

Evolution of the plasma proteome of divers before and after a single SCUBA dive.

By

Jacky Lautridou¹, Vianney Pichereau², Sébastien Artigaud², Benoit Bernay³, Otto Barak⁴, Ryan Hoiland⁵, Andrew T Lovering⁶, Ingrid Eftedal⁷, Zeljko Dujic⁸, François Guerrero¹

*Corresponding author

Jacky Lautridou

Université de Bretagne Occidentale

6, avenue Le Gorgeu 29200 BREST

Jacky.lautridou@gmail.com

Affiliations

¹Université de Bretagne Occidentale, Laboratoire ORPHY EA 4324. ²Université de Bretagne Occidentale, LEMAR UMR 6539. ³Université de Caen, Proteogen SF ICORE 4206. ⁴University of Novi Sad School of Medicine, Serbia. ⁵University of British Columbia, Okanagan Campus, Kelowna, British Columbia, Canada. ⁶University of Oregon, USA. ⁷Norwegian University of Science and Technology, Trondheim, Norway. ⁸University of Split School of Medicine, Croatia.

Abbreviations

DCS: Decompression Sickness; TTR: Transthyretin.

Keywords

Proteomics, decompression sickness, human, SCUBA, plasma, proteome

Word count

3785

Clinical Relevance

DCS is a poorly understood systemic pathology displaying a wide variety of symptoms, some of them having long term consequences. A previous study of ours found changes of the plasma proteome of rats that were linked with DCS occurrence. The aim of the present study is to assess whether if a single dive inducing significant stress but without inducing DCS symptoms elicits changes in the plasma proteome of human divers. This may provide further evidence of the link between changes in the plasma proteome and DCS occurrence. Consequently, this work may show that changes of the plasma proteome could be used as biomarkers of DCS and lead to better patient care. Results from this study are therefore relevant both to fundamental and applied research.

Abstract

Purpose: Decompression sickness (DCS) is a poorly understood and complex systemic disease caused by inadequate desaturation following a reduction of ambient pressure. A previous proteomic study of ours showed that DCS occurrence but not diving was associated with changes in the plasma proteome in rats, including a dramatic decrease of abundance of the tetrameric form of Transthyretin (TTR). The present study aims to assess the impact on the human blood proteome of a dive inducing significant decompression stress but without inducing DCS symptoms.

Experimental design: Twelve healthy male divers were subjected to a single dive at a depth of 18m of sea water (msw) with a 47-min bottom time followed by a direct ascent to the surface at a rate of 9msw/min. Venous blood was collected before the dive as well as 30mn and 2h following the dive. The plasma proteomes from four individuals were then analyzed by using a two-dimensional electrophoresis-based proteomic strategy.

Results: No protein spot showed a significantly changed abundance ($fdr < 0.1$) between the tested times.

Conclusion: These results strengthen the hypothesis according to which significant changes of the plasma proteome measurable with two-dimensional electrophoresis may only occur along with DCS symptoms.

Introduction

Decompression sickness (DCS) is the most serious danger for Self-Contained Underwater Breathing Apparatus (SCUBA) divers. It is a systemic pathology displaying a wide range of symptoms including minor ones such as skin rashes up to more serious clinical outcomes like neurological damage, cardiac collapse and death[1]. It is thought that venous gas emboli (VGE) formed during decompression trigger DCS but that other mechanisms are also important for its development such as vascular damage and inflammatory response[2]. Previous studies suggested that an increase of oxidative stress correlates with the severity of

decompression[3], -however its involvement in DCS is not clear[4]. An activation of the coagulation cascade has also been shown[3,5] as well as a release of circulating microparticles[6,7]. The innate immune system is also affected by scuba diving, as shown by the activation of neutrophils, monocytes, and macrophages[5–8]. However, although these changes are considered putative mechanisms involved in the development of DCS, they have also been reported in divers with no signs of DCS, suggesting important asymptomatic acute deteriorations in important physiological variables induced by SCUBA diving. Proteomics offers a modern tool to explore protein responses to changes occurring during the dive, especially in terms of post-translational regulations or modifications[9]. In general, plasma proteins are synthesized in the liver, but they also come from other various tissues and cells. Plasma protein levels may thus reflect an integrative whole body responses to a specific stress such as diving [10]. In this context, plasma can be used for analysis since it interacts with the whole organism and a sample of venous blood can be easily collected. In a previous study, we investigated potential changes of plasma proteome of rats after a simulated air dive[11]. We identified changes among 4 proteins (Apolipoprotein A1, α 1-antitrypsin, SerpinA3K and Transthyretin) in animals which suffered DCS only but no significant modification in asymptomatic animals. Interestingly, one spot, identified as the tetramer of Transthyretin, almost disappeared among rats displaying symptoms of DCS. Therefore, the aim of the current study was to assess whether single air dive which induces significant decompression stress as indicated by VGE formation, but without symptoms of DCS, elicits changes in the plasma proteome of human divers. **In this present study we used the same analysis methods as previously used in the rat study to allow comparison.**

