Doctoral theses at NTNU, 2018:322

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Commercial Saturation Divers:

Blood biochemistry and perceptions of acclimatization to oxygen and oxidative stress; in saturation and back to surface

NTNU
Norwegian University of
Science and Technology

Doctoral thesis

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Thesis for the Degree of
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Kommersiell Metning Dykkere:
Blodbiokjemi og oppfatninger av akklimatisering til oksygen og oksidativt stress; i metning og tilbake til overflaten.

Metningsdykking for olje- og gassvirksomhet i Nordsjøen har gjennomgått stor utvikling siden starten på 60-tallet. Dykkernes sikkerhet er forbedret, og det blitt registrert svært få dekompresjonssykdommer i løpet av de siste 20 årene, takket være mer konservative dykkeprosedyrer. Vår kunnskap om helsevirkningen av metningsdykking, dvs. å leve og arbeide under trykk og forhøyet oksygenpartialtrykk (ppO2) i lengre perioder, kan fortsatt forbedres.

I dette prosjektet har vi undersøkt metningsdykkere under arbeid på norsk og britisk kontinentalsokkel gjennom delstudier av genuttrykk i blodceller, biomarkører for vaskulær funksjon, virkninger av vitamin C og E-inntak, blodnivåer av hemoglobin (Hb) og erytropoietin (EPO) samt dykkernes oppfatning av egen fysisk tilstand.

Vi fant flere indikasjoner på at dykkere fysiologisk akklimatiseres til det hyperbar miljøet. I metning fikk de en reduksjon av kapasiteten for oksygentransport i blod, som økte i løpet av det første døgnet direkte etter metning. Globalt genuttrykk og blodnivåer av biomarkører indikerte at deres vaskulære funksjon returnerte til en tilstand som ligner pre-metning i det dykkerne kom ut av metning; og det var en økning av dykkernes eget antioksidantforsvar som potensielt gjorde inntaket av ekstra antioksidant-kosttilskudd unødvendig.

Navn kandidat: Fatima Zohra Kiboub
Institutt: Institutt for sirkulasjon og bildediagnostikk
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Contents

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My warmest thanks go to my academic supervisor Ingrid Eftedal. Ingrid's continuous and tireless support helped me to go through all the work with more confidence, every step of the way. I never thought it would be possible to find in one person a mentor, a moral support, a faithful friend and a real role model of strong researcher female. It has been a real pleasure and privilege to work with her.

My thanks go also to my industrial supervisor Øyvind Loennechen, who not only hired me in Technip Norge in 2011 and was my manager for a while; but had faith in me and did all he could to make this PhD project possible. Even in the downtime of the oil and gas industry in Norway, he was always supportive of the project continuation and answered patiently to my continuous flow of questions whenever he could.

I would also never forget all the work done by the project administrator, my current manager now and hopefully for many years to come; Morten Hinna. He had trust that I could accomplish my work duties alongside with the PhD since the idea of the project first came in. His support and understanding were precious to me, especially when things didn't go as I have planned or wished them to.

Working alongside Andreas Møllerløkken, Astrid Hjelde, Costantino Balestra, Jean Pierre Imbert has not only been fruitful, but a real pleasure. I know I will always have good friends and collaborators in them in the future.

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Last but not least, all my love goes to my dear family in Algeria. My parents Yahia Kiboub and Dada Aicha; and my siblings Mustapha, Ali, Djihane and Abdelhak have always been with me in thoughts and prayers throughout all my endeavours. I could not wish for a better family who gives me unconditional love and always encourage me to further my education, even so far away from my homeland.

Fatima Zohra Kiboub, Stavanger, August 2018

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Norsk sammendrag – Summary in Norwegian

Profesjonelle metningsdykkere har arbeidet olje- og gassindustrien i Nordsjøen siden 1960-tallet. Disse dykkerne jobber under krevende forhold; de utfører arbeidsoppgaver som kan være komplekse og fysisk tunge, på gjørmete havbunn, og i mørkt og kaldt vann. Samfunnets behov for energi har vært en drivkraft i utvinning av olje og gas. Dette har ført med seg behov for å flytte grenser for dybde og varighet i metningsdykking. Kunnskap om fysiologiske effekter av endret omgivelsestrykk har vært i utvikling siden Haldanes tid, og det samme har utstyr for undervannseksplorasjoner. Men det er ennå mye vi ikke vet om hvordan kroppen reagerer på de ekstreme miljøene i dykking. Trykkfallsyke (TFS) har ikke vært observert i offshoredykking i Nordsjøen på flere tiår etter at konservative dykkerprosedyrer ble obligatorisk for alle operatører på norsk kontinentalsokkel, gjennom et samarbeid mellom myndigheter, industri og fagforeninger. Dette trepartssamarbeidet har bidratt til innebygd sikkerhet. Men det finnes noen unike stressfaktorer som det må tas hensyn til i metningsdykking; oksidativt stress er en utfordring fordi dykkerne puster høyrer partialtrykk av oksygen (ppO_2) enn det som fins i vanlig luft. Bruken av økt oksygen er en balanse, der høy ppO_2 i pustegassen gir raskere eliminasjon av inertgass under dekompresjon etter dykking.

Denne avhandlingen presenterer og diskuterer effekter av langvarig eksponering for økt ppO_2 i kommersiell metningsdykking. Dykkerne i de tre delstudiene i avhandlingen var på kommersielle arbeidsoppdrag på undervannsinstallasjoner for olje- og gassindustrien offshore, der de bodde om bord på et dykkesupportskip på norsk og britisk sektor. Effekter av langvarig eksponering for økt oksygen ble studert ved hjelp fullgenom genuttrykksanalyse og biomarkører for vaskulær funksjon; i.e. inflammasjon, endotelfunksjon og fibrinolyse. Analysene ble gjort på blod samlet inn på norsk kontinentalsektor før og etter en arbeidsperiode i metning; og med og uten daglig inntak av antioksidant vitaminer C og E i anbefalte doser (Paper I). Deretter ble evolusjon av hemoglobin (Hb) og erytropoietin (EPO) i blod rett etter og et døgn etter en avsluttet arbeidsperiode undersøkt i metningsdykkere på britisk sektor (Paper II). Dykkernes subjektive vurdering av egen fysiske tilstand etter en arbeidsperiode i metning ble undersøkt ved hjelp av et spesielt tilpasset spørreskjema i løpet av flere offshore dykkeoperasjoner i både norsk og britisk sektor (Paper III).

Akklimatisering til høyt ppO_2 ble påvist i form av nedregulering av gener involvert I oksygentransport; i.e. heme, hemoglobin, røde blodceller og EPO, og oppregulering av kroppens primære antioksidanter; i.e. superoksid dismutase 1, katalase og glutation syntetase. Gener involvert i immunaktivitet og inflammasjonssignaler økte i uttrykt etter dykking. Vitamintilskuddene hadde ingen effekt verken på genuttrykk eller biomarkører etter sammenlignet med før metning. Akklimatisering til høyt ppO_2 under metning ble også observert i form av lav Hb rett etter fullført dekompresjon, fulgt av en rask øking av EPO i løpet av de neste 24 timene i det kroppen oppfatter vanlig luft som en relativ hypoksi (Paper II). Dykkerne selv ga rapporterte fysiske (blekhet og fatigue), og mentale effekter (hodepine og konsentrasjonsproblemer) (Paper III); som knyttes opp mot funnene i de to foregående artiklene (Papers I og II), og kan forklares ved redusert kapasitet for oksygentransport i blod etter langvarig eksponering for høyt ppO_2.

Dette arbeidet bidrar med ny kunnskap om sammenhengen mellom de tegn og symptomer dykkerne selv opplever, og biokjemiske mål på akklimatisering til omgivelsene i metning.

Vellykket akklimatisering til hyperoksi manifesterte seg i form av redusert oksygentransport i blodet under og like etter metning. Biomarkører for vaskulær funksjon var uendret før og etter metning; og økingen av kroppens egen antioksidantforsvar kan forklare at inntak av ekstra antioksidanter som vitamin C og E ikke hadde målbar effekt etter metning. Vi håper resultatene av disse studiene bidrar til økt forståelse av fysiologiske effekter av metningsdykking i operative settinger.

Executive summary

Professional saturation divers were partly behind the development of the offshore oil and gas industry in the North Sea since the 60ies. The saturation divers face challenging conditions, including the execution of often complex tasks in difficult conditions; on muddy sea-beds and in pitch black cold waters. The increasing need for energy has been driving the offshore oil and gas exploration to continuously push the limits of depth and pressure. Knowledge about the effects of elevated pressure on the human body, and the equipment for subsea exploration have improved tremendously since the days of Haldane; but we are far from presuming to know everything just yet. Decompression sickness (DCS) incidents haven't been reported in Norway for several decades, as a conservative diving procedure framework was made normative for all companies operating on the Norwegian Continental Shelf (NCS) in a cooperation between Authorities, Industry and Unions. The tri-part cooperation has ensured that the diving industry became intrinsically safer. Nonetheless, oxidative stress still presents a challenge to the divers' health due to the higher than normal partial pressure of oxygen (ppO_2) in their breathing gas. Saturation procedures use of elevated oxygen content is a trade-off; as high ppO_2 in the breathing gas mixture accelerates the inert gas elimination during decompression.

What is discussed in this present work, is the effect of a long exposure to elevated ppO_2 on the divers. Saturation divers working in real offshore oil and gas subsea construction projects; onboard a dive support vessel in the NCS and the UK sector were examined to study the effect of prolonged exposure to elevated oxygen levels. Genome wide gene expression and plasma biomarkers of vascular function (i.e. for inflammation, endothelial function and fibrinolysis) were assessed after a period of saturation; with and without daily intake of vitamin C and E supplements (Paper I). For that, blood samples were taken immediately pre- and post-saturation during operations in the NCS. Also, the evolution of haemoglobin (Hb) and erythropoietin (EPO) was investigated in saturation divers during operations in the UK sector (paper II). The parameters were measured in blood samples taken immediately pre- and post-saturation; then 24-hours post-saturation. The divers' perception of their own physical state after a period in saturation was investigated using specifically developed questionnaires that were answered during several offshore campaigns in both sectors (Paper III).

Acclimatization to the elevated ppO_2 was demonstrated by a down regulation of the genes involved in oxygen transport (i.e. heme, haemoglobin, erythrocytes and EPO) and upregulation of primary endogenous antioxidant genes (i.e. superoxide dismutase 1, catalase and glutathione synthetase); while there was an increased expression of genes involved in immune activity and inflammatory signalling pathways. The antioxidant vitamin supplements had no effect on either the gene expression profiles nor the levels of plasma biomarkers post-saturation comparing to pre-saturation values (Paper I). Acclimatization to the elevated ppO_2 during saturation could also be seen in the decrease of EPO and Hb levels immediately post-saturation and the rapid increase of EPO 24-hours

post-saturation as the body perceives breathing normobaric air as relative hypoxia (Paper II). Finally, the divers expressed their perception of some physical changes (relative pallor and fatigue) and mental changes (headaches and difficulty to focus) (Paper III); which could be directly linked to our previous findings (paper I and II) and could be explained by the reduction of blood transport capacity that occurs after a long exposure to elevated ppO2.

This work allows us to assert that the changes and symptoms that most of the divers perceive during and/or after surfacing can be the result of acclimatization to the saturation conditions. A successful acclimatization to hyperoxia translates in the reduction of blood transport capacity during saturation and immediate increase post-saturation, the return of the vascular function to a state similar to pre-saturation condition by the time they surface; and the increase of endogenous antioxidant defence that potentially renders the intake of antioxidant supplements unnecessary. We hope that this work has contributed to improve our understanding of the effects of saturation diving in real operations.

Abbreviations

CNS:	Central nervous system
CO_2:	Carbon dioxide gas
CRP:	C-reactive protein
DCI:	Decompression illness
DCS:	Decompression sickness
DDC:	Deck decompression chamber
DP:	Dynamic positioning
DNA:	Deoxyribonucleic acid
DSV:	Diving support vessel
ECG:	Electro-cardiogram
ELISA:	Enzyme-linked immune-sorbent assay
EPO:	Erythropoietin
FMD:	Flow mediated dilation
Hb:	Haemoglobin
Hct:	Haematocrit
HDL:	High density lipoprotein
HIF-1α:	Hypoxia inducible factor-1 alpha
ICAM-1:	Intra-cellular adhesion molecule-1
IL-6:	Interleukine-6
LDL:	Low density lipoprotein
LSS:	Life support supervisor
LST:	Life support technician
mRNA:	messenger ribonucleic acid
msw:	Meter of sea-water
NCS:	Norwegian continental shelf
NO:	Nitric oxide
O_2:	Oxygen gas
PAI-1:	Plasminogen activator inhibitor-1
ppO_2:	Partial pressure of oxygen
ROS:	Reactive oxygen species
ROV:	Remotely operated underwater vehicle

SDC:	Submersible decompression chamber (Diving bell)
SPHL:	Self-propelled hyperbaric lifeboat
TNF-α:	Tumour necrosis factor-alpha
TUP:	Transfer under pressure
VO_{2max}:	Maximum oxygen uptake

Definitions

Biomarkers: quantifiable factors that can be detected and measured in the blood or tissues and serve as indicators of a biological or pathological state, e.g. proteins, cells, genes, enzymes or hormones.

