biother Radiopharmaceut* 13: 275–290, 1998.
36. Nordberg J, Arnér ESJ. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31: 1287–1312, 2001.
37. Obad A, Marinovic J, Ljubkovic M, Breskovic T, Modun D, Boban M, Dujic Z. Successive deep dives impair endothelial function and enhance oxidative stress in man. *Clin Physiol Funct Imaging* 30: 432–438, 2010.
38. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. *Apoptosis* 12: 913–922, 2007.
39. Pavlidis P, Li Q, Noble WS. The effect of replication on gene expression microarray experiments. *Bioinformatics* 19: 1620–1627, 2003.
40. Peng YM, van de Garde MD, Cheng KF, Baars PA, Remmerswaal EB, van Lier RA, Mackay CR, Lin HH, Hamann J. Specific expression of GPR56 by human cytotoxic lymphocytes. *J Leukoc Biol* 90: 735–740, 2011.
41. Peng Z, Ren P, Kang Z, Du J, Lian Q, Liu Y, Zhang JH, Sun X. Up-regulated HIF-1 $\alpha$  is involved in the hypoxic tolerance induced by hyperbaric oxygen preconditioning. *Brain Res* 1212: 71–78, 2008.
42. Pontier JM, Gempp E, Ignatescu M. Blood platelet-derived microparticles release and bubble formation after an open-sea air dive. *Appl Physiol Nutr Metab* 37: 888–892, 2012.
43. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21: 2067–2075, 2005.
44. Thom SR. Oxidative stress is fundamental to hyperbaric oxygen therapy. *J Appl Physiol* 106: 988–995, 2009.
45. Thom SR, Milovanova TN, Bogush M, Bhopale VM, Yang M, Bushmann K, Pollock NW, Ljubkovic M, Denoble PJ, Dujic Z. Microparticle production, neutrophil activation, and intravascular bubbles following open-water SCUBA diving. *J Appl Physiol* 112: 1268–1278, 2012.
46. Thom SR, Milovanova TN, Bogush M, Yang M, Bhopale VM, Pollock NW, Ljubkovic M, Denoble PJ, 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.
47. 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.
48. van Velzen EJJ, Westerhuis JA, van Duynhoven JPM, van Dorsten FA, Hoefsloot H CJ, Jacobs DM, Smit S, Draijer R, Kroner CI, Smilde AK. Multilevel data analysis of a crossover designed human nutritional intervention study. *J Proteome Res* 7: 4483–4491, 2008.
49. Vann RD, Butler FK, Mitchell SJ, Moon RE. Decompression illness. *Lancet* 377: 153–164, 2011.
50. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44–49, 2011.
51. Warren BA, Philp RB, Inwood MJ. The ultrastructural morphology of air embolism: platelet adhesion to the interface and endothelial damage. *Br J Exp Pathol* 54: 163–172, 1973.