Methods

Subjects. Twelve healthy male divers negative for patent foramen ovale (PFO) volunteered to participate in the study. Diving experience ranged from 5 to 25 years with 100 to 3,000 dives. Written informed consent was acquired on the first visit when anthropometric measures were taken, which was done a week before the experimental protocol (Table1). All subjects were healthy non-smokers, with no current medication and no record of respiratory or circulatory disease. All human experiments were conducted in agreement with the University of Split School of Medicine Ethics Committee, n° 2181-198-03-04 [PubMed](#) -15-0016.

Diving protocol. The study was performed at a military base of the Croatian Navy Force. The dive site was about 30m from the location where the experiments took place. All divers performed a single dive at a depth of 18m of sea water (msw) with a 47-min bottom time. Decompression was performed with direct ascent to the surface at a rate of 9msw/min. Sea temperature at the bottom was 16°C. Throughout the dives divers performed swimming of moderate intensity. Each diver was accompanied by a safety diver, and both of them were equipped with a diving computer (Uwatec Galileo sol, Johnson outdoors, Inc., Racine, WI, USA) interfaced with a lab computer for later verification of the diving profile.

Transthoracic echocardiography (TTE). Within 15 min after surfacing, the divers were placed in the supine position and a dual frequency (1.5–3.3 MHz) ultrasonic probe connected to a Vivid q echocardiographic scanner (GE, Milwaukee, WI, USA) was used to obtain a clear apical 4-chamber view of the heart. VGE were monitored at 30 min after surfacing, and bubble grades were recorded and graded on a scale of 0–5 with grade 4 being subdivided into 4A, 4B and 4C, according to the method described by Eftedal & Brubakk[12], and later modified by Ljubkovic et al.[13]. In addition to monitoring bubble grade at rest, VGE were graded after two different movements, i.e. arm and leg contractions, to mobilize bubbles that may be lodged in the venous circulation.

Sampling

Blood was drawn into test tubes before, 30 minutes after and 2 hours after surfacing. The volume drawn per sample was approximately 5mL.

Chemicals

Thiourea (VWR Chemicals, ref.M226), Urea (GE Healthcare/Plus One, ref.17-1319-01), CHAPS (ARESCO, ref.M127), DTT (GE Healthcare/Plus One, ref.17-1318-02), Bromophenol Blue (Sigma, ref.B0126), Bradford (BioRad ref.500-0006), Acrylamide

(Applichem, ref.A3626), SDS (Merck Millipore, ref.8.22050), APS (Interchim, ref.UP306098), TEMED (Sigma, ref.T9281), Agarose (Interchim, ref31772L), IodoAcetate (GE Healthcare/Amersham, ref.RPN6302), EDTA (Amresco, ref.0245), Glycerol (VWR Chemicals, ref.24387-292), Coomassie Blue (GE Healthcare/Plus One, ref.PhastGel Blue R-350/17-0518-01), Methanol (VWR, ref.20847.368), Acetic Acid (Sigma, refA6283), CuSO₄ (Acros Organic, ref.1977110000)

IEF and SDS-PAGE analysis

For proteomic analysis, approx. 10 μ L of plasma (500 μ g of total protein) taken before, 30mn and 2h after the dive on 4 divers were added to 250 μ L of Destreak rehydration solution (GE Healthcare) with 1% of IPG buffer pH 4-7 (GE Healthcare). IPG strips (pH 4–7, 13 cm; GE Healthcare) were passively rehydrated with the protein solution in IEF wells for 14 h. Isoelectric focusing was conducted using an Ettan IPGphor3 IEF (GE Healthcare) with the following protocol: 250 V for 15 min, 500 V for 2 h, gradient voltage increased to 1 000 V for 1 h, gradient voltage increased to 8 000 V for 2.5 h, 8 000 V for 3 h, and finally reduced to 500 V. IPG strips were then placed in an equilibration solution (SDS 2%, urea 6M, EDTA 0.5M, bromophenol blue 20mg, Tris-HCL 1.5M, Glycerol 30%) with DTT (10mg/mL) for 15 min, and in the same equilibration solution with IAA (48mg/mL) for the next 15 min in order to trigger the carbamidomethylation of cysteine residues. IPG strips were placed on top of 12% polyacrylamide gels, which were run in TGS 1X (Tris 250mM, Glycine 1920 mM, SDS 1%) in thermo-regulated electrophoresis unit at 10°C (SE 600; Hoefer Inc.), at 10mA per gel for 1 h and then 30mA per gel until complete migration. Gels were subsequently stained with Coomassie Blue (PhastGel R350, GE Healthcare) and unspecific coloration was destained with an aqueous solution containing 30% methanol and 7% acetic acid. The resulting gels were scanned with a transparency scanner (G:BoxChemi XL 1.4; SynGene) in gray scale with 16-bit depth and a resolution of 100 dpi. This bidimensional electrophoresis protocol is technically biased toward the most abundant proteins, with a cut-off of 100ng per sample.

