Immune and inflammatory responses to freediving apnea calculated from leukocyte gene expression profiles

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Abstract

Freedivers hold their breath while diving, causing blood oxygen levels to decrease (hypoxia) while carbon dioxide increases (hypercapnia). Whereas blood gas changes are presumably involved in the progression of respiratory diseases, less is known about their effect on healthy individuals. Here we have used gene expression profiling to analyze immune and inflammatory responses in elite freedivers. Blood was collected before, 1 and 3 h after a series of static and dynamic apneas during a freediving competition, and peripheral blood gene expression was mapped on genome-wide microarrays. Fractions of phenotypically distinct immune cells were computed by deconvolution of the gene expression data using Cibersort software. Apnea-induced changes in gene activity and associated biological pathways were determined using R and GeneGo software. The results indicated a temporary increase of neutrophil granulocytes, and a decrease of cytotoxic lymphocytes; CD8+ T cells and resting NK cells. Biological pathway associations indicated possible protective reactions: genes involved in anti-inflammatory responses to proresolving lipid mediators were upregulated, whereas central factors involved in granule-mediated lymphocyte cytotoxicity were downregulated. While it remains unresolved whether freediving alters the immune system's defensive function, these results provide new insight into leukocyte responses and the protection of homeostasis from apnea-induced stress in healthy athletes.

New & Noteworthy

Freedivers hold their breath while diving; causing blood oxygen to fall while carbon dioxide rises. Blood gas changes presumably affect respiratory diseases, yet freedivers remain unharmed. We have studied immune cell activity in elite freedivers through their peripheral blood transcriptomes, finding that freediving triggered neutrophil activity while suppressing certain lymphocytes. The data also implied defensive responses, possibly resolving

inflammation and limiting cytotoxicity. These novel findings may promote our understanding of physiological responses to non-pathological apnea.

Introduction

Freedivers dive on a single breath, and their performance hinge on their ability to voluntarily suppress breathing while floating face down (static apnea) or swimming horizontally (dynamic apnea) or vertically (constant weight apnea, free immersion apnea, no-limits apnea). In order to improve their performance, they use adaptive techniques that increase their lung capacity, reduce metabolic rates and improve their tolerance to apnea, i.e. to hypoxia and hypercapnia (22). Freediving performance is often further enhanced by hyperventilation to reduce carbon dioxide levels prior to the dive, and by glossopharyngeal insufflation ("lung packing") for extra volumes of air or breathing pure oxygen to add to the body's oxygen reserves (19). During dives, an initial easy-going phase is followed by a physiological breaking point after which the urge to breath causes a struggle phase with displays of involuntary movements of the respiratory muscles that are thought to increase cardiac output (12, 31). This effect restores oxygen supply to the vital organs, such as the brain and the heart. Still, hypoxia can be severe at the end of a dive; oxygen levels that are considered pathological in untrained individuals have been measured in freediving athletes' first expired breaths and arterial blood after diving (21, 30, 52). Adaption to hypercapnia also permits freedivers to prolong their apnea times, and the carbon dioxide first expired after breaking off a dive is considerably elevated (30). In light of an emerging understanding of the role of the immune system and inflammatory signaling in maintaining tissue and organ homeostasis (24), it is of interest to understand the responses of the white blood cells; leukocytes, to physiologically stressful changes in blood gas during voluntary apnea.

The possibility of genome-wide measurements of gene expression on microarrays has expedited research into the molecular basis of biological states and responses. For studies of the immune system, peripheral blood is an obvious choice for gene expression analysis (5). Blood is a highly heterogeneous tissue. Of its formed elements: the erythrocytes, platelets and leukocytes, only leukocytes have chromosome-containing nuclei; genome-wide gene expression in peripheral blood therefore ideally represent the biological state of its leukocytes. However, the interpretation of gene expression data from blood is complicated by the heterogeneity of the leukocyte compartment, which consists of a number of phenotypically different cell types. The main leukocytes: the neutrophils, eosinophils, basophils, lymphocytes and monocytes, are further divided into subset of cells with different function in the immune system. Each leukocyte subtype derives its phenotype from the particular set of genes it expresses, and the cell types are present in blood in variable amounts (51). In practical terms, this means that a measured change in the abundance of any transcript in blood does not immediately tell us whether the activity of its gene has changed, or whether there has been a change in the relative abundance of cells in which this gene is expressed (39).

Traditional microarray analysis does not take sample composition into account, but recent papers have presented methods where transcriptome contributions from phenotypically distinct cell types are separated by signal deconvolution on basis of cell type-specific gene expression (1, 25, 38). Deconvolution of microarray signals extracts cell type-specific information from system-wide data, and has been found to corroborate results from flow cytometric phenotyping. Also, since deconvolution is done after the genome-wide data is collected, it eliminates the need for fractioning of samples, and facilitates unbiased detection of cell types for which the patterns of gene expression are known.

In this study we examine the effects of voluntary apnea on cells of the immune system. Genome-wide cDNA microarrays were used to analyze the peripheral blood transcriptome of

elite freedivers who performed a series of static and dynamic apnea dives in a pool with their respiratory tract immersed. The proportions of major immune cell types in the participant's blood before and after dives were calculated by cell type-specific deconvolution of the microarray data. Changes in biological pathways in response to apneas were predicted on basis of differentially expressed genes.

Materials and Methods

Research ethics

The experimental procedures were performed in compliance with the declaration of Helsinki ethical principles for human experimentation, and approved by the Ethics Committee of the University of Split School of Medicine (No. 2181-198-03-04-14-0011), and the Norwegian Regional Committee for Medical and Health Research Ethics (No. 2015/200). Prior to inclusion, the subjects were informed about the study's purpose and scope, and of possible risks associated with participation. Inclusion into the study was based on signed informed consent.