Decompression: slow reduction of ambient pressure to allow the body to get rid of the inert gas dissolved in the tissues, while returning to surface (normobaric) pressure.

Decompression illness (DCI): systemic or local clinical manifestations that may occur after decompression. It includes decompression sickness, arterial gas embolism and barotrauma.

Decompression sickness (DCS): a disease caused by gas bubbles formed by excess inert gas coming out of solution in the tissues and blood vessels.

Dynamic positioning (DP): a system that maintains the DSV in a relative stationary position without the need for mooring with anchors by using several reference points; e.g. transponders placed on the seabed and a global positioning satellite. The computer-controlled vessel propellers and thrusters are then operated to ensure the position is kept with regard to the transponders. For diving operations, class 2 (DP2) or class 3 (DP3) control systems are commonly used.

Flow-mediated dilation: dilation of the blood vessels caused by the release of nitrogen oxide (NO) by the vascular endothelium and consequent increase of blood flow. Measurement of the FMD is conducted by measuring the brachial artery dilation following a certain period of forearm ischemia; using ultrasound.

Gene expression: the production of mRNAs transcribed from the DNA; and eventually manifested as gene products, e.g. proteins, hormones, enzymes...etc.

Genome-wide gene expression profiling: the simultaneous measurement of the expression of all genes in a sample.

Hyperbaric: increased ambient pressure.

Hyperoxia: increased partial pressure of oxygen in a breathing gas relative to that of normobaric air.

Hypoxia: decreased partial pressure of oxygen in a breathing gas relative to that of normobaric air.

kPa: kilopascal. 100 kPa = 1 bar.

Oxidative stress: imbalance between the production of reactive oxygen species and the body's antioxidant defences, that can damage cells and tissues and disrupt normal cellular signalling.

Saturation dive: a dive in which the body tissues become completely saturated with inert gas equal to the surrounding pressure (depth), allowing for unlimited bottom time.

Storage depth: the depth at which the divers are stabilized post compression. The diver's storage depth can be altered as long as the blowdown rates, holds and system decompression rules are adapted.

Preface

This study was carried out between 2014 and 2018 at the Department of Circulation and Medical Imaging, the Norwegian University of Science and Technology NTNU, Trondheim, Norway. It was based on an Industrial PhD agreement between the Norwegian Research Council (NRC); TechnipFMC in Norway (formerly Technip Norge AS until January 2017) and NTNU. The Industrial PhD program's target is to encourage the involvement of the industry in research and innovation and link between the industrial and academic fields.

The candidate was already employed at TechnipFMC in Stavanger and was enrolled at NTNU for the duration of the PhD. The PhD was funded by the NRC and TechnipFMC, with a technical supervisor from TechnipFMC and a supervisor and co-supervisor from NTNU. The candidate was also awarded a grant for an Overseas Research Collaboration with fellow researchers from Belgium and France to combine respective research results in the same field (parallel research funded separately by TechnipFMC).

The papers below will be referred to in their respective numbers throughout this study.

List of papers

I. Blood gene expression and vascular function biomarkers in professional saturation diving
Fatima Zohra Kiboub, Andreas Møllerløkken, Astrid Hjelde, Arnar Flatberg, Øyvind Loennechen, Ingrid Eftedal

Published at Frontiers in Physiology / Environmental, Aviation and Space Physiology, 16 July 2018.

II. Hemoglobin and erythropoietin after commercial saturation diving
Fatima Zohra Kiboub, Costantino Balestra, Øyvind Loennechen, Ingrid Eftedal
Published in Frontiers in Physiology / Environmental, Aviation and Space Physiology, 21 August 2018.

III. Commercial divers' subjective evaluation of saturation
Jean Pierre Imbert, Fatima Zohra Kiboub, Ingrid Eftedal, Øyvind Loennechen, Costantino Balestra
Submitted to Frontiers in Psychology / Environmental Psychology, 17 September 2018.

Contributions

All the authors contributed to the manuscripts with drafting, critical reviews and approval of final versions.

Paper I: F.K., A.M., Ø.L. and I.E. conception and design of research; F.K. I.E., A.H., A.F., collection, analysis and interpretation of data.

Paper II: F.K., I.E., C.B. and Ø.L. conception and design of research; F.K., Ø.L. collection of data; F.K., C.B. and I.E. analysis and interpretation of data.

Paper III: J.P.I., C.B. and Ø.L. conception and design of research; J.P.I., F.K. and Ø.L. collection of data; J.P.I., F.K., I.E. and C.B. analysis and interpretation of data.

1 Introduction

Commercial offshore saturation divers work and live in closed hyperbaric chamber systems on specialized construction vessels for consecutive periods of approximately 3-4 weeks. The work consists of 12 hours shifts with a maximum of 6 hours in the water, the rest of the time is spent in the dry hyperbaric chamber onboard the vessel and inside the diving bell that transfers them from the diving support vessel (DSV) to the seabed. The divers are exposed to elevated ambient pressure with frequent pressure changes, as well as higher partial pressure of oxygen (ppO_2) compared to a normobaric environment. Saturation refers to the state when the diver's tissues become saturated with the dissolved breathing gas at equilibrium with the surrounding pressure such that increased exposure time does not alter the required decompression duration.

The commercial saturation divers need to be physically fit and in good health to live and work under hyperbaric conditions.

This study was initiated with the sole purpose to improve our understanding of the effects of saturation diving on the divers' health during real offshore operational settings. It investigates the effects of the hyperbaric work environment, as well as antioxidant vitamin C and E supplements intake on the levels of genetic activity in the peripheral blood cells and biomarkers for vascular function in professional saturation diving (paper I), acclimatization to hyperoxia through the assessment of the haematological status during the initial 24 h after a period of saturation (paper I and II), and how the divers perceive the saturation and its mental and physical effects on them with emphasis on fatigue and headaches at the end of the decompression phase (paper III).

1.1 Brief history and applications of saturation diving in Norway

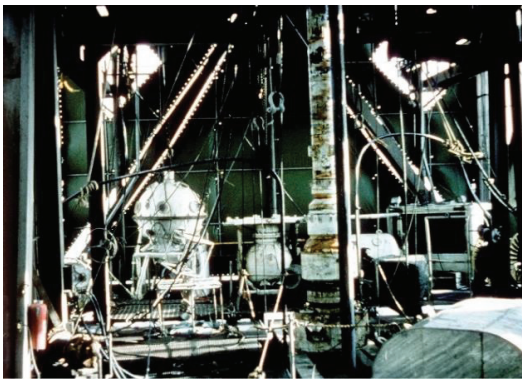
The first experimental saturation dives were initiated in December 1938 by Edgar End and Max Nohl, but were interrupted by the second World War. The experiments were resumed by the end of the 50ies in both France and the USA (1). At that time, the effects of pressure were poorly understood, but knowledge was built gradually from the experimentation of different breathing gas mixtures and diving tables to increasing depths. Indeed, in 1966; when diving started in the Norwegian Continental Shelf (NCS) to depths from 70 msw or deeper, commercial saturation diving was still at its experimental stages. Most of the

advancements recorded in the diving systems and equipment were led by the Navy and private oil and gas companies in the USA.

In Norway, oil and gas-related manned underwater operations started with the drilling of the first exploration well in 1966 for Esso Exploration. The American company Ocean Systems was contracted to conduct the diving operations from the Ocean Traveler drilling platform (2). According to one of the Norwegian pioneer diver, Leif-Tore Skjerven, the first dives were conducted in June 1966 using a Yokohama recirculation helmet, breathing a gas mixture composed of helium and oxygen. Closed diving bells were introduced some years later. The first drilled well in block 8/3 was dry. In the second well in block 25/11, a Norwegian diver named Idar Johnsen Myrset went down to 130 msw, setting a record in the Norwegian sector at that period. But there was little success in the wells that were drilled the first years; and exploration drilling was almost ended until a successful discovery was made in the Ekofisk field in 1969 (2). For the divers thermal conductivity of helium was an issue and hypothermia a real risk; until the use of warmed breathing gas and hot water diving suits was introduced in 1972 (2, 3). In 1974, the Norwegian-built Arctic Surveyor was the world's first dynamically positioned (DP-equipped) DSV; especially designed for subsea construction and equipped with a saturation diving system. In 1975, it was approved for operating in the NCS and used in the construction of the Ekofisk field.

A

B



C

D

Figure 1. A. Skjerven and Jentoft ready to dive with a Yokohama heliox mixed gas diving helmet onboard the Glomar North Sea, 1969. B. Ocean Traveler drilling platform and the first Ocean Systems diving bell used in the NCS for bounce diving, 1966. C. Yokohama diving helmet. D. DSV Arctic Surveyor (Photos: Norsk Oljemuseum).

Different techniques of diving were used offshore depending on the depth, type of operation and equipment available at that time for oil and gas-related diving; i.e. surface-supplied diving, wet bell diving, bounce diving with a closed bell, lock out submarines, scuba diving and 1 atmosphere armoured suites. Bounce diving was the most used at great depths, but it was not very efficient. I.e. for an hour's work on the Ekofisk field seabed in 1975, 4 hours and 25 minutes were needed for decompression before the next dive could be conducted, which gave only 4 hours of work on the seabed during a workday. This also took a toll on the divers' health. With saturation diving, the divers could work for up to 18

hours per day for several days/weeks, and decompress only one time when their tasks were performed. With the development of the DSV Dynamic Positioning (DP) systems and organization of the dive support teams; diving activities could eventually be performed offshore 24 hours a day (2).

1.2 Commercial saturation diving accidents in the NCS

The first years of saturation diving were far from safe for the pioneer divers that ventured into the field in the 1970ies. When asked how it felt to be a diver in the 1960-70ies, Leif Tore Skjerven described diving in the North Sea to be “dark and lonely”.

Since the 1970ies vast amounts of research and development have led to significant improvements in diver education, procedures, equipment and facilities utilizing the latest within industry automation process technology.

According to the Norwegian Petroleum Safety Authority, the last time a fatality occurred during a commercial saturation dive on the NCS was in 1987. The last case of decompression sickness (DCS) was registered in 2002, whereas most of the cases that occurred in the past were registered between 1987 and 1993. From 1990 to the present day, saturation diving was conducted according to standardized frameworks, and most of the incidents related to it were muscles/joints injuries, minor wounds and mostly; microbial infections (4).

1.3 Physiological challenges during saturation diving

A Norwegian study has previously shown that divers in the North Sea; who started their working careers before the 1990ies and experienced DCS, had significant reduction in health-related quality of life (5). DCS is a rare event (6), but even in the absence of symptoms, diving causes vascular phenotype changes associated with functional impairment of the vascular endothelium (7, 8). The endothelium forms a barrier between the bloodstream and other tissues, and acts as a key sensor of physical and chemical changes in the bloodstream. It is essential for the regulation of fluid transport into the extravascular tissue; a determinant of cellular trafficking and a regulator of coagulation and blood pressure (9). Repeated exposure to elevated ppO_2 for prolonged periods of time can impair the endothelial function (10). Ambient pressure reduction during decompression from a dive causes inert gas bubble formation in the blood stream, and

these bubbles may also affect the endothelium (7, 11, 12). The combined effects of environmental stressors experienced during diving have been shown to promote pro-inflammatory development; with expression of cytokines and adhesion molecules, increased coagulation and elevation of circulating micro-particles (8, 13, 14).

1.3.1 Oxidative stress and redox biology

In normal conditions, the body's endogenous antioxidant system scavenges ~~the~~ excess reactive oxygen species (ROS) and other free radicals in redox reactions to preserve the homeostasis (15). Higher than normal oxygen levels are a major cause of oxidative stress (16); the latter is caused by the disturbed balance between oxidants and antioxidants (17), resulting in the increased presence of ROS in the tissues. Excess ROS can cause damages to the tissues, especially in the lungs and the central nervous system (CNS), by directly interacting with and damaging DNA (18), peroxidation of lipids, and oxidation of proteins and other peptides (16, 19). A previous study conducted during an experimental deep saturation dive suggested that the use of antioxidant vitamins could prevent impairments caused by the oxidative stress (20).

1.3.2 Antioxidant supplements

Taking antioxidant food supplements has become more and more popular, as an effort to boost the body's endogenous antioxidant system. In our study, we chose to supply some of the divers with antioxidants in the form of commercially available vitamin C and E. These vitamins are potent antioxidants with previously reported positive effects on the endothelial function in divers and the general population. Indeed, it has been demonstrated that the acute use of a combination of Vitamin C and E attenuates the negative effects on the heart, pulmonary and brachial artery in air-diving males (10, 21-23). This effect depends on the user's fitness level.

1.3.2.1 Vitamin C

Vitamin C, also known as L-ascorbic acid; is one of the most commonly known vitamins. It is water-soluble and naturally present in a multitude of fruits and vegetables. As it is not produced by the body's endogenous antioxidant system, it is important to consume the quantities required for maintaining healthy bodily functions. With its ability to donate one or two electrons, L-ascorbic acid is a potent antioxidant that scavenges ROS and other free

radicals; and is regenerated by reduction with glutathione, NADH or NAD-PH (24). Lack of vitamin C is the cause of scurvy, a disease that sailors were exposed to due to the lack of fresh produce during their long trips. Excess of vitamin C can cause gastrointestinal disturbances. The recommended intake dosages vary by age and gender.