Gel image analysis and statistical analysis of protein's abundance

Images were aligned and spots were detected and quantified using the ProgenesisSameSpots software (version 3.3, Nonlinear Dynamics) with manual alignment completed by automated algorithm. All detected spots were manually checked and artifact spots were removed. Data were exported as volume raw values and statistical analyses were conducted in R[14] using the package prot2D[15] from the Bioconductor suite[16]. Data were normalized (quantile normalization) and the samples from each time point were compared between each other (n=4 for 3 groups). For comparisons, we used a moderated t-test, which is a modified t-test, for which the standard errors were moderated across spots, increasing the reliability of the test[15,17]. Once the values of moderated t-test were calculated, p-values were corrected for false discovery, in order to take into account multiple comparison issues. Spots with a false discovery rate (fdr) threshold lower than 0.1 were considered as differentially expressed.

Results

Bubble scores: VGE bubble grade 30 min postdive was rather high, with the majority of divers at grades 3 or 4A. (Median 4A, Max 4B, Min 1). None of the divers suffered any DCS symptom during the experiment.

2-DE Proteomic analysis of plasma proteins: Plasma proteins extracted from each group were separated by 2-DE.

Electropherograms were analysed by using progenesis SameSpot software (version 3.3). In all, 454 proteins spots could be aligned on all gels, and their relative intensities (normalized volumes) were extracted for statistical analysis by using the recently developed prot2D R package. No protein spot showed a significantly changed abundance (fdr < 0.1) between the tested times. A shown in table 2, even among the 7 protein spots which were the most modified, fdr was not lower than 0.1.

Discussion

The aim of this study was to investigate potential plasma proteome changes within asymptomatic divers. As VGE grade testify, the SCUBA dive profile to 18 msw on compressed air induced saturation levels high enough to observe high VGE grades, even if a wide inter-individual variability was observed. 2-DE proteome analysis successfully aligned 454 distinct proteins spots. However, this experiment failed to identify any significant protein changes in asymptomatic divers.

Our previous study analyzed the plasma proteome of rats exposed to a simulated hyperbaric protocol, the animals being separated in 3 groups; a non-diving group, an asymptomatic group and a group of animals displaying DCS symptoms. No variation of the plasma proteome was found between the control and asymptomatic group, but the plasma proteome of DCS rats was significantly different from that of the asymptomatic group. As a matter of fact, 9 protein spots showed significantly changed abundance. One protein, identified by mass spectrometry as the tetrameric form of transthyretin, almost disappeared in DCS rats. In addition to the near disappearance of transthyretin tetramers in DCS rats, Apolipoprotein A1 (APoA1), Alpha-1-antiproteinase or alpha-1-antitrypsine (A1AT) and Alpha-1-antichymotrypsin (Serpina3K) displayed a mild change in abundance between the rats with and without DCS. In this previous study, only the plasma sampled 30 minutes after the dive were compared for the control and asymptomatic groups. In the present study, each diver was his own control for three separate sampling times; before diving, 30 minutes and 2h after surfacing. Therefore, this protocol would enable detection of smaller differences than in our previous study by taking into account inter-individual differences. It also allowed us to check for any slower changes which may occur further from the dive. As previously cited, Dujic et al. showed that divers developed asymptomatic physiological modifications following this dive profile[2,18]. Interestingly, this current study identified no changes in the diver's plasma proteome. Proteins that had altered abundance in the previous rat study did not appear modified among asymptomatic human divers.