Study subjects

The study group consisted of ten elite free-diving athletes; one female and nine males aged 20 – 48 yrs (30 ± 9 yrs, mean ± SD), height 160 – 191 cm (183 ± 8, mean ± SD) and weighing 51 -100 kg (82 ± 14, mean ± SD) were included on basis of the following criteria: the athlete should be a former or present members of a national free-diving team, with participation in at least one world or European championship in the last three years resulting in a placement among the top ten competitors in at least one discipline. All subjects were healthy non-smokers. Two individual blood samples were discarded on basis of RNA quality; the study was therefore performed on material from eight participants.

Apnea protocol

The experimental apnea was conducted in a swimming pool with a water temperature of 27°C at the Sports Park Mladost in Zagreb, Croatia. All experiments were done in the afternoon, limiting the impact of circadian variation in gene expression. On the day of the experiment, the subjects were instructed not to consume alcohol, caffeine or other stimulants. The subjects started one by one on a series of 8 repeated apneas; one dynamic apnea without fins followed by seven bouts of static apneas, with the aim of provoking physiological stress. The subjects were allowed individual preparations prior to the first and fifth apnea. Most chose to prepare by moderate hyperventilation and/or glossopharyngeal insufflation. The second, third and fourth apnea continued until the subject experienced 10 involuntary breathing movements, i.e. involuntary respiratory muscle contractions. The experimental protocol with the order, mode and duration (means ± SD) of apneas and intermitting rests is outlined in Table 1.

Blood sampling and pre-analytic handling

For each subject, peripheral blood (2.5 mL) was drawn on PAXgene tubes (PreAnalytix, Hombrechtikon, Switzerland) by standard venipuncture three times on the day of experiments: the first sample was taken shortly before the first apnea, and the second and third samples were taken 1 and 3 h after completion of the experimental protocol (Table 1). In order to minimize technical variation, all samples were collected on a single batch of PAXgene tubes by the same technician. The filled tubes were kept at room temperature for 4 h for fixation of the gene expression profile, transported to the microarray laboratory on dry ice, and kept at -80 °C until RNA extraction.

Microarray processing

Total RNA was extracted from the blood samples using PAXgene Blood RNA kit version 2 (PreAnalytix). RNA concentration and quality was measured on a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA): RNA concentrations of the included samples ranged from 100 to 400 ng/μL, and the RNA integrity number (RIN) ranged from 8.2 to 9.2. Two samples were excluded based on low RNA concentration, resulting in the exclusion of two male athletes from downstream analyses. For *in vitro* preparation of material for microarrays, total RNA was amplified using the TotalPrep RNA amplification kit (Ambion Inc., Austin, TX, USA), first and second strand cDNA synthesis was performed by reverse transition and replication, and cRNA was synthesized by transcription. Gene expression measurements were done on the Illumina humanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA, USA), which provide genome-wide measurement of the expression of more than 31,000 genes with over 47,000 probes. Scanning of the microarrays was done on the Illumina HiScan System.

Gene expression profiling

Microarray data background signal subtraction with Benjamini Hochberg false discovery *p*-value correction was performed in the Illumina GenomeStudio software, version 1.7.0, before the data was exported to R (http://www.r-project.org/) for further analysis using the lumi Bioconductor package version 1.1.0 (11). Negative signal intensities were flagged and set to zero after log2 transformation, and inter-sample differences were normalized by quantile transformation. We excluded probes with detection *p*-values outside of the 0.01 threshold in more than 50% of the samples. An exploratory analysis was performed using multilevel partial least squares regression to determine sensible choices for blocking structure in the subsequent differential testing, and to obtain a global visualization of sample relations.

Differential gene expression was estimated by a moderated paired *t*-test. A false discovery adjusted *p*-value of < 0.05 was considered significant.

Leukocyte cell-type enumeration

Determination of the relative numbers of different leukocyte types in each sample was done by deconvolution of the gene expression data using the Cibersort software (25), with a default input matrix of cell-type specific gene expression signatures. The LM22 signature matrix contains 547 genes that distinguish 11 leukocytes subtypes: B cells, plasma cells, cytotoxic T cells (CD8+), helper- and regulatory T cells (CD4+), γδ T cells, natural killer cells (NK cells), monocytes and macrophages, dendritic cells, mast cells and eosinophil and neutrophil granulocytes. These can be further divided into 22 phenotypic variants. In the present study, cut-off was set so that only cells with a mean relative fraction across all samples of ≥ 5 % of the calculated leukocyte population were included in comparisons across time-points. Further statistical analyses were done in IBM SPSS statistics software version 21.0 (http://www-01.ibm.com/software/uk/analytics/spss/). Normal distribution of the calculated leukocyte fractions at each time-point was verified by Kolmogorov-Smirnov tests and Q-Q normal probability plots. Kruskal-Wallis tests were used to compare means over all time-points, and to subsequently identify significant changes in pairwise comparison between time-points. *p*-values < 0.05 were considered significant.

Enrichment analysis for biological pathways

Functional clustering of gene expression data was performed using the MetaCore GeneGo software release 6.21 (http://thomsonreuters.com/metacore/), with the differentially expressed gene lists from the gene expression analysis as input. The enrichment analyses were ranked according to the probability of a particular pathway or process occurring by chance in the

gene list in comparison to a background consisting of all probes on the Illumina humanHT-12 v4 Expression BeadChips. The absolute threshold for transcription change was set to 0.5, and false discovery adjusted *p*-values < 0.05 were considered significant.

Microarray data availability

The microarray data has been submitted for open access in the EMBL-EBI ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/) according to MIAME standards. The accession code is E-MTAB-3547.

Results

Experimental procedure

Peripheral blood samples were collected from elite participants at a freediving competition. The participants provided base-line blood samples before start, and proceeded to give two additional samples 1 and 3 h after completion of the freediving protocol outlined in Table 1. All participants successfully completed the protocol, and there were no reports of adverse effects caused by the apneas or blood collection.