1.3.2.2 Vitamin E

Vitamin E, refers to compounds including tocopherols and tocotrienols. α-tocopherol is the most biologically active form of vitamin E in humans. It is fat-soluble and acts as an oxygen scavenger too. Deficiency in vitamin E is very rare in healthy people, even when the intake through diet is low. Excess of α-tocopherol was shown to cause haemorrhage and blood coagulation interruption in animals. The recommended intake dosages vary with the age.

1.3.3 Functional and biochemical markers of vascular function

The state of the vascular endothelium has been linked to venous gas embolism generated after diving (25-27), but with variable results showing both; reduction of endothelial function, and no effect from the diving activity on the endothelial function, measured by flow mediated dilatation (FMD). In this study, FMD measurements were performed before the commencement of saturation diving, and after decompression was finished.

While FMD measurements are direct functional assays, blood biomarkers may also be used as indicators of vascular function, and the measurement of multiple biomarkers may provide a comprehensive assessment of vascular health. Analysing biomarkers across a range of functions may also allow for the comparison of the relative contributions of distinct biological pathways (28). In this study, blood samples obtained from divers immediately before and after hyperbaric saturation dives were analysed with the aim of detecting and interpreting significant changes in biomarker levels (29-33).

1.3.4 Gene-wide gene expression profiling:

DNA microarrays allow the measurement of the expression levels of each gene in the genome (34). This method was used in our study to measure the effects of saturation on the divers and determine which genes were up or downregulated as an effect of hyperbaric hyperoxia and the antioxidants vitamin C and E intake. It was also used to assess the

involvement of specific biological functions and pathways in acclimatization to the environmental stress in saturation diving.

2 Aims and hypotheses

In this study, professional divers' fitness level, vascular endothelial function, blood biomarkers, blood genome expression, haematological status and the divers' perceptions were examined with the aim of providing better understanding of the effects of environmentally imposed stress caused by elevated ambient pressure, frequent pressure changes and oxygen enriched environment in the saturation divers' living atmosphere. The study also investigated the effects of oral antioxidant intake, i.e. vitamin C and E in relation to living in a hyperbaric hyperoxic atmosphere.

The study objectives were:

1) To measure endothelial function as flow mediated dilatation (FMD) in offshore saturation divers.
2) To measure blood biomarkers of endothelial function and vascular health in offshore saturation divers.
3) To assess and interpret the changes in blood cell gene expression after a period of saturation.
4) To determine whether a daily combination of vitamin C and E supplementation during a period of saturation, affects post-saturation endothelial function and biomarkers of vascular health.
5) To measure the changes in the levels haemoglobin (Hb) and erythropoietin (EPO), in saturation divers at surfacing and 24 hours post-surfacing.
6) To link the findings from the different measurements with what the divers perceive during and post-saturation, when submitted to pressure and breathing gas changes.

It was hypothesized that in the divers who did not receive antioxidant supplements while diving, the biomarkers levels post-saturation would indicate reduced vascular function due to increased ambient oxidative stress. This would be indicated by the analysis and comparison of a set of selected biomarkers in the blood samples pre- and post-saturation.

The specific hypotheses were:

- The flow mediated dilation was expected to decrease in the divers who had not received vitamin C and E supplementation during the dive. This would be a direct indicator of the endothelial stress level increase during diving.

- The intake of antioxidant vitamin C and E should contribute to the improvement of the divers' endothelial function. This would have been indicated by changed levels of vascular health blood biomarkers in the divers who received vitamin C and E supplementation comparing to the control group (no vitamin C and E intake). The changes would be reflected on the gene expression patterns.
- The levels of Hb and EPO were expected to decrease in the blood post-saturation comparing to pre-saturation. During the initial 24 hours after decompression, we expected to see an increase in hematological parameters as the divers were acclimatizing back to breathing normobaric air.
- The divers were expected to acclimatize to the changes in living conditions, pressure and breathing gas. As the levels of Hb and EPO would be changing by the end of the saturation period and during the first 24 hours post-surfacing; the acclimatization effects would be manifested in relative pallor, headaches and general fatigue.

3 Methodology

3.1 Ethical approval

All the work in this study was performed on material collected from commercial saturation divers during work assignments on the Norwegian and UK Continental Shelfs in 2015 and 2016 respectively. The study protocol was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, approval reference number 2015/351; and TechnipFMC's Diving and Health services in UK and Norway divisions (the latter known as Technip Norge AS until January 2017). Data and samples collections were conducted according to the Declaration of Helsinki principles for ethical human experimentation (35). All participants were individually informed and provided written consents before inclusion. The Norwegian Petroleum Safety Authority and Operators were informed prior to the study start.

3.2 Study population

The targeted population for this study were saturation divers onboard the DSV Deep Arctic (known as the Skandi Arctic until January 2016); all males, certified and approved for diving in the Norwegian and/or the UK sector. The divers were employed and managed by TechnipFMC, the diving contractor. In order to be eligible to participate in our study, participants had to be cleared for diving by the vessel's hyperbaric nurse after passing through the pre-dive medical examination; and not have a current infection.

Figure 2. DSV Deep Arctic

3.3 Compression and saturation period

The divers were organized in 4 groups of 3 men, each team on a shift of 12 hours overlapping the other teams. Thus, the 4 groups of divers rotate throughout the day to ensure continuous diver "in-water" intervention. After passing the pre-dive medical examination, they were compressed (or blown down) with the rate of 1 msw/min to the storage depth. The latter varied depending on the project and location of the field offshore. The storage depths during the offshore campaigns varied from 100-115 msw in the Norwegian waters (Paper I); 80-90 msw in the UK sector (Paper II) and 110 and 136 msw in the Norwegian and UK sectors respectively (Paper III). During the shift, the divers had 8 hours of bell runs; with maximum 6 hours spent in the water. Both the UK and Norwegian regulations require 20-30 minutes of restitution break between the 3rd and 4th hours of the excursion; inside the bell with the helmet off. During the excursions, two of the divers work in the water while the 3rd diver remains in the bell (bellman) as a rescue and standby diver for monitoring and assisting the divers in the water. Regardless of the above water depths, the maximum duration the divers could stay in saturation was 21 days in the Norwegian sector as per HSE regulation requirements (36), and 28 days in the UK sector as per TechnipFMC Group Diving procedures. Decompression was done according to Table 12 in the NORSOK U-100 Standard in the Norwegian sector (37) and according to TechnipFMC's procedures in the UK sector.

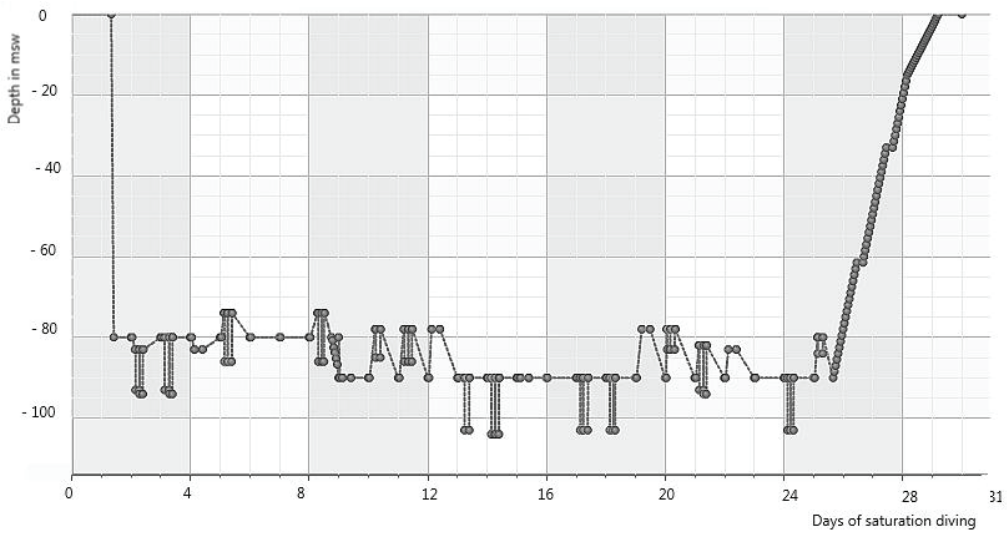


Figure 3. Example of a typical UK saturation period. Compression to a storage depth of 80 msw took 2 hours. The storage depth was increased to 90 msw 8 days after the saturation commenced. The maximum diving depth was 104 msw. Decompression back to surface took 5 days.

3.4 Vessel diving system

The Deep Arctic is equipped with a 24-man saturation diving system, rated to a maximal pressure corresponding to 350 msw. The system includes 6 chambers: four 3-men living chambers (DDC2 to 5) and two 6-men (de)compression chambers (DDC1, DDC6); in addition to 2 Transfer Under Pressure units (TUP 1 and 2). The vessel is also equipped with two closed diving bells (SDC) to transport the divers from the vessel to the seabed, one located at the port-side and the other at the starboard side; and two self-propelled hyperbaric lifeboats (SPHL) connected directly to the living chambers with a capacity to take 18 men each. The diving system is continuously monitored by life support technicians (LSTs) and a supervisor (LSS) working on shifts, from the saturation control room. In operational mode, when in the bell and during work in the water; the divers are monitored and directed by diving supervisors operating from the dive control room, located on the vessel's main deck.

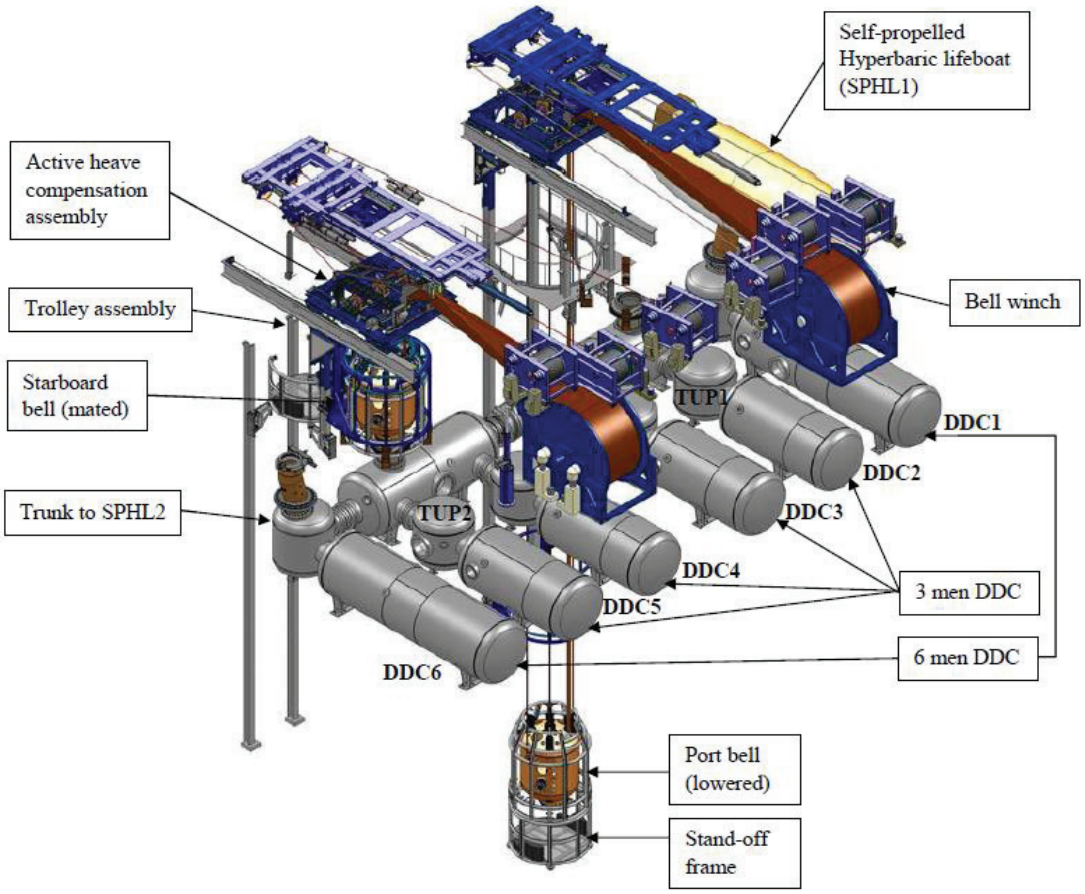
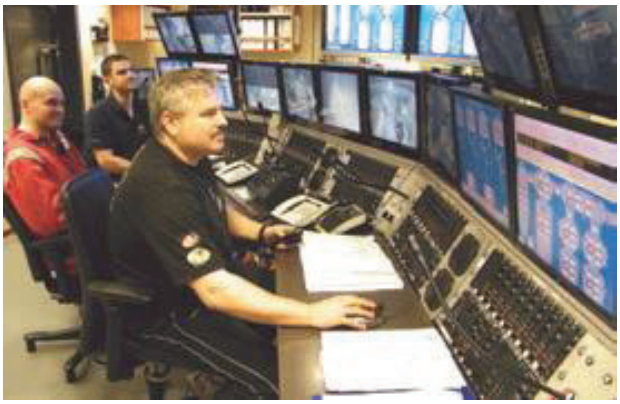


Figure 4. Schematic of the diving system layout onboard the Deep Arctic.