Apolipoprotein ensures lipoprotein solubilization in the blood. Cholesterol metabolism is affected by variations in ApoA1, and may have an impact on the risk of heart diseases. ApoB/Apo1 blood ratio is a common marker of cardiovascular risk, Apo1 being considered as an antioxidant and an anti-inflammatory protein, constitutive of High Density Lipoproteins (HDL). HDL protective activity may however be altered during acute inflammation, HDL being converted into a dysfunctional and proinflammatory particle unable to prevent Low Density Lipoprotein (LDL) oxidation[19,20]. Amyloid pathologies have also been related to variations in Apo1 abundance[21]. In the present study, Apo1 abundance was not modified among asymptomatic human divers. Alpha-1-antiproteinase or alpha-1-antitrypsine (A1AT), is described as a protease inhibitor belonging to the serpin (Serum Protease Inhibitor) family, protecting tissues from damage caused by inflammatory processes[22]. Its blood abundance may increase during inflammatory events. Serpina3K (alpha-1-antichymotrypsin), a protein also belonging to the serpin family and having a protective effect on the lower respiratory tract[23], have seen its abundance decreased among rats in DCS in the previous study. We previously hypothesized that this protein would have protected asymptomatic animals from pulmonary inflammation, their Serpina3K abundance being 2.3 fold superior compared to DCS group. In previous studies, Transthyretin (TTR) has been described as a blood carrier for thyroxin (T3), triiodothyronine (T4) and retinol[24], and we showed that the abundance of its tetrameric (active) form strongly decreased in animals suffering from DCS [9]. Such decreased abundance has been described in various amyloid pathologies such as familial polyneuropathy, amyloidotic cardiomyopathy, and senile systemic amyloidosis[25]. Interestingly, this protein is also used as a negative acute-phase marker of inflammation, TTR concentration sharply decreasing in case of acute inflammation[26]. **Recently, Domoto et al. showed an elevation of TTR plasma abundance during saturation diving before returning to pre-dive levels after the dive[27].**

Eftedal et al. have already showed that gene expression, and particularly the blood transcriptome was changed in similar dives[8]. These findings imply that some blood proteins should also be altered also among our divers, if possibly at low amounts. In the present study, we used the same 2-DE methods that were used in our previous study on rats. As cited in our previous publication, the proteins that were identified then are rather abundant in the plasma. Bidimensional electrophoresis is associated with a variable loss of proteins[28] and is technically biased toward abundant proteins that are likely to represent general responses to physiological stress[29]. **Domoto et al. recently used a precipitation step with 1:10 cold acetone[27]. However, preliminary**

experiments preceding our first study on the plasma proteome of rats showed that the use of a precipitation step led to biased and non-conclusive results and therefore, has been used neither in the first study nor in the present work, for the sake of comparison between the two studies. However, it should be noted that this protocol has still successfully separated 454 protein spots that have not been cloaked by the most abundant proteins. Coomassie blue staining was chosen over silver staining to ensure maximum reproducibility of our protocol[30]. However, even if this staining technique is reproducible, it leads to less sensitive results. Taken into account these considerations, changes in gene expression that were showed by Eftedal et al. during diving[8] may occur in this study but without inducing abundance changes for the proteins that have been spotted in 2-DE, given the fact that these experiments are less sensitive than gene expression analysis. This could explain why we did not observe any change of the blood proteome among these divers, even if inflammatory processes may have taken place during this dive. However, these results might be explained by the use of an animal model in our previous study, which leads to a bias in our interpretation. These findings show nevertheless that the search for blood biomarkers on asymptomatic divers, even with a high bubble grade, are unlikely to be successful. However, as our previous proteomic study suggested, DCS could elicit changes in the blood proteome that are strong enough to be used as an early biomarker of DCS. **Given the wide variability of correlation between a high bubble grade and DCS occurrence[31], a transthoracic echocardiography is not discriminant enough to assess an early development of DCS. In this regard, an early biomarker of DCS would be a tool which would allow discriminating DCS versus non-DCS, and would be useful in differential analysis of doubtful cases.** Thus, future studies will investigate the impact of DCS on human diver's plasma proteome. This would enable to check the value of the potential biomarkers we previously identified, especially TTR, and thus lead to a better medical care of divers. **If a particular involvement of the tetramer of TTR is confirmed, we will focus then specifically on this protein.**

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Conflict of interest

The authors declared no conflict of interest.

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Table 1. Anthropometric data

Parameter	Mean±SD
Age, years	39.3±9.2
Height, cm	186±6.0
Weight, kg	88.0±11.6
BMI	25.3±2.8

Table 2: Changes in protein abundance. These results take into account the seven proteins that displayed the highest changes in their abundance. None of these changes are significantly different between observed groups ($fdr < 0.1$).

Protein spot	Before diving/30min after diving Ratio	Before diving/2h after diving Ratio	30min after diving/2h after diving Ratio	Modified p-value ANOVA (F-test)
92	3,63	1,90	0,52	0,13
349	1,94	1,47	0,75	0,13
125	0,42	1,81	4,33	0,15
27	0,80	2,38	2,98	0,21
88	0,76	0,55	0,73	0,22
117	1,44	1,58	1,10	0,22
463	0,68	1,27	1,86	0,22