Table 1.

Sample relations in the microarray data

After microarray data processing and signal filtering, an exploratory multilevel partial least squares regression analysis was performed on the data in order to visualize sample relations. As shown in Figure 1, data from individual samples fell into distinctively separable classes according to time of blood collection relative to the apneas. Freediving apnea hence

outweighed other sources of variation in this supervised analysis, and differential gene expression was considered to be apnea-induced in the subsequent analyses.

Fig 1.

Apnea-induced gene expression changes

Gene expression profiling of the processed and filtered microarray data was done in order to determine the effects of apneas on the global peripheral blood transcriptome. As summarized in the Venn diagram in Fig 2A, the analysis revealed differential expression of 5,353 probes representing 4,585 genes in measurements performed 1 h after apneas, and 2,321 representing for 2,054 genes in the later measurement 3 h after apneas. Ninety-one % of all differentially expressed genes were present after 1 h, whereas the remaining 9% were unique to the later dataset. The signature overlaps comprised in the intersection between the 1 and 3 h data consisted of one third of apnea-induced gene expression changes, and approximately of 60% of the genes were no longer present after 3 h. Complete lists of differentially expressed genes at both time points are presented in the Appendix.

Fig 2.

In an initial assessment of apnea-related effects, we considered the genes with the most pronounced expression changes. The top upregulated genes pointed to temporary activation and recruitment of neutrophil granulocytes: 1 h after apneas, the single most upregulated gene codes for matrix metallopeptidase 9 (MMP9) (Fig 3). MMP9 facilitates the migration of neutrophils between the endothelium and underlying tissues by degrading collagens in the basement membrane (10). MMP9 is activated in neutrophil granulocytes by interleukin 8 (IL-

8) inflammatory signaling via Toll-like receptors 2 and 4 (TLR2 and TLR4) and the adapter myeloid differentiation factor 88 (MyD88) (26, 34), all of which were upregulated. The expression of *MMP9* and *IL-8* was no longer upregulated 3 h after apneas, whereas the *TLRs* and *MyD88* still were.

On the other end of the expression scale, the most downregulated genes comprised a striking number of factors involved in the mediation of lymphocyte cytotoxicity, i.e. CD8+ T cells and NK cells. Along with markers for cytotoxic lymphocytes; natural killer cell granule protein 7 (NKG7) and G protein-coupled receptor 56 (GPR56), all major components of cytolytic granules; the membrane-destroying proteins perforin (*PRF1*) and granulysin (*GNLY*), and the apoptosis-inducing serine proteinase granzymes (*GZMs*) *GZMA*, *GZMB*, *GZMH*, *GZMK* and *GZMM* were downregulated 1 h after apnea. In the 3 h dataset only *GZMB* and *GZMM* were still downregulated, indicating that the effect was temporary.

Fig 3.

Calculated changes in immune cell fractions

In order to determine whether apneas affected the composition of immune cells in peripheral blood, we performed signal deconvolution of the transcriptome data using the Cibersort software with leukocyte-specific gene expression signatures in the input matrix (25). The deconvolution returned 14 phenotypically distinct immune cell types, listed in Table 2. Comparison of the calculated fractions across and between time-points revealed apnea-associated changes in three immune cell types 1 h after apneas; neutrophil granulocytes (p=0.001) increased, whereas CD8+ T cells (p=0.007) and resting NK cells (p=0.011) decreased. Naïve CD4+ T cells and M2 macrophages came close, but were not significantly

changed. The effects of apneas on immune cell fractions appeared to be temporary, as no significant effects were found 3 h after apneas.

Table 2.

In Fig 4, immune cell types that on average were found to represent > 5% of all leukocytes are shown as box plots. While the analysis did not identify sustaining differences in immune cell fractions 3 h after apneas, the data in Fig 4 indicates that they were not fully restored to before apnea-levels.

Fig 4.

Biological pathway associations

In order to further investigate the biology of immune cell responses, we used MetaCore GeneGo software to identify biological pathway associations for apnea-induced gene expression changes. The 1 and 3 h data for differentially expressed genes were first analyzed together, on merged data (1 h ∪ 3 h); i.e. all genes in the Venn diagram in Fig 2, and on the intersection between the datasets (1 h ∩ 3 h); i.e. genes contained within the Venn overlap. Both analyses returned almost identical results, and no pathways were identified in subsequent analysis of the contrast between the 1 and 3 h data (1 h Δ 3 h). Individual analysis of the 1 and 3 h data returned similar pathway associations at both time points, with different significance and some variation in order; whereas the order of pathways in the 1 h data were essentially the same as for the merged data, the top pathway association in the 3 h data was that for Toll-like receptor TLR2 and TLR4 signaling. The top five pathways associated with the up- and downregulated gene expression respectively are shown in Table 3.

Biological pathways involved in the development, migration and activity of neutrophil granulocytes in response to inflammatory stimuli are highly represented among those associated with upregulated gene expression, most so at the earliest time point (1 h) after apneas. The top pathway association was that for inhibition of neutrophil migration by proresolving lipid mediators; an anti-inflammatory process that limits airway inflammation caused by trans-endothelial migration and accumulation of neutrophils in chronic obstructive pulmonary disease (20). The bottom part of Table 3 contains pathways of cytotoxic lymphocyte receptor-mediated signaling and pre-apoptotic activity. Whereas most of the genes associated with NK cell-mediated immunity were downregulated; including the inhibitory killer cell receptors *KLRD1* and *KIR2Dl3* and intracellular signal transmitting kinases *ZAP70* and *FYN*, the pattern of gene expression changes was more variable in the granzyme B signaling pathway: the cytotoxic granule-associated *PRF1* and *GZMB* were strongly downregulated, but several other genes acting further downstream in intercellular apoptotic signaling were upregulated.