A

B



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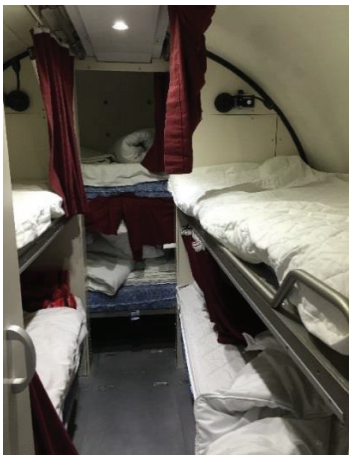

D

Figure 5. A. Diving bell. B. Diving chambers monitored from the saturation control room. C. Sleeping area in a 6 men decompression chamber. D. Recreational area in a 6 men decompression chamber.

3.5 Diving standards, procedures and risk assessments

All manned underwater operations were conducted in accordance with the Norwegian standard requirement for Manned Underwater Operations NORSOK U-100 (38) in the Norwegian sector, and TechnipFMC Group Diving procedures in the UK sector. Project specific procedures that include dive plans, specifying the tasks that the divers should conduct at each dive; were prepared during the engineering phase of the project. The work procedures were risk-assessed to ensure the work was to be conducted in the safest manner

possible, and control measures were taken to ensure that any potential hazards were mitigated in order to prevent incidents and injuries to the men working on the seabed.

3.6 Activities during the diving excursions

The activities that are conducted by the divers during saturation usually include one or more of the following; usually in cooperation with remotely controlled underwater vehicles (ROVs): pipelines tie-ins and hyperbaric welding, inspections of platforms/pipelines and wellheads, cleaning of platforms from marine growth, rigging/lifting and shifting of equipment, anodes installation and replacement, risers' installation, manual or hydraulic bolts-tensioning and removal, pressure barrier testing and access into structures where ROVs are not able to fit in or where they lack movement dexterity and depth of vision.

Handling of heavy equipment on the seabed or in mid-water is generally the most physically demanding task as the divers must carry and install/remove heavy shackles and slings or heavy permanent equipment such as bolts, nuts, seal rings or tooling like sledge hammers, large torque wrenches and bolt tightening equipment. The divers' work is made even harder on soft seabed with deep layers of mud or clay (39). Sometimes conditions are even more difficult with limited visibility or when working in strong currents.

In some areas, where there is a risk that the divers could be exposed to hazardous substances (i.e. hydrocarbons, condensate, drill mud/cuttings, any other chemicals or potential microbial or radioactive contaminants); the divers must wear protective coveralls over their diving suits that are removed before the divers return to the bell after an excursion.

3.7 Decompression

When the maximum duration at bottom depth was reached or when the project operations were concluded, the divers were decompressed back to surface. They were transferred from the 3 men living chambers to the 6 men decompression chambers, where the pressure was decreased according to the relevant procedures. After surfacing, the divers had to stay on the vessel for bend watch, which consisted of monitoring for any signs of decompression illness; the duration is at least 24 hours in the Norwegian sector, and 12 hours in the UK sector (4 hours if the divers are within 2 hours reach of a re-compression chamber. Note that a restriction of 24 hours must be observed before flying).

4 Experimental protocol

4.1 Pre- and post-saturation medical examinations

Pre-dive medical examinations were conducted by the vessel hyperbaric nurse shortly prior to saturation, for collection of information such as: age, weight, pulse, blood pressure, allergies (if any), any previous or current injuries and/or illnesses, signs of drug/alcohol and medication use.

Post-saturation, the hyperbaric nurse conducted another medical examination in addition to the examinations conducted pre-saturation; an external body examination was conducted to check for any disorders, reflexes and response changes including skin and ear canal infections. Records of the dates, saturation start and end; and the maximum diving depth were registered.

The information from the pre- and post-dive medical examinations was collected for use in our study. In addition, information about the use of substances that could affect vascular contractility (i.e. tobacco, nicotine tablets or vasopressors/dilatators) was recorded.

4.2 Maximum oxygen uptake

Maximal oxygen uptake (VO_{2max}) is commonly used for evaluating aerobic performance since the amount of oxygen a healthy individual can utilize is highly dependent on the cardiac output. Some oil and gas operators require the saturation divers to meet a minimum specified threshold in their oxygen uptake to be considered fit for diving. Hence, measures of the divers' VO_{2max} prior to going into saturation are conducted on a yearly basis onboard the DSVs for some projects. The system and methodology used to conduct the VO_{2max} onboard the Deep Arctic are described in paper I.

The limits imposed by the Norwegian Health Directorate (40) for male saturation divers were:

- Divers less than 30 years old = minimum 45 ml/(kg.min)
- Divers over 30 years old = minimum 40 ml/(kg.min)
- Divers over 50 years old = minimum 35 ml/(kg.min)

The divers who had not passed a VO_{2max} test during the last 12 months had to run the test during the pre-dive medical check. In the UK sector, additional VO_{2max} measurement was

not required by the operators, so the results from the latest annual test were used (if available). But since these could not be obtained for some divers that had not worked on the vessel previously, the VO_{2max} results were incomplete and therefore; could not be used (paper II).

4.3 Flow mediated dilation

The divers' endothelial function was assessed by measuring FMD on the brachial artery during the pre- and post-saturation medical checks, prior to blood sampling. The measurements were done according to current guidelines (41, 42). Interpretation of the data was done independently by two trained operators, and the results were compared retrospectively. But the results could not be used, as the post-saturation FMD examinations could not be conducted at the appropriate times; rendering the measurements non-comparable. Indeed, the main reason was the lack of accommodation on the vessel for the examiner; who could not be present during most of the offshore periods when the divers surfaced. The examiner could get access to them only when the vessel was back to shore, which represented up to 5-days post-saturation time for most of the divers.

4.4 Antioxidant vitamins C and E

Vitamin C and E are potent antioxidants. It has been demonstrated by previous studies that the acute use of a combination of Vitamin C and E ameliorates negative effects on the heart, pulmonary and brachial artery in scuba divers (10, 21, 22); and when combined with tea catechins, prevents hepatic disturbances in experimental deep saturation divers (20).

In this study, the experimental group was given vitamin C and E supplements to be ingested with lunches and dinners for the duration of their stay in the hyperbaric chambers. Lunch time on the Deep Arctic was from 11:30 to 13:00 and dinner time from 18:00 to 19:30. But as the different groups of divers were on different shifts, the lunch and dinner times differed.

The brand of vitamin C used was NYCOPLUS® tablets, 250 mg per tablet. The producer recommended the ingestion of ½ to 4 tablets (125 mg - 1g) per day for adults. The divers were instructed to ingest 1 tablet with lunch and 1 with dinner for a total of 500 mg/day.

The brand of vitamin E used was NYCOPLUS® Multi E-vitamin capsules with a dosage of 30 mg per capsule. The producer recommended the ingestion of 10 to 30 mg Vitamin E per day, equivalent to 1 capsule per 1 or 2 days; depending on the diet's Vitamin E content. The divers were instructed to ingest 1 capsule with dinner.

Ingestion of higher dosage than 2 g of vitamin C and more than 1g of vitamin E can increase the risk of adverse effects (including diarrhoea, nausea, abdominal cramps and other gastrointestinal disturbances). As the divers' diet was not controlled, and their intake of vitamins C and E from their food could not be measured during saturation, the doses used in this study were kept within the maximum recommended values, to avoid any risk of adverse effects during operations.

4.5 Blood sampling

All the divers cleared for diving by the vessel hyperbaric nurse and with no current infection were invited to take part in the study. The blood sampling always occurred during the pre- and post-dive medical examinations, held immediately before the divers go into saturation and after they surfaced from decompression. The pre- and post-dive medical examinations always took place between 08:00 and 24:00 as there was only one hyperbaric nurse on the vessel at a time.

4.5.1 Paper I

4.5.1.1 Blood biomarkers analyses

The first blood sampling campaign was conducted in spring 2015 in the NCS. Blood samples were taken from 20 divers. The methodology is explained in paper I.

At the St. Olav's University Hospital laboratory for clinical biochemistry, the following parameters and biomarkers were measured: total cholesterol, LDL, HDL, triglycerides, creatinine, fibrinogen and high sensitivity CRP.

The following blood biomarkers were measured at the NTNU hyperbaric laboratory using pre-packed kits for immunochemical detection micro-titration plates; combined with ELISA technique for results reading: Interleukin-6 (IL-6), Intra-Cellular Adhesion Molecule-1 (ICAM-1) and Platelet Activator Inhibitor-1 (PAI-1). The kits were procured from R&D Systems®.

4.5.1.2 Gene expression profiling

For the gene expression study, 2.5 ml of blood was drawn in PaxGene tubes from the divers pre- and post-saturation. The blood sampling and gene expression assessment were done according to the methodologies described in paper I. Total mRNA was analysed to determine global genes expression levels. Gene expression changes were analysed using bioinformatic tools.

4.5.2 Paper II

During the first offshore campaign, haematocrit (Hct) levels were found to be significantly reduced post-saturation comparing to pre-saturation (43). Assuming the hydration levels of the divers were maintained during saturation, a decrease in Hct levels could indirectly indicate a decrease in the erythrocytes count that could represent an acclimatization response to the hyperoxic conditions during saturation (see in paper I).

Therefore, another blood sampling campaign was organized during offshore operations that were ongoing in the UK sector, late 2016. Blood samples were taken from 13 saturation divers onboard the Deep Arctic. The aim was to investigate the haematological changes during saturation by measuring Hb and EPO immediately pre- and post-saturation and 24 h post-saturation. The samples were collected according to the methodology described in paper II.

4.5.3 Paper III

During the 2015 and 2016 offshore campaigns, parallel research was being conducted onboard the same vessel by other researchers sponsored by TechnipFMC. Although not originally planned for this PhD, this research aligned with and complemented the biomedical studies. This led to a collaborative study that is included in this thesis as a 3rd paper.

A questionnaire was developed in order to determine how the divers perceived the effects of repeated saturation manifesting in the form of fatigue and headaches, and to compare if these effects differed between the Norwegian and UK sectors. A total of 51 divers responded to the questionnaire during offshore operations conducted in both sectors.

Transthoracic ultrasound examinations were conducted post-saturation. The tests did not reveal bubbles in any of the participating divers in the two sectors; indicating comparable stress exposure during decompression. The hyperbaric breathing gases were similar in the two operations. Therefore, the results from the two sectors were merged despite the difference of diving and storage depths.

The response rates and results are described in paper III.

5 Results and discussion

5.1 Paper I

5.1.1 Blood biomarkers changes

There are few prior studies that have addressed the effects of antioxidant supplements in diving. The study from Ikeda et al. (20) was the only one, to our knowledge, to be conducted in a dry deep experimental saturation setting (400 msw for 40 days), with ingestion of vitamin C, E and tea catechins. The experiment concluded that hepatic disturbance due to oxidative stress was prevented by the antioxidants ingestion. A second experiment (10) was conducted by breathing oxygen at surface (oxygen content 21% and 100%), and demonstrated that hyperoxic vasodilatation and endothelial impairment were reversed by vitamin C intake. In a third, fourth and fifth studies (21, 22, 44); experiments were conducted on air divers who went to 30 msw, with vitamin C and E intake at different dosages and times. Brachial artery flow mediated dilation (FMD) and bubble loads measurements were found to be reduced in all the experiments. The last experiment (45) included scuba divers (one dive to 30 msw) with ingestion of dark chocolate. The antioxidant effect of the chocolate lead to the increase of the FMD measurements, and nitric oxide (NO) levels.

In this present study, no effects of antioxidant supplements were observed on neither the blood biomarkers of vascular function nor the gene expression.

5.2 Paper II

5.2.1 Haemoglobin and erythropoietin changes

During saturation, the divers are living in a hyperoxic environment; that is expected to cause elevated ROS levels. The divers' acclimatization to the high ppO_2 is apparent by a decrease in their Hb and Hct levels as was shown in previous experiments (46). The reduction of Hb in the blood is controlled by the hormone erythropoietin (EPO).

5.2.2 Mechanism of EPO regulation

The mechanism by which EPO is regulated is reviewed in (47). A link is suggested between the ROS concentration and the availability of glutathione (GSH); which scavenges the excess ROS in hyperoxic hyperbaric environments (48).

When returning to normobaric normoxia, or in the case of our divers to a relative hypoxia; the GHS level increases. The extra GSH will neutralize ROS and start a chain of reactions. These ultimately lead to triggering a cascade for transcription and production of EPO; thus, increasing the haemoglobin and erythrocytes production (49, 50). This phenomenon is known as the normobaric oxygen paradox (NOP) (48). It seems that very small variations in oxygen can induce the NOP (51).