In all major pathways associated with differential gene expression, the direction of gene expression changes was identical across all data. Overall, the results indicated that the same immune and inflammatory responses were reflected in the data measured both 1 and 3 h after apneas. However, as seen in Table 3, the number of involved objects was higher in the earliest (1 h) measurement. The log-fold change of differentially expressed genes was also consistently larger in earliest dataset (Appendix). The responses thus appeared to be temporary, diminishing within hours of the apnea exposure.

Table 3:

Discussion

Freedivers maximize their performance by optimal oxygen use and heightened tolerance to CO_2. They emerge from dives hypoxic and hypercapnic. Prior studies have addressed the effects of physiological stress in freediving on specific leukocytes, demonstrating e.g. altered antioxidant responses in mononuclear cells (44, 45) and increased tolerance to oxidative stress in neutrophil granulocytes (42, 43). In this study we have demonstrated that experimental apnea extensively alters the global peripheral blood transcriptome of elite freediving athletes. Deconvolution of transcriptomes indicated a temporary increase in neutrophil granulocytes, and a decrease of CD8+ T cells and resting NK cells. Biological pathway analysis showed that genes involved in neutrophil responses to anti-inflammatory proresolving lipid mediators were upregulated, whereas those coding for components of granule-mediated lymphocyte cytotoxicity were downregulated. This is to our knowledge the first study that addresses the effects of freediving apnea across the complete leukocyte compartment.

In diseases that are associated with chronic or intermittent hypoxia and hypercapnia, such as chronic obstruction pulmonary disease and sleep apnea, systemic inflammation is thought to contribute to pathological progression and sequelae (16, 49). The inflammatory activation progresses via TLR signaling, and results in increased amounts of circulating neutrophils (2, 35). There is an intimate connection between hypoxia, inflammation and innate immunity that is evident on the level of gene expression (7, 9, 27). Two transcription factors are of particular importance; HIF-1α is considered the master switch of transcription in hypoxia (36), whereas NF-kB regulates the transcription of genes in inflammatory signaling (29). The two act in concert to control the genetic activity of immune cells (33). Hypoxia-driven gene expression affects different immune cells in different ways; whereas innate immune cells are triggered, it appears that some features of adaptive immunity are suppressed (40). A possible explanation for this lies in our evolutionary history. The development of cellular defenses against pathogen invasion in early eukaryotes took place when the atmosphere's oxygen was low

(23). The current descendants of early immune cells remain quiescent under normal oxygen tensions, but they have retained the capacity to switch to anaerobic metabolism in response to inflammatory signaling, thus stimulating their function in hypoxic tissues (17). Neutrophil granulocytes are essential constituents of the innate immune system, and experimental hypoxia in healthy individuals has been shown to boost both the cytotoxicity and the survival of neutrophils (46, 50). The adaptive immune system emerged in an oxygenized atmosphere, and NK cells - although considered effectors of innate immunity - are also late arrivers on the evolutionary scene (4, 13). The response of lymphocytes to changes in oxygen tension is more complex than that of neutrophils, but hypoxia has been shown to decrease the survival of human lymphocytes *in vitro* (6, 41, 47). However, whereas the number of lymphocytes is reduced in hypoxia, it has been reported that their effector function, i.e. the ability of CD8+ T cells and NK cells to kill target cells by granule-mediated apoptosis, increases (3).

CO_2 is also a modulator of inflammatory gene expression (48). Therapeutic hypercapnia has been shown to inhibit lung and systemic inflammation (14), which may be explained by the ability of CO_2 to suppress NF-κB-dependent gene transcription (8, 28). Hypoxia and hypercapnia may therefore have opposite effects on inflammatory processes, and while we could not separately assess effects of hypoxia and hypercapnia in this study it appears likely that the changes in immune cell fractions after apneas were triggered predominantly by hypoxia.

In this study there was a strong association between differentially expressed genes and the biological pathway involved in inhibition of neutrophil migration by proresolving lipid mediators. Endogenous proresolving lipid mediators are highly conserved structures with essential functions in resolution of acute inflammation, and their synthetic counterparts are potent anti-inflammatory agents when administered *in vivo* (37). This may imply that there was activation of endogenous anti-inflammatory proresolving lipid mediators in reaction to

the neutrophil activation, possibly protecting the divers' own cells from inflammatory injury. Similarly, the downregulation of genes coding for components of cytolytic granules may confer some self-protection against a hypoxia-induced increase in lymphocyte cytotoxicity. As the study participants were all elite freedivers, these findings may imply that frequent apnea exposure causes an adaption. Similar studies of healthy apnea-naïve individuals might elucidate this.

Limitations

The interpretations of results in this study are based on assumed matches between gene expression levels and the presence and activity of phenotypically distinct leukocytes in peripheral blood. However, there are some caveats. First, the deconvolution method uses reference profiles for gene expression that are derived from purified or enriched leukocyte subsets. These profiles are reported to be of high sensitivity and specificity, but they do not make allowance for possible deviations in gene expression from cells that are phenotypically abnormal due to e.g. biological plasticity or disease (25). Second, there is the assumption that the peripheral blood transcriptome originates exclusively from leukocytes. Other formed elements of blood; immature erythrocytes (reticulocytes) and platelets, though devoid of cell nuclei still contain mRNAs from their precursor cells (15, 18). Any contribution they may have given to the gene expression profiles is disregarded. Third, transcriptome changes are not necessarily reflected in phenotypes. However, since the principal differences in transcriptome measurements before and after apneas are contributed by genes with well-established roles in cell-specific immune responses and inflammatory signaling, we believe that our results represent normal leukocyte biology. It should also be noted that this study does not control for water immersion alone. As immersion causes blood volumes to shift from the extremities to the trunk, and drainage of fluids from cells into the vascular lumen (32), it is

conceivable that leukocytes might be affected. The gene expression changes identified in this study were compatible with transcriptional responses to hypoxia, but we cannot decidedly rule out effects triggered by the water surrounding the participants during the apneas.

Conclusion

Immune and inflammatory responses to apnea in freediving may be derived from peripheral blood gene expression profiles. Changes in the leukocyte compartment in experienced freedivers; a temporary increase of neutrophil granulocytes and reduction of CD8+ T cells and resting NK cells, are likely triggered by hypoxia. The biological pathways most associated with apnea-induced gene expression point to possible compensatory reactions that limit the injury from altered immune and inflammatory activity. While it remains unknown whether the immune system's defensive function is affected, our findings provide novel insight into the effects of non-pathological apneas on phenotypically distinct leukocyte types, and the protection of homeostasis from physiological stress in freediving.

Acknowledgements

The freedivers are gratefully acknowledged for their participation in the study. Ivana Banic at the University of Split Medical School performed the collection and preprocessing of blood samples. Microarray processing was done by Tom Wheeler and Sten Even Erlandsen at the NTNU Genomics Core Facility (GCF). GCF is run by the Faculty of Medicine at NTNU and the Central Norway Regional Health Authority.

Grants

This work was funded by the Norwegian Research Council; Petromaks2 project No 220546/E30 (Ingrid Eftedal), the NTNU Faculty of Medicine and the Central Norway

Regional Health Authority (Arnar Flatberg) and the Croatian Science Foundation; grant No IP-2014-09-1937 (Ivan Drvis and Zeljko Dujic). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosures

There are no competing interests to declare.

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Figure Captions

Fig 1. Partial Least Square (PLS) analysis for dimensional reduction of freedivers' peripheral blood transcriptome data. In this plot, the small equilateral triangles represents the position of data from individual samples, colored according to time of sampling, and positioned in the plot by the first two principal components of a PLS model derived from a naïve Bayesian classifier on the transformed subspace spanned by the two first principal components (PC1 and PC2). The larger, protruding triangles represent normalized fractions of the cell types that explain the majority of observed differences; neutrophil granulocytes, cytotoxic T cells and resting NK cells. Class probabilities are indicated by background colors. The plot shows distinctive separation of samples according to classes; i.e. to time relative to blood sample collection.

Fig 2. Apnea-induced differential gene expression. Venn diagram of the differentially expressed genes that were identified by GeneGo analysis 1 and 3 h after experimental apneas. Out of > 5,000 unique genes, the majority (91%) were present in, and almost 60% unique to, the earliest (1 h) dataset, whereas only 9 % were unique to the later (3 h) dataset, indicating that the effects of the experimental apneas on gene expression in leukocytes were predominantly transient.

Fig 3. Forest-plot of selected genes differentially expressed 1 h after experimental apneas. The upper part of the plot includes genes with essential function in neutrophil granulocyte activation and recruitment, all of which were upregulated in the 1 h dataset. The bottom part comprises central factors in lymphocyte cytotoxicity, all downregulated 1 h after apneas. Whereas the direction of change was consistently the same 1 h and 3 h after apneas,

595 only a few genes still showed significantly different expression levels from pre-apnea
596 measurements at the latter time point.
597
598 **Fig 4. Major immune cell fractions in peripheral blood calculated by transcriptome**
599 **deconvolution.** Cell types that on average contributed ≥ 5% of all leukocytes are included in
600 the box plot In the 1 h dataset, neutrophil granulocyte fractions were increased (p=0.001),
601 whereas CD8+ T cells (p=0.007) and resting NK cells (p=0.011) decreased. In the 3 h dataset,
602 the cell fractions were no longer significantly different from before the experimental apneas.
603 Horizontal bands, bars and whiskers indicate median values, lower and upper quartiles and
604 min/max respectively. Outliners are shown as dots.
605
606

Tables

Table 1. Experimental freediving protocol, with apnea types and durations.

Step	Mode	Duration [s]	
		Mean (SD)	Range
	Individual preparation		
1	Dynamic apnea without fins	97 (26)	71-150
	Rest 10 min – passive exhale		
2	Static apnea until 10 involuntary breathing movements	73 (29)	42-144
	Rest 3 min		
3	Static apnea until 10 involuntary breathing movements	162 (51)	105-257
	Rest 7 min		
4	Static apnea until 10 involuntary breathing movements	203 (58)	150-321
	Rest 7 min		
	Individual preparation		
5	Maximal static apnea	286 (74)	206-430
	Rest 1 min – maximal inhale		
6	Maximal static apnea	209 (45)	160-294
	Rest - two breaths, maximal inhale		
7	Maximal static apnea	130 (27)	100-169
	Rest - two breaths, maximal inhale		
8	Maximal static apnea	126 (22)	93-164

Table 2. The effect of experimental apnea on relative fractions of major immune cell types in peripheral blood. Cell types are sorted according to mean relative fractions over all samples.