5.3 Paper III

Many of the divers declared having headaches near or just after surfacing from a saturation. Most of them reported a post-saturation fatigue that took 1 to 10 days to recede. The divers' acclimatization to living and working in high ppO_2 for long periods translates in the haematological changes observed post-saturation. This correlates with the decrease in Hb levels post-saturation, or the state of relative hypoxia described in paper II. Headaches may also in part be ascribed to pressure changes during the last phases of decompression, and/or dehydration due to insufficient fluid intake.

6 Study limitations

The experimental work in this study was conducted in operational settings, during commercial offshore projects. It was essential not to disturb the operations more than needed. The dive teams went into- and out of saturation at different times of the day (4 shifts as operations on the vessel were running 24/7 until the end of the project). This had an impact on our access to the divers for the blood sampling, as these could not be done at controlled/similar times to avoid any differences related to the circadian rhythm. Also, it rendered our FMD measurements useless as it was not possible to monitor many divers immediately post-surfacing as per the protocol (41, 42).

Getting the blood samples sent to the laboratories depended on the frequency of crew change and/or project equipment mobilization/demobilization. This was done on an as needed/planned basis by helicopter (Stavanger heliport in the Norwegian sector and Aberdeen in the UK sector) or sailing back to a specific quayside (port calls to Haugesund in Norway and Inverness in the UK). The samples had to be kept on the vessel for 14 days before they could be sent to shore. In the 2015 offshore campaign, the plasma and PAXgene samples were kept refrigerated at 4ºC, transported at 4ºC then kept at -80ºC and thawed right before use. In order to verify that the quality of the samples did not degrade, blood samples were taken from two healthy males at St. Olav hospital in Trondheim. A part of their plasma was refrigerated at 4ºC for 14 days, then frozen at -80ºC. The other part was frozen directly at -80ºC. The samples were tested with the divers' plasma for all the parameters; and the results were the same for the samples frozen after 14 days or immediately after sampling. This indicated that the quality of the plasma samples was not significantly reduced due to the refrigeration. In the 2016 offshore campaign, Hb was measured directly with the same apparatus to limit technical errors. The plasma was kept in the vessel' deep freezer at -18 ºC for 14 days then sent in a 4ºC cooling box to be stored at -80ºC in the biobank. Although the transportation from vessel to biobank did not exceed 18 hours, the samples might have been partly thawed before freezing.

Previous studies indicated that the oral intake of antioxidants could reduce the levels of endothelial stress biomarkers in the blood after a period of experimental saturation (20). We found no significant changes in the levels of biomarkers monitored either with or without vitamin C and E intake in the recommended doses. But the measurements were

conducted only pre- and post-saturation; so, a possible significant effect of the antioxidants during the period of saturation could not be ruled out.

The responses to the questionnaires were incomplete in some instances, leading to different response rates to the different questions.

7 Conclusions

I. Acclimatization to professional saturation diving was associated with extensive downregulation of genes involved in oxygen transport, and upregulation of endogenous antioxidant system, immune activity and inflammatory signalling. Daily antioxidant vitamin C and E intake in recommended doses had no effect on the outcome of either genetic activity or vascular function biomarkers.

II. Blood Hb was reduced immediately after decompression from commercial saturation diving, compatible with acclimatization to the hyperbaric hyperoxia the divers experienced during saturation. These results were compatible with the findings in paper I, as the Hb reduction reflects the downregulation of the genes involved in heme, haemoglobin and erythrocytes production. A marked increase of EPO levels 24 h after decompression suggested ongoing re-acclimatization back to breathing normobaric air.

III. The effects of diving on headaches and fatigues post-saturation include a dimension related to acclimatization to the higher than normal levels of oxygen experienced in saturation. Initially, the divers in saturation acclimatize to high levels of oxygen. Secondly, at the end of the final decompression when returning to normoxic conditions; the divers experience a relative hypoxia caused by the changes of ppO_2. Our assumption is consistent with the haemoglobin concentration changes measured in similar conditions (in paper II) and supported by the subjective evaluation of saturation by the divers as assessed by our questionnaire. These effects are reversible post-decompression through the increase of EPO levels (as shown in paper II).

8 Future research and way forward

For future research, it could be beneficial to conduct blood samplings during compression, before and after excursion(s) and during decompression to see the evolution of the biomarkers of endothelial function in the different phases of saturation. The challenge here would be to have the samples collected by competent personnel and decompressed properly to ensure their quality is preserved; and obtain enough samples. Measuring relevant parameters in other tissues than blood (i.e. saliva, urine, stools…etc.), or using capillary blood; could be considered as possible alternatives.

Measurement of hydration levels should be taken into consideration as it can impact the diver's general state and certain haematological parameters.

For ultrasound examinations, the time of the day when the examinations are conducted matters, as the circadian cycle can affect the FMD. Any re-examinations should be conducted around the same time to limit biases. Readings should be done by trained personnel, and it would be preferable that blinded second readings are conducted by 3rd parties to check the quality of the results.

It must be noted that in real conditions, accommodation on the vessel might not be readily available. Training the hyperbaric nurses on the vessel to conduct the examinations themselves could be a solution, but training is time consuming and pricy; so, should be planned for in advance if this option is chosen.

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10 Appendices

10.1 Appendix 1

Individual study population demographics and basic information

n	Total diving [year]	Sat. diving [year]	Sat. period [day]	Age [year]	Height [cm]	Allergy	Injuries/ illnesses	Alcohol [Unit][1]	Medication/ supplement	Tobacco/ Nicotine	Coffee/ Tea intake	VO_{2max} [ml/kg.min]
1	14	6	14	45	170	No	History of artery blockage	0	Aspirin bisoprol	No	No	44
2	18	8	21	45	176	No	No	1	No	No	No	46
3	17	7	21	44	179	No	No	0	No	No	Important[2]	46
4	35	25	21	54	180	No	No	0	No	No	Important	36
5	34	26	14	53	175	Hay	No	5	No	No	No	51
6	27	16	21	51	182	No	No	0	No	No	No	40
7	15	21	14	46	180	No	No	2	No	No	Important	45
8	27	16	21	48	180	No	No	3	Fish oil	No	Moderate[3]	41
9	43	29	21	55	158	No	No	3	No	Yes	Moderate	37
10	12	7	21	38	188	No	No	3	No	No	Important	41
11	7	1	21	35	180	No	No	0	No	No	Moderate	49
12	9	8	21	34	183	No	No	2	Vitamin mix	No	Important	51
13	33	29	21	54	166	No	No	3	No	No	Moderate	50
14	17	8	21	44	180	No	No	2	No	No	Important	52
15	8	1	21	37	197	No	No	3	No	No	Important	50
16	25	14	21	50	172	No	Previous ear infection	0	Cilox	No	Moderate	36

n	Total diving [year]	Sat. diving [year]	Sat. period [day]	Age [year]	Height [cm]	Allergy	Injuries/ illnesses	Alcohol [Unit][1]	Medication/ supplement	Tobacco/ Nicotine	Coffee/ Tea intake	VO_{2max} [ml/kg.min]
17	9	3	21	35	186	No	No	4	No	No	Important	49
18	19	12	21	45	183	No	No	1	No	No	Moderate	56
19	13	9	21	36	187	No	No	0	Nail fungi	No	Moderate	45
20	5	2	21	40	173	No	No	0	No	No	No	50

[1] Average alcohol consumption per week only during time off at home, [2] Important: more than 3 cups of coffee/tea per day, [3] Moderate intake: 1-3 cups of coffee/tea per day

	Pre-saturation				Post-saturation			
n	**Weight [kg]**	**Pulse at rest [Beat/min]**	**Blood Pressure [mmHg]**	**Haematocrit [%]**	**Weight [kg]**	**Pulse at rest [Beat/min]**	**Blood Pressure [mmHg]**	**Haematocrit [%]**
1	76	65	102-71	46	76	86	140-90	42
2	87	95	132-88	45	87	76	120-80	40
3	80	67	134-89	40	80	82	120-90	41
4	93	83	140-90	40.5	90	82	130-85	39
5	70	69	119-77	38	70	64	115-75	36.5
6	85	53	121-72	43	85	60	120-75	38
7	87	66	128-84	39.5	86	64	130-75	39
8	93	81	111-78	38	92	76	125-85	39
9	92	80	140-90	40	92	88	140-85	35
10	110	81	121-86	39	108	100	120-80	38
11	99	63	139-84	40	99	76	125-75	39.5
12	72	80	110-80	38	73	76	120-83	39
13	70	76	110-70	39	72	76	125-80	40
14	81	60	120-70	40	79	68	130-85	39

15	82	64	120-70	37	84	64	115-80	38
16	90	60	120-80	44	90	72	120-85	40
17	86	74	120-80	37	86	98	120-85	38
18	90	72	140-80	42	91	96	138-83	35
19	93	67	119-84	39.5	93	109	111-85	40
20	75	60	135-75	44	74	71	114-76	35

Appendix 2

Individual study population base information

n	Total diving [years]	Saturation diving [years]	Saturation duration [days]	Age [years]	Height [cm]	Injuries/illnesses	Medication/ supplement	VO_2max [ml/(kg.min)]
1	10	5	25d 15h	41	178	No	No	51
2	25	18	25d 15h	44	192	No	No	36
3	6	3	25d 15h	41	175	No	No	45
4	30	27	25d 15h	49	178	No	No	None
5	35	34	27d 6h	59	183	Heart surgery	Aspirin	45.4
6	35	26	27d 6h	54	182	No	No	None
7	14	10	27d 6h	37	186	Face infection	Flucloxacillin 500mg x 7d	44
8	31	26	27d 6h	53	183	No	No	None
9	28	17	27d 6h	48	180	No	No	41
10	8	5	27d 21h	34	171	No	No	52
11	18	12	27d 21h	44	174	No	No	44
12	18	9	27d 21h	45	180	No	No	52
13	20	13	27d 21h	46	183	No	No	58

	Pre-saturation					Post-saturation 0h					Post-saturation 24h	
n	**Weight [kg]**	**Pulse [Beat/min]**	**Blood Pressure [mmHg]**	**Haemoglobin [g/dl]**	**Haematocrit [%]**	**Weight [kg]**	**Pulse [Beat/min]**	**Blood Pressure [mmHg]**	**Haemoglobin [g/dl]**	**Haematocrit [%]**	**Haemoglobin [g/dl]**	**Haematocrit [%]**
1	80	66	110-70	15.8	46	75	70	115-70	14.9	44	14	41
2	112	75	120-70	15.6	46	113	75	130-70	14.9	44	14.3	42
3	72	65	130-80	14.2	42	72	68	120-85	14	41	13.2	39
4	94	66	160-80	17	50	90	96	135-80	16.8	49	15.9	47
5	104	83	125-80	15.4	45	105	83	120-80	15	44	15.2	45
6	90	67	120-80	14.2	42	92	63	125-80	14.5	43	13.1	39
7	90	91	120-80	16	48	92	86	120-80	14.5	43	14.5	43
8	78	65	110-70	16	47	78	71	105-70	15.2	45	14.5	43
9	88	66	125-80	16.6	49	89	58	105-70	14.8	44	14	41
10	69	56	125-80	15.9	47	65	79	110-70	14.4	42	14.8	44
11	84	61	120-70	15.8	46	83	67	100-82	16.9	50	15.7	46
12	78	54	130-80	15.1	44	78	76	130-80	14.2	42	14.3	42
13	90	60	140-80	15.5	46	90	88	140-82	14.6	43	14.2	42