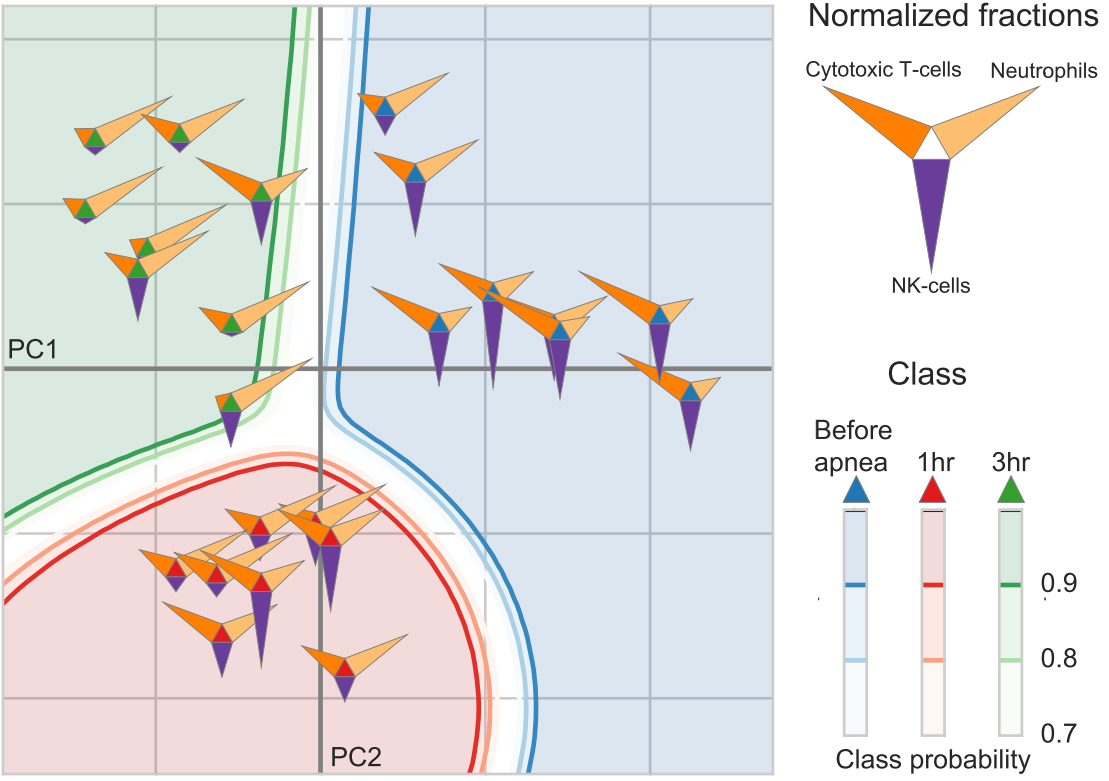
Cell type	Mean fraction	*p*-values for change[1]			
		Across all classes	Before vs 1 h after apnea	Before vs 3 h after apnea	1 h vs 3 h after apnea
Neutrophil granulocytes	0.401	0.002	0.001	0.111	0.442
CD8+ T cells	0.122	0.009	0.007	0.198	0.688
Monocytes	0.115	0.595			
Naïve CD4+ T cells	0.096	0.05			
Resting NK cells	0.091	0.015	0.011	0.537	0.359
Regulatory T cells	0.046	0.269			
Memory B cells	0.044	0.193			
Activated CD4+ memory cells	0.027	0.378			
Resting mast cells	0.026	0.512			
M0 macrophages	0.016	0.141			
Activated dendritic cells	0.005	0.690			
Activated NK cells	0.004	0.399			
M2 macrophages	0.002	0.045	0.051	1	0.219
Plasma cells	0.002	0.713			

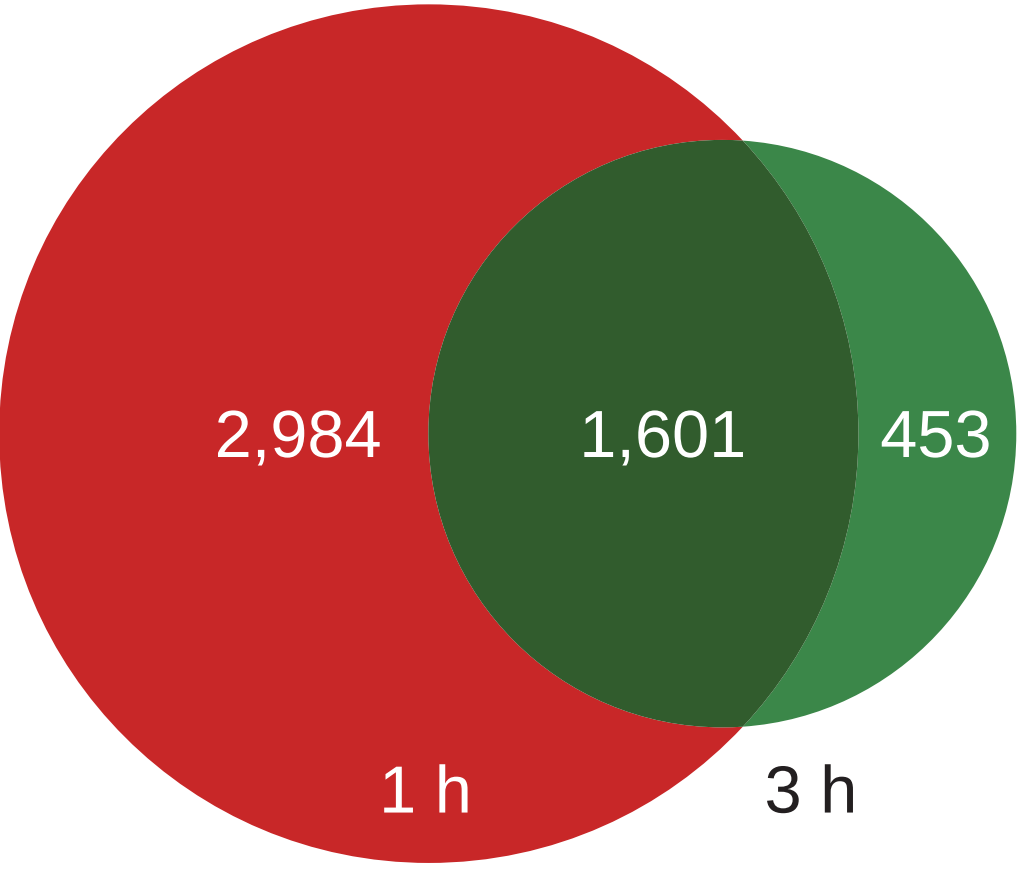
[1]Adjusted *p*-values for pairwise comparisons between classes were calculated only when Kruskal-Wallis tests showed significant differences across all classes.