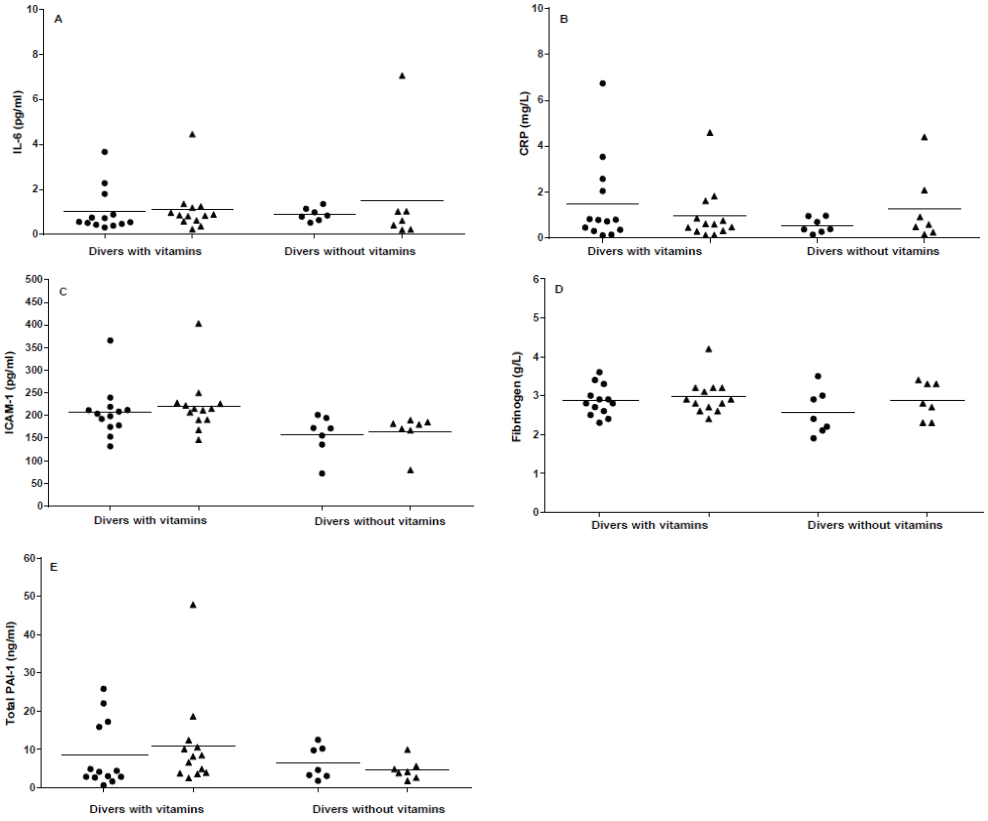
10.2 Appendix 3

Individual biochemical and biomarkers analyses result pre- and post-saturation

	Pre-saturation								Post-saturation							
n	**PAI-1 [ng/ml][1]**	**IL-6 [ng/ml][2]**	**ICAM-1 [pg/ml][3]**	**Chol [mmol/l][4]**	**Trigly [mmol/l][5]**	**CRP [mg/l][6]**	**Fib [g/l][7]**	**Creat [µmol/l][8]**	**PAI-1 [ng/ml]**	**IL-6 [ng/ml]**	**ICAM-1 [pg/ml]**	**Chol [mmol/l]**	**Trigly [mmol/l]**	**CRP [mg/l]**	**Fib [g/l]**	**Creat [µmol/l]**
1	2.62	0.75	192.40	2.9	1.51	0.31	2.5	117	8.42	0.81	206.53	2.7	1.16	0.76	2.9	98
2	2.98	0.54	174.51	5.1	1.18	0.83	3.4	125	3.69	0.23	189.89	5.4	0.96	0.86	3.2	115
3	2.82	0.72	239.50	5.0	1.04	6.74	2.4	108	18.61	0.62	214.70	5.1	1.12	0.62	2.6	110
4	4.36	0.88	365.71	6.3	1.78	2.58	3.0	99	12.36	0.96	403.38	5.5	3.02	1.62	3.2	108
5	2.82	0.39	131.81	6.1	0.64	0.12	2.3	93	3.53	4.46	146.25	6.0	1.11	0.13	2.7	112
6	3.02	1.35	171.47	4.7	1.11	0.97	3.5	94	3.81	7.07	166.94	4.3	1.07	0.59	3.3	90
7	9.72	0.84	194.44	4.4	1.41	0.28	2.2	86	4.12	0.22	181.82	5.2	4.58	0.25	2.3	119
8	12.48	1.14	135.57	4.8	1.06	0.70	3.0	91	9.89	0.41	170.18	4.6	1.88	0.49	3.3	88
9	4.12	1.8	219.09	5.6	1.30	0.46	2.8	95	2.50	1.36	227.88	3.8	1.63	1.83	3.2	85
10	0.58	2.28	211.87	4.3	1.03	2.05	2.9	123	3.89	1.24	249.86	4.4	1.25	0.60	2.8	118
11	1.76	0.98	71.53	5.8	2.28	0.38	2.4	108	1.76	0.20	79.29	5.4	4.69	0.92	3.3	105
12	22.00	0.56	177.96	6.5	1.28	0.80	2.6	93	8.10	0.88	190.52	6.0	1.91	0.46	2.3	87
13	25.80	0.47	211.56	5.2	1.23	0.36	2.8	87	10.54	0.58	225.68	5.1	0.89	0.32	3.3	72
14	3.25	0.52	155.62	4.5	0.63	0.15	1.9	82	5.56	0.61	179.88	5.1	1.61	0.16	2.3	76
15	1.60	0.51	153.16	4.3	0.66	0.15	2.9	87	480	0.36	211.24	4.4	1.03	0.14	2.9	103
16	15.82	0.43	203.71	4.4	2.05	0.73	3.3	95	47.79	0.83	214.70	4.4	1.34	4.60	4.2	94
17	4.60	0.64	172.12	5.3	1.19	0.39	2.9	90	2.58	1.02	189.59	5.0	1.44	4.40	3.4	85
18	4.84	3.67	198.37	3.9	1.73	3.54	3.6	93	6.56	0.85	167.92	4.9	1.56	0.29	2.4	86
19	17.21	0.31	208.42	6.7	3.37	0.79	2.7	94	10.05	1.19	221.92	7.3	4.44	0.48	2.8	105
20	10.17	0.79	200.91	5.8	0.97	0.96	2.1	85	4.80	1.03	185.06	4.7	0.93	2.09	2.7	85

[1] Plasminogen Activator Inhibitor-1, [2] Interleukin-6, [3] Inter-cellular Adhesion Molecule-1, [4] Total Cholesterol, [5] Triglycerides, [6] C-Reactive Protein, [7] Fibrinogen, [8] Creatinine

Blood biomarkers changes pre- and post-saturation, with and without vitamin C and E intake.



10.3 Appendix 4

Individual Hb and EPO data

	Haemoglobin			Erythropoietin		
n	**Pre-sat**	**0h Post-sat**	**24h Post-sat**	**Pre-sat**	**0h Post-sat**	**24h Post-sat**
1	15,8	14,9	14	6,5	15,6	22,5
2	15,6	14,9	14,3	5,6	6,5	10,1
3	14,2	14	13,2	8,5	10,1	17,8
4	17	16,8	15,9	9,0	9,5	10,5
5	15,4	15	15,2	9,8	5,4	15,5
6	14,2	14,5	13,1	6,0	9,9	14,7
7	16	14,5	14,5	8,4	9,1	13,2
8	16	15,2	14,5	7,0	8,4	13,4
9	16,6	14,8	14	20,5	17,9	23,6
10	15,9	14,4	14,8	20,8	9,2	37,7
11	15,8	16,9	15,7	7,0	13,8	15,7
12	15,1	14,2	14,3	12,8	12,8	29,9
13	15,5	14,6	14,2	9,4	7,2	21,8

11 Papers

Paper I

Blood gene expression and vascular function biomarkers in professional saturation diving

Fatima Zohra Kiboub, Andreas Møllerløkken, Astrid Hjelde, Arnar Flatberg, Øyvind Loennechen and Ingrid Eftedal

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Blood Gene Expression and Vascular Function Biomarkers in Professional Saturation Diving

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Saturation diving is an established way to conduct subsea operations with human intervention. While working, the divers must acclimatize to the hyperbaric environments. In this study, genome-wide gene expression and selected plasma biomarkers for vascular function were investigated. We also examined whether antioxidant vitamin supplements affected the outcome. The study included 20 male professional divers, 13 of whom took vitamin C and E supplements in doses of 1,000 and 30 mg daily during saturation periods that lasted 7–14 days. The dives were done in a heliox atmosphere with 40 kPa oxygen partial pressure (ppO_2) to a depth of 100–115 m of sea-water (msw), from which the divers performed in-water work excursions to a maximum depth of 125 msw with 60 kPa ppO_2. Venous blood was collected immediately before and after saturation. Following gene expression profiling, post-saturation gene activity changes were analyzed. Protein biomarkers for inflammation, endothelial function, and fibrinolysis: Il-6, CRP, ICAM-1, fibrinogen, and PAI-1, were measured in plasma. Post-saturation gene expression changes indicated acclimatization to elevated ppO_2 by extensive downregulation of factors involved in oxygen transport, including heme, hemoglobin, and erythrocytes. Primary endogenous antioxidants; superoxide dismutase 1, catalase, and glutathione synthetase, were upregulated, and there was increased expression of genes involved in immune activity and inflammatory signaling pathways. The antioxidant vitamin supplements had no effect on post-saturation gene expression profiles or vascular function biomarkers, implying that the divers preserved their homeostasis through endogenous antioxidant defenses.

Keywords: antioxidant vitamins, oxidative stress, hyperbaric, hyperoxia, gas saturation

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INTRODUCTION

Professional saturation divers live onboard Diving Support Vessels (DSVs) in hyperbaric pressure chambers during work assignments. Their assignments may last up to 3 weeks in Norwegian waters, and even longer in international waters. When commuting from the hyperbaric living chambers onboard the DSV to work on the seabed, the divers are transported in pressurized diving bells.

They leave the bell through a door in the bell floor to perform physically demanding underwater work, comprising handling of tools and equipment, rigging and welding of pipelines. In order to remain fit, the divers must acclimatize to the hyperbaric environment, including elevated partial pressures of oxygen (ppO_2), and to breathing heliox – a mixture of oxygen and helium – instead of air. Health risks in saturation diving are managed through procedures initially established in the 1960s, which have since developed with accumulated research and empirical knowledge.

In the relatively small volume of data published on saturation divers since the 1990s, there is no indication of persistent health injury in the absence of acute symptoms (Brubakk et al., 2014). However, even without disease or injury, diving has been associated with altered vascular function, inflammatory changes, increased coagulation and elevation of circulating microparticles (Ersson et al., 2002; Brubakk et al., 2005; Thom et al., 2012). These responses have been attributed to excess reactive oxygen species (ROS) triggered by factors in the hyperbaric environment. It has been demonstrated that elevated ROS levels activate the body's own defense mechanisms through production of endogenous antioxidants after a single non-saturation dive (Sureda et al., 2012; Thom et al., 2012), and that extensive surface-oriented (non-saturation) diving may cause divers to become acclimatized to oxidative stress as a result of persistent changes in biological pathways that control, e.g., inflammation (Eftedal et al., 2013).

Administering antioxidant supplements with the aim of enhancing the body's antioxidant defenses is an intuitive measure against excess oxidative stress. Prior studies have reported different ROS-related effects due to antioxidants administration in experimental saturation and non-saturation diving. The intake of antioxidants in the form of vitamin C and/or E has been reported to diminish hepatic disturbance (Ikeda et al., 2004), increase the brachial artery's flow-mediated dilation (FMD) and reduce the bubble loads (Mak et al., 2002; Obad et al., 2006, 2007a,b).

In this study, we have examined the outcome of a professional saturation dive on genetic activity in peripheral blood, and plasma biomarkers of vascular function; with and without antioxidant vitamin supplements. The analyses were focused on genes and pathways associated with acclimatization to oxidative stress in the hyperbaric environment, and plasma proteins involved in modulation of inflammation, fibrinolysis, and endothelial function. To our knowledge, this is the first study where antioxidant effects have been assessed in professional saturation diving.

MATERIALS AND METHODS

Ethics

This study was performed on material from professional saturation divers on the DSV Skandi Arctic (renamed Deep Arctic in 2016) during work assignments on the Norwegian Continental Shelf in 2015. The protocol was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REK), approval reference number 2015/351, and by the Norwegian Petroleum Safety Authority. The study subjects were individually informed and provided written consents before inclusion, and all procedures were conducted according to the Declaration of Helsinki principles for ethical human experimentation.

Study Subjects

The study subjects were 20 healthy male divers, who held valid health certificates for working as saturation divers. All subjects underwent the diving contractor's standard medical examinations before and after saturation diving, including measurements of weight, height, pulse, and blood pressure. Information regarding allergies, smoking, previous/current injuries or illnesses and current medication were also registered.

Professional saturation divers working for certain Norwegian Operators are required to pass an annual maximum oxygen uptake test (VO_{2max}) to determine if they fulfill requirements for aerobic fitness by use of direct oxygen uptake measurement. In this study, the diving contractor used a Woodway PPS Med treadmill (Woodway Inc., Waukesha, WI, United States); combined with a METALYZER® 3B analyzer and MetaSoft® Studio software (Cortex Biophysik GmbH, Leipzig, Germany). The oxygen uptake tests were supervised by the hyperbaric nurse on duty, following a standardized protocol (Thompson et al., 2013).

Prior to saturation diving, the subjects were randomly divided into two groups: the antioxidant vitamin group ($n = 13$) and the control group ($n = 7$). **Table 1** describes the participating divers' subgroups demographics, experience, VO_{2max} and body-mass index (BMI).

Antioxidant Vitamin Intervention

Vitamin C and E supplements (Nycomed, Asker, Norway), were supplied to the vitamin group during the pre-saturation medical examination. The divers in the vitamin group consumed two tablets of vitamin C (500 mg/tablet) and one capsule of vitamin E (30 mg/capsule) with dinner for the duration of saturation and decompression; i.e., every day from entry to exit from the pressure chambers. All the divers in the vitamin group reported that they took the vitamins as instructed. Apart from the vitamin intervention, no constraints were put on the divers' diet or life-style choices as part of this study.

Saturation Diving

Diving operations took place in June–July 2015, approximately 200 km west of the Stavanger coast in Norway, conducted in accordance with the NORSOK U-100 requirements (Standards Norway, 2014). The divers went into the pressure chambers shortly after their mandatory pre-dive medical examination, where they were compressed at a rate of 1 msw/min until reaching a storage depth of 100–115 msw. The compression took 2 h with an additional 2 h period at bottom depth for acclimatization. ppO_2 was kept at 40 kPa in the pressure chambers for the duration of the saturation period. The divers were organized in teams of 3, with each team working daily 12 h shifts. A bell dive takes up to 8 h; with the diver locked out and working in the water for maximum 6 h, including a mandatory 30 min restitution and

TABLE 1 | Study group demographics, diving experience, aerobic fitness, body-mass index, and resting heart rate.

	Divers with vitamins (n = 13)		Divers without vitamins (n = 7)	
	Pre-saturation	**Post-saturation**	**Pre-saturation**	**Post-saturation**
Age (year)	44.6 (34–55)		42.7 (35–51)	
Total diving (year)	22 (8–43)		15 (5–27)	
Saturation diving (year)	14 (1–29)		10 (1–21)	
VO_{2max} (ml/kg.min)	45 (36–56)		47 (40–52)	
BMI (kg/m^2)	27.0 (21.1–36.9)	27.0 (21.6–36.9)	26.7 (24.9–30.6)	26.4 (24.4–30.6)
Pulse at rest (beat/min)	74 (60–95)	82 (64–109)	65 (53–81)	73 (60–98)

Data are shown as mean (range). VO_{2max} (maximal oxygen uptake), BMI (body mass index).

rehydration break in the bell between the third and fourth hour of work. The maximal diving depth was 125 msw.

During the bell runs, the ppO_2 was increased to 60 kPa. In order to protect the divers from hypothermia from work in 4°C water, seawater was heated onboard the DSV and pumped down, through the diving bell and out into the divers' wet suits. After a rotation of one (3 divers) or two (17 divers) weeks in saturation, the divers were decompressed following table 12 in NORSOK U-100 requirements (Standards Norway, 2014) until they reached atmospheric pressure. Decompression took 5–6 days depending on the maximum storage depth, followed by 24 h of "Bend Watch" for symptoms of decompression sickness (DCS). See **Figure 1** for an example of a dive profile of one of the participating divers.

Blood Samples

Blood samples were collected by standard phlebotomy of the median cubital vein at two time-points: the first before the divers entered the hyperbaric living chambers (pre-saturation), and the second shortly after completed decompression back to surface (post-saturation). For plasma, 3.5 ml venous blood was collected on citrated and heparinized tubes (Greiner Bio-One, Radnor, PA, United States). After filling, the tubes were gently inverted 8–10×. Hematocrits were measured with the citrated blood samples, using capillary tubes and a Haematokrit 200 centrifuge (Hettich GmbH and Co. KG, Tuttlingen, Germany). To prepare plasma, the blood tubes were centrifuged (Hettich GmbH and Co. KG) for 10 min at 1,800 × g. The separated plasma transferred into 2 ml cryogenic storage tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and kept refrigerated at 4°C.

For RNA, 2.5 ml blood was collected in PAXgene blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland). A single batch of PAXgene tubes was used in order to limit technical variation. After filling, the tubes were kept upright at room temperature for 4 h before they were refrigerated at 4°C. All samples were transported in temperature-controlled cubes (VeriCor Medical Systems, Holmen, WI, United States) at 4°C to NTNU in Trondheim, where they were frozen at −80°C until all material was collected for analysis.

For reasons of logistics on the DSV, and uniformity in the analyses, all samples were kept refrigerated at 4°C for exactly 14 days prior to freezing at −80°C. In order to determine whether this affected the quality of the subsequent analyses, two control samples from healthy males were added to each plasma set-up: one that had been frozen at −80°C on the day of collection, and another from the same donor that was kept at 4°C for 14 days and then frozen at −80°C. These controls gave similar results for all biomarkers, indicating that the plasma collected on the DSV was unlikely to be deteriorated. The PAXgene tubes were expected to sufficiently stabilize the RNA profiles during the cold storage.

Gene Expression Profiling

Total RNA was extracted from thawed blood using the PAXgene Blood RNA kit version 2 (PreAnalytix). RNA quantity and quality were measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), giving RNA concentrations of 52–357 ng/l, and RNA integrity numbers (Perk et al., 2012) ranging from 6.3–8. Amplification of RNA was done using the Ambion TotalPrep RNA amplification kit (Ambion Inc., Austin, TX, United States), and cDNA was synthetized by reverse transcription and replication. Finally, cRNA was synthesized by transcription, and hybridized onto Illumina humanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA, United States) according to the manufacturer's protocol, providing genome-wide RNA measures for >47,000 probes. After hybridization, the microarrays were scanned using the Illumina HiScan array scanner, and the data exported to the Illumina GenomeStudio software, version 1.7.0 for background filtration prior to further analysis.

The filtered microarray data was transferred to the R statistical computing software[1] for gene expression analysis using lumi Bioconductor package version 1.1.0 (Du et al., 2008). Inter-sample normalization, exclusion of low-significance probes and multilevel partial least squares (PLSs) regression analysis was done as described previously (Eftedal et al., 2016). Genes that were differentially expressed after saturation diving were identified using a moderated paired t-test comparing pre-and post-saturation data. Biological pathway associations were identified from functionally clustered genes that were differentially expressed in post-dive samples, using the MetaCore GeneGo software (release 6.21[2]). An absolute threshold for

[1]http://www.r-project.org/

[2]https://clarivate.com/products/metacore/

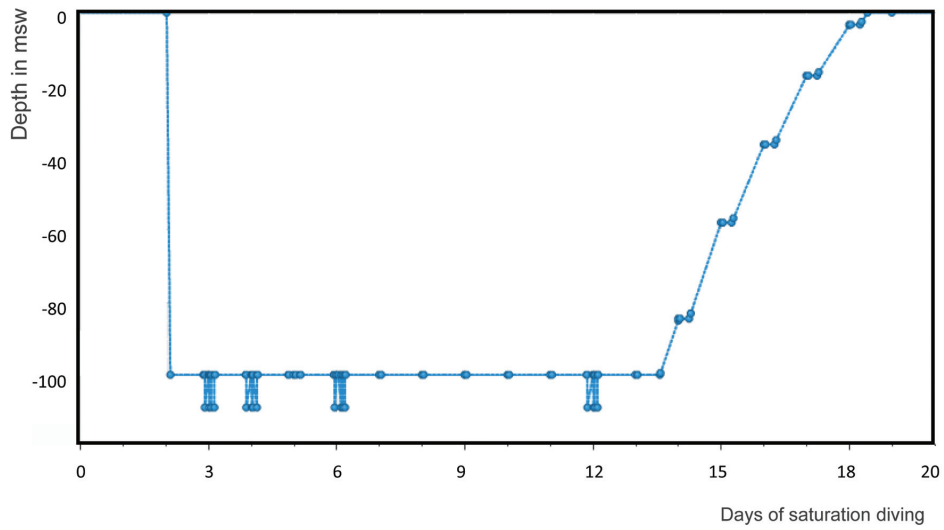

FIGURE 1 | Saturation period profile of a participating diver. Compression took 2 h to 100 msw storage depth. The bell runs went down to 110 msw; and decompression took 5 days.

transcription change was set to 0.5. The pathways were ranked according to the probability of a chance occurrence on the background of all probes on the Illumina humanHT-12 v4 Expression BeadChips microarray. In all analyses, the significance level was set at $P < 0.05$, using false discovery-adjusted *P*-values.

Plasma Biomarker Analysis

Prior to plasma analyses, the expected power was assessed on the SISA online statistical power calculator (Uitenbroek, 1997), using biomarker data predictive of atherosclerosis in healthy males as a reference (Tzoulaki et al., 2005). While the size of the antioxidant vitamin group was sufficient to obtain a power of 80%, with an error probability of 0.05, the control group met the size requirements only for one biomarker (IL-6).

In order to determine appropriate statistical tests for group-wise comparison of the plasma data, the normality and homogeneity of variances in the data were examined using Kolmogorov–Smirnov and Levene's tests. When biomarker data were normally distributed or became so after transformations, a one-way ANCOVA test was applied. In cases where data were not normally distributed, a non-parametric Mann–Whitney test was applied. All analyses were performed in IBM SPSS Statistics software (version 21, IBM Corp., Armonk, NY, United States), with $P < 0.05$ considered significant.

Heparinized plasma was used for analysis of high-sensitivity CRP (Pearson et al., 2003), total cholesterol, HDL, LDL (Perk et al., 2012), triglycerides, and creatinine (Rustad et al., 2004) on a Roche Modular P (Diamond Diagnostics Inc., Holliston, MA, United States). Fibrinogen was measured using an ACL Top 750 LAS (Werfen Instrumentation Laboratory, Bedford, MA, United States) (Yudkin et al., 2000). The analyses were conducted by an IEC 17025 accredited laboratory at St. Olavs Hospital, Trondheim, Norway.

Commercial ELISA immunoassay kits (catalog numbers: HS600B, DCD540, and DTSE100, respectively) from R&D systems™ (Bio-Techne Ltd., Minneapolis, MN, United States) were used to quantify interleukin 6 (IL-6) (Gaines Das and Poole, 1993), intercellular adhesion molecule 1 (ICAM-1), and plasminogen activator inhibitor-1 (PAI-1). The IL-6 assay was run on undiluted plasma, whereas samples were diluted 20× for the ICAM-1 assay and 10× for the PAI-1 assay. All samples were run in duplicates on the same plate, along with medium and high controls from the same producer (catalog numbers: QC41 for IL-6, QC105 for ICAM-1, and QC209 for PAI-1). Optical density (OD) values were measured on a Biochrom® Asys Expert Plus microplate reader (Biochrom, Holliston, MA, United States). All controls were within the target ranges set by the manufacturer, and plasma protein concentrations were calculated from the standard curves using the sample mean OD values with blank correction.

The normal ranges indicated by the producer for CRP, ICAM-1, IL-6, fibrinogen, and PAI-1 were, respectively: <5 mg/L, 0.435–9.57 pg/ml, 106–337 ng/ml, 1.9–4.2 g/l, and 2.66–69.3 ng/ml.

Data Repository

The microarray data are openly available in the EMBL-EBI ArrayExpress repository [3] in accordance with MIAME standards. The access code is E-MTAB-4491 (Eftedal et al., 2017).

[3]http://www.ebi.ac.uk/arrayexpress/

RESULTS

The saturation diving operations were conducted according to plan, and blood samples were collected from all study subjects as per the protocol. All data for total cholesterol, HDL, LDL, triglycerides, and creatinine were within normal range (Cox and Garcia-Palmieri, 1990; Hosten, 1990), and unaltered post-saturation compared to pre-saturation for both groups of divers (**Table 2**). Hematocrit levels were within the normal range (Billett, 1990) pre-saturation, and slightly below normal post-saturation for both groups (**Table 2**).

Sample Relations in the Microarray Data

In order to examine the relations between the samples collected from saturation divers with and without antioxidant vitamins, and before and after diving, we performed an exploratory multilevel PLSs regression analysis on the microarray data. As illustrated in **Figure 2**, sample relations for the primary (PC1) and secondary (PC2) PLS components revealed two major traits. First, the samples were almost completely separated along PC1 according to whether they had been collected before or after saturation. This implied that saturation diving was the predominant cause of variation in the data. Second, intake of antioxidant vitamins had no effect on the two primary PLS components. In further gene expression analysis, data from both groups of divers were therefore merged.

Responses to Saturation Diving on the Level of Genome-Wide Gene Expression

Genome-wide effects of saturation diving on transcription were determined by comparing the pre- and post-saturation microarray data. A total of 12,201 transcripts were identified as differentially expressed: 5,452 were downregulated, and 6,749 were upregulated after saturation diving. Complete lists of differentially expressed transcripts and their corresponding genes are presented as **Supplementary Table S1**.

Differentially Expressed Genes Indicated Reduced Blood Oxygen Transport and Increased Endogenous Antioxidant Activity After Saturation Diving

For biological interpretation of the gene expression data, we first considered the genes that displayed the largest fold change post-saturation. Among those with established function, the data comprised a disproportionally large number of genes involved in oxygen transport, all of which were downregulated. Further inspection revealed more downregulated genes with similar function. In **Figure 3**, log fold expression changes for genes involved in central aspects of blood oxygen transport are plotted according to their placement in the process; from the synthesis of oxygen-carrying heme molecules, the synthesis and activity of different hemoglobin types into which heme is built, through the final step of erythropoiesis in which reticulocytes released from the bone marrow are differentiated into erythrocytes, and finally the activity of mature circulating erythrocytes in the blood stream.

Several gene coding for antioxidant factors were differentially expressed after saturation. Among them, all three primary endogenous cytosolic antioxidants; superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione synthetase (GSS), were upregulated, whereas the mitochondrial superoxide dismutase, SOD2, was downregulated (**Supplementary Table S1**). Genes involved in glutathione turnover, GSR and GPX, showed variable results.

In order to identify biological pathways that may be affected by saturation diving, we performed functional clustering analysis of genes that were differentially expressed. As predictable from the inspection of genes with the largest fold-change in expression, the downregulated genes were largely associated with anemic conditions. The upregulated genes were primarily involved in immune responses and inflammatory signaling, indicating that there was increased inflammatory activity in the divers' blood at the time they completed their hyperbaric saturation (**Supplementary Table S1**).

Vascular Function Biomarkers Were Unaffected by Antioxidant Vitamin C and E Supplements

There was no difference between the two diving groups, with and without antioxidant vitamin supplementation, for CRP (P = 0.279), IL-6 (P = 0.968), ICAM-1 (P = 0.588), fibrinogen (P = 0.464), and PAI-1 (P = 0.536) (**Table 3**).