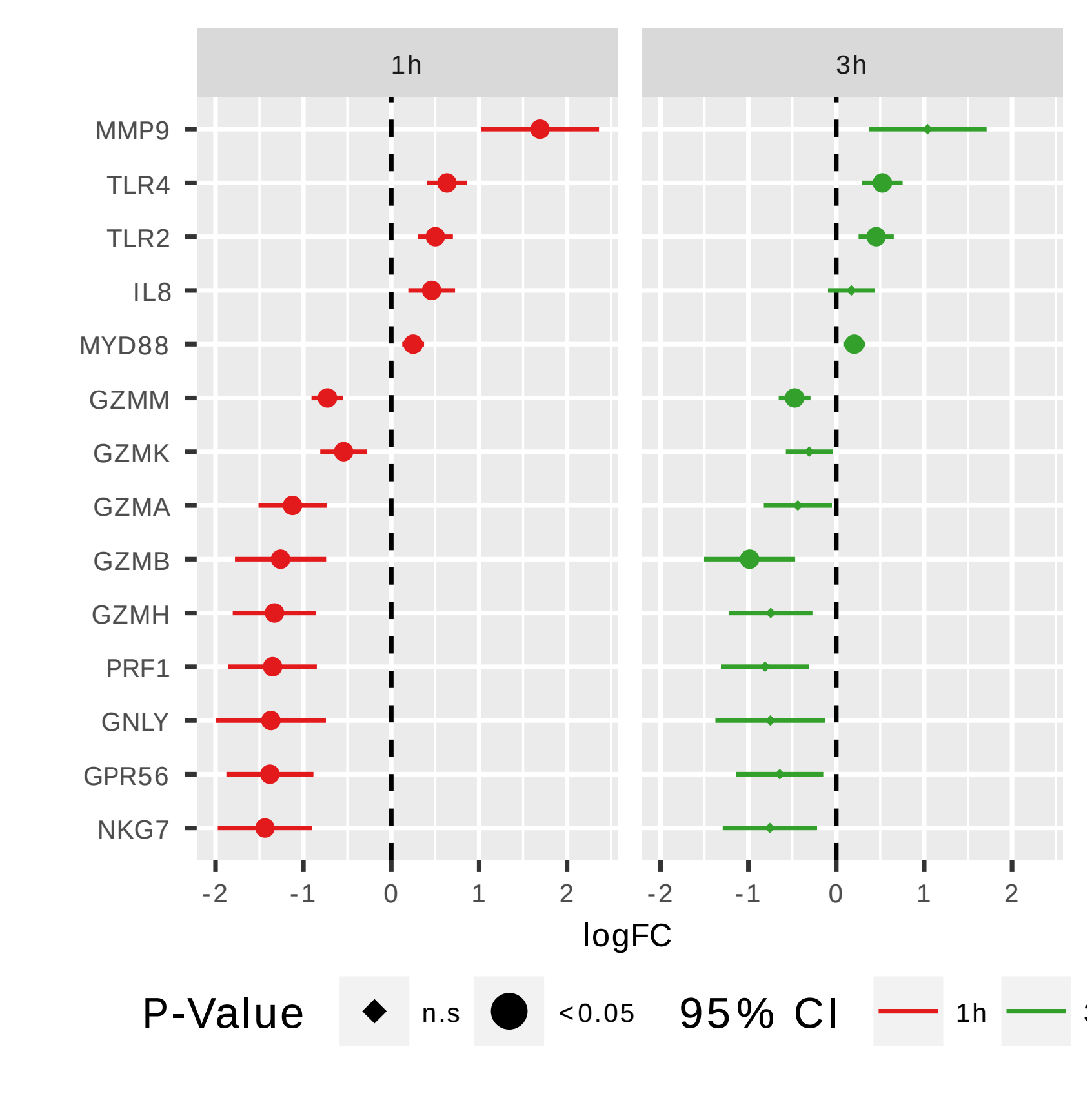
Table 3. Top biological pathway associations for effects of the experimental apneas. Objects associated with an absolute log fold expression change >0.5 in the 1 h dataset are included; comprehensive lists of genes and apnea-associated pathways are presented in the Appendix.

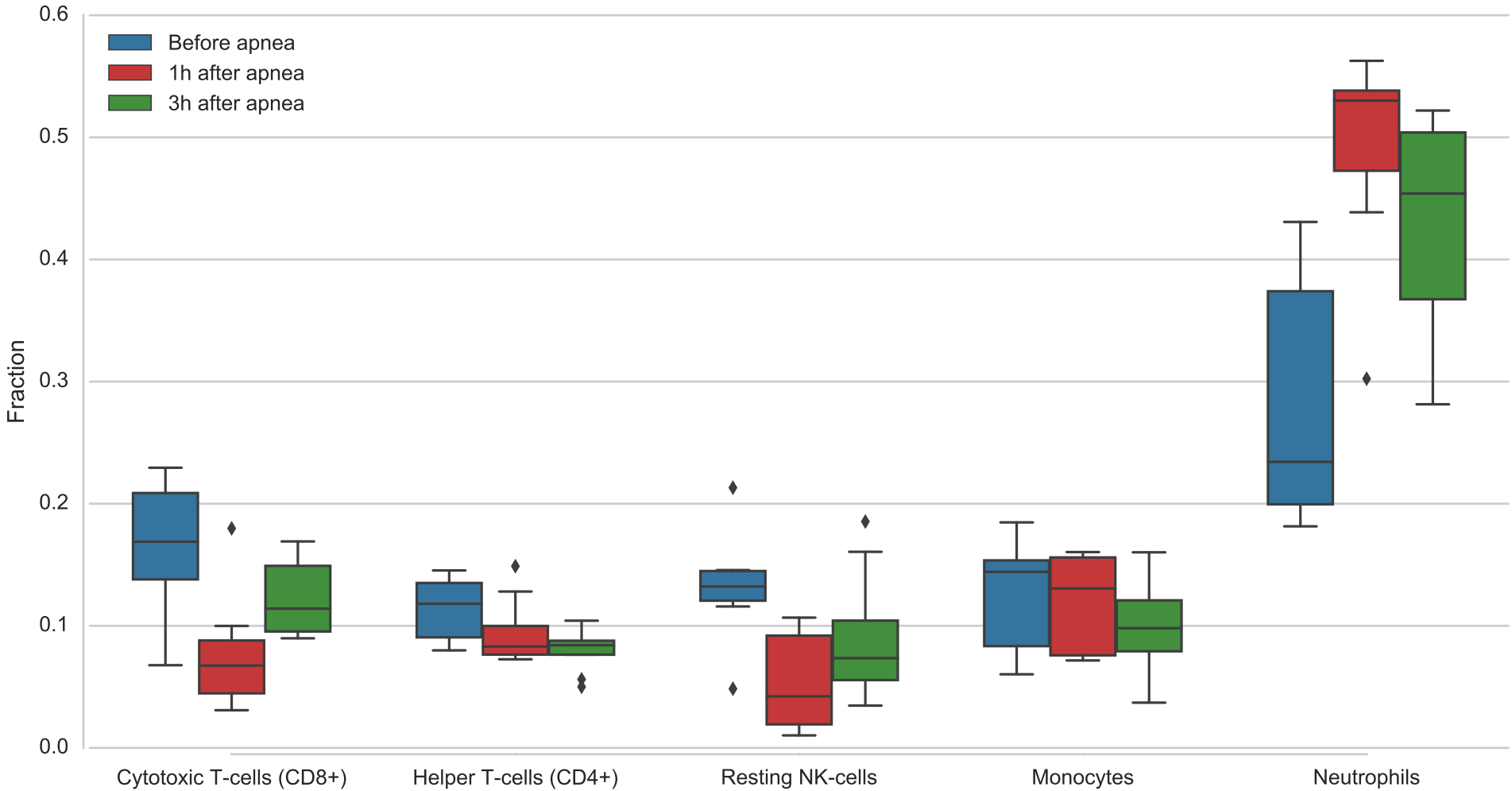
Biological pathway associations	1 h after apnea		3 h after apnea	
	p*-value	**Significant objects**	***p*-value***	**Significant objects**
Top upregulated				
Inhibition of neutrophil migration by proresolving lipid mediators in COPD	$1.7\ 10^{-7}$	C5AR, TLR4, FPRL1, LTBR1, IL8RA, FPR, TLR2, PAK1, L-selectin	0.013	C5AR, TLR4, FPRL1
Transcription regulation of granulocyte development	$2.2\ 10^{-6}$	G-CSF receptor, MAD, CD13, p47-phox, p67-phox, LRG	0.001	CD45, p67-phox, MAD
Inhibitory action of lipoxins on superoxide production induced by IL-8 and Leukotriene B4 in neutrophils*	$2.4\ 10^{-6}$	FPRL1, LTBR1, IL8RA, p47-phox, p40-phox, PAK1, p67-phox	0.048	FPRL1, p67-phox
TLR2 and TLR4 signaling pathways	$5.9\ 10^{-6}$	COX-2, TLR1, TLR2, TLR4, TLR6, Pellino 1, Pellino 2	$2.7\ 10^{-6}$	COX-2, TLR4, TLR6, MEK4, Pellino 1, Pellino 2
TLR ligands	$3.2\ 10^{-6}$	TLR1, TLR2, TLR4,TLR6, TLR8, HSP70	$8.9\ 10^{-5}$	TLR4, TLR6, TLR8, HSP70
Top downregulated				
Role of DAP12 receptors in NK cells	$1.1\ 10^{-9}$	KIR3DL1, KIR2DS2, KIR2DL3, Fyn, PLC-gamma 1, ZAP70, LAT, CD94	0.046	Fyn
T cell receptor signaling pathway	$2.9\ 10^{-8}$	CD3 zeta, IP3 receptor, Fyn, PLC-gamma 1, ZAP70, CARD11, LAT	0.045	Fyn
CXCR4 signaling via second messenger	$1.8\ 10^{-6}$	IP3 receptor, Fyn, PLC-gamma 1, ZAP70, LAT	0.029	Fyn
Differentiation and clonal expansion of CD8+ T cells	3.710^{-6}	CD8, CD27, Granzyme B, STAT4, Perforin	0.033	Granzyme B
CD16 signaling in NK cells	$3.8\ 10^{-6}$	CD3 zeta, IP3 receptor, PLC-gamma 1, ZAP70, LAT, CD94	0.058	Calcineurin B

* *p*-values are from GeneGo pathway analysis.


PC1
PC2
Normalized fractions
Cytotoxic T-cells
Neutrophils
NK-cells
Class
Before apnea
1hr
3hr
0.9
0.8
0.7
Class probability


2,984
1,601
453
1 h
3 h


1h
3h
MMP9
TLR4
TLR2
IL8
MYD88
GZMM
GZMK
GZMA
GZMB
GZMH
PRF1
GNLY
GPR56
NKG7
-2
-1
0
1
2
logFC
P-Value
n.s
<0.05
95% CI
1h


Before apnea
1h after apnea
3h after apnea
Fraction
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Cytotoxic T-cells (CD8+)
Helper T-cells (CD4+)
Resting NK-cells
Monocytes
Neutrophils