DISCUSSION AND CONCLUSION

This study had two objectives. The first was to identify and characterize indicators of acclimatization to the hyperbaric environments in professional saturation diving, on genetic activity level and plasma biomarkers of vascular function. The second objective was to assess whether daily intake of antioxidant vitamins C and E affected the outcome of either of the above.

For the first objective, peripheral blood gene expression profiling revealed extensive downregulation of genes involved in oxygen transport, including the production and activity of heme, hemoglobin and erythrocytes, at the time when the divers had completed their saturation diving assignments. At that time, they had been exposed to elevated ppO_2 for 14–21 days; from 21 kPa in the normal ambient air to a range of 40–60 kPa in the heliox gas mixture they were breathing in saturation and on work excursions. It is reasonable to conclude that a reduction in blood oxygen transport represents acclimatization against the hyperoxia. Our results are in agreement with reports of decreased hemoglobin levels and erythrocyte counts in experimental saturation diving (Nakabayashi et al., 1991; Thorsen et al., 2001; Hofso et al., 2005). Hyperoxic acclimatization in saturation is also in line with reports of transient symptoms of hypoxia when the divers must acclimatize back to breathing normal air (Balestra et al., 2004; Hofso et al., 2005).

During work excursions, saturation divers experience dehydration due to physical activity, the use of seawater-heated diving suites, and in some cases failure to rehydrate (Hope et al., 2005). A loss of fluids and electrolytes can trigger fatigue and

TABLE 2 | Divers' blood lipids, hematocrit and creatinine.

	Divers with vitamins (n = 13)		Divers without vitamins (n = 7)	
	Pre-saturation	**Post-saturation**	**Pre-saturation**	**Post-saturation**
Total cholesterol (mmol/l)	5.1 (2.9–6.7)	5.0 (2.7–7.0)	5.0 (4.4–5.0)	4.9 (4.3–5.4)
LDL (mmol/l)	3.0 (1.2–4.3)	3.0 (1.3–4.5)	3.2 (2.5–3.9)	2.8 (2.1–3.2)
HDL (mmol/l)	1.47 (0.82–2.40)	1.24 (0.75–2.01)	1.32 (1.15–1.55)	1.07 (0.86–1.32)
Triglycerides (mmol/l)	1.45 (0.64–3.37)	1.65 (0.89–4.44)	1.24 (0.63–2.28)	2.31 (0.93–4.69)
Hematocrit (%)	41 (37–46)	39 (35–42)	40 (37–44)	38 (35–40)
Creatinine (μmol/l)	100.7 (87.0–125.0)	99.5 (72.0–118.0)	90.9 (82.0–108.0)	92.6 (76.0–119.0)

Data are shown as mean (range). LDL (low-density lipoprotein), HDL (high-density lipoprotein).

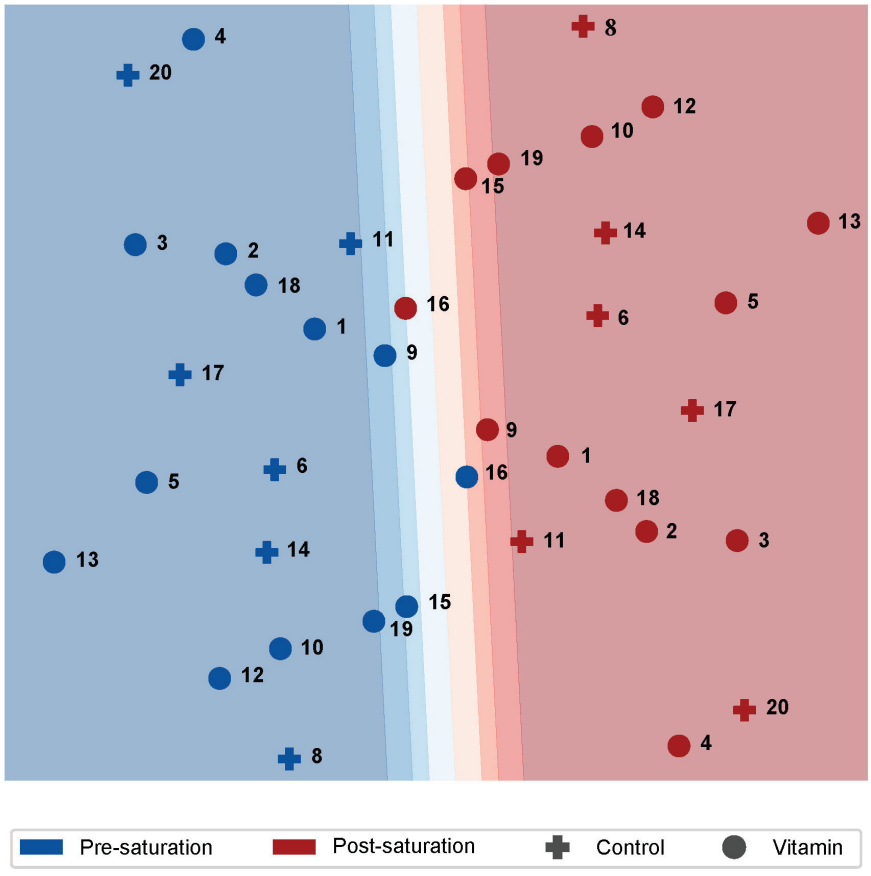

FIGURE 2 | Two-dimensional plot of sample relation after PLS analysis of paired cDNA microarray data from professional saturation divers. Individual diver numbers are shown next to each data point. Separation of samples along the first primary component (PC1) was predominantly explained by the time of sample collection; i.e., pre- or post-saturation diving. The antioxidant vitamin supplements had no effect on either of the two first PLS components. Responses to saturation diving on the level of gene expression.

reduced mental performance; hence, there are mandatory water breaks (Hope et al., 2001). Dehydration alone would cause a relative increase in hematocrit as a consequence of the reduction of the aqueous phase in the blood (Billett, 1990), but in this study the divers' hematocrit levels were decreased post-saturation. This further supports the conclusion that they were acclimatized

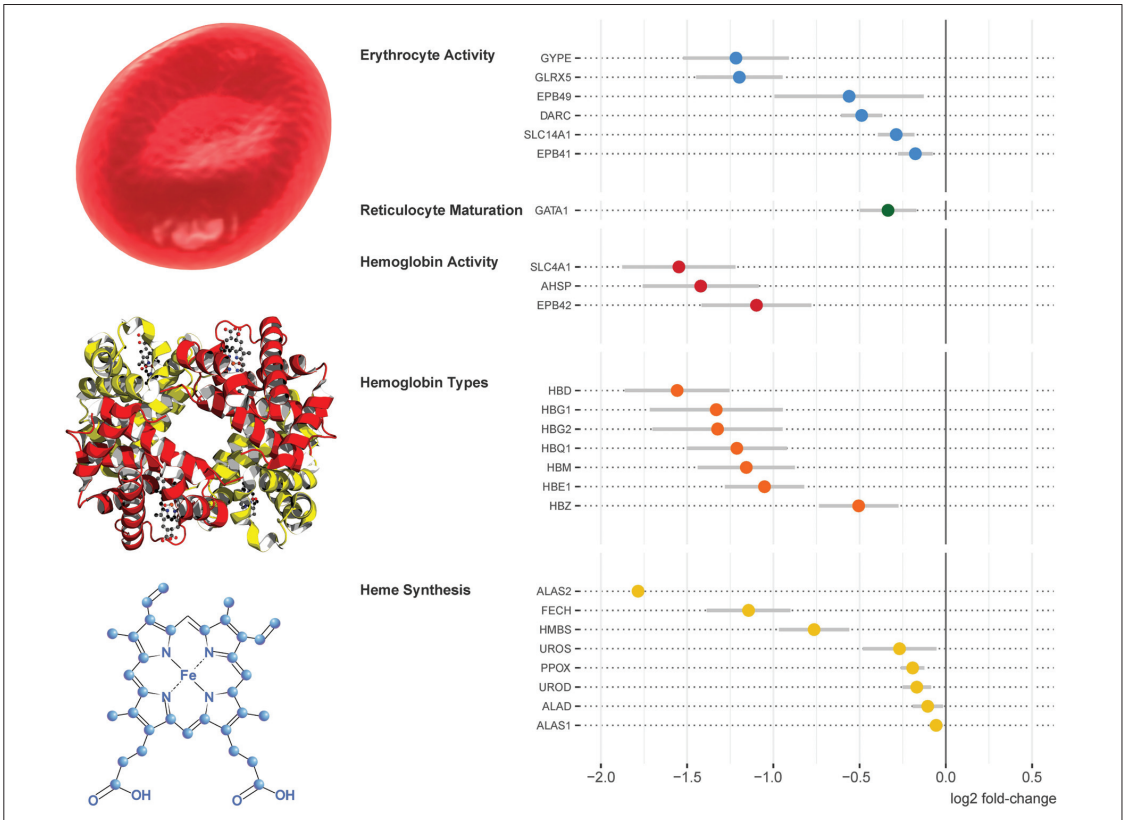

FIGURE 3 | Genes involved in oxygen transport were downregulated on multiple levels after saturation diving. Reading from the bottom up, the plot on the right hand side presents differential gene expression on the levels of heme synthesis, hemoglobin synthesis and activity, reticulocyte maturation, and erythrocyte activity. The gene expression data are shown as means and 95% confidence intervals.

TABLE 3 | Plasma biomarker levels pre- and post-saturation with and without antioxidant vitamin C and E supplements.

	Divers with vitamins (*n* = 13)		**Divers without vitamins (*n* = 7)**	
	Pre-saturation	**Post-saturation**	**Pre-saturation**	**Post-saturation**
CRP (mg/l)	1.50 (0.12–6.74)	0.98 (0.13–4.60)	0.55 (0.15–0.97)	1.27 (0.16–4.40)
IL-6 (ng/ml)	1.02 (0.31–3.67)	1.11 (0.23–4.46)	0.89 (0.52–1.35)	1.51 (0.20–7.07)
ICAM-1 (pg/ml)	206.77 (131.81–365.71)	220.81 (146.25–403.38)	157.38 (71.53–200.91)	164.68 (79.29–189.59)
Fibrinogen (g/l)	2.86 (2.30–3.60)	2.96 (2.30–4.20)	2.57 (1.90–3.50)	2.94 (2.30–3.40)
PAI-1 (ng/ml)	8.27 (0.58–25.80)	10.83 (2.50–47.79)	6.43 (1.76–12.48)	4.65 (1.76–9.89)

Data are shown as mean (range).

to hyperoxia, demonstrated by the decrease in the erythrocytes count due to the higher availability of oxygen. The divers' BMI did not change post-saturation, indicating that they kept their energy balance and hydration levels stable (Deb et al., 2016).

For the second study objective; the assessment of antioxidant vitamin supplement effects, we found no changes in the blood collected after saturation diving for either gene expression or plasma biomarker levels.

Effective management of oxidative stress is important for health preservation. While this is normally provided by the body's endogenous redox systems, diving challenges the physiological balance by inducing ROS production in excess of normal doses. In saturation diving, hyperoxia along with absolute and partial pressure changes and inert gas exchange during decompression are likely sources of excess ROS (Brubakk et al., 2014). In the present study, we found increased expression of genes

involved in inflammatory signaling after saturation diving, as would be expected if ROS levels increased. Previous studies have concluded that high oxidative stress in divers may be reversed through antioxidant vitamin supplementation. In a previous experiment, 600 mg of vitamin C, 150 mg of α-tocopherol (vitamin E) and 600 mg of tea catechins given to divers during a 400 msw deep saturation dive, every day for 40 days; prevented hepatic disturbances (Ikeda et al., 2004). A recent publication demonstrated that divers' endogenous anti-oxidant mechanisms counteracted the effects of hyperbaric hyperoxia after 200 msw saturation diving (Domoto et al., 2016).

Sparse data are available showing the effect of antioxidant vitamins on vascular function in relation to diving, but many experiments were conducted on the general population. However, reports on the latter are conflicting. A 2015 systematic review of vitamin C and E supplementation concluded that while either vitamin C or E alone appeared to improve endothelial function in healthy individuals, their combination might have little effect (Ashor et al., 2015). There is also cause for caution concerning antioxidant types and dosages, as a 2014 Cochrane review reported that some vitamin supplements – vitamin E, but not C, amongst them – might even be harmful (Bjelakovic et al., 2012). The antioxidant vitamin C and E doses in our study were at the upper limits of the daily intake recommended for healthy adults, beyond which adverse effects such as stomach cramps, nausea, and diarrhea have been reported (Institute of Medicine Panel on Dietary Antioxidants Related Compounds, 2000). The supplements were combined with a diet that was already rich in antioxidants through fruits and vegetables, so that the total doses of antioxidants were unknown. As this study was conducted in an offshore operational setting, the risk of significant discomfort from the vitamins would be unacceptable; we were therefore not at liberty to increase the dosage, and do not know whether larger doses would have altered the outcome. However, as apparent from a recent review of antioxidant effects in saturation diving subjects, care is warranted when choosing whether and how to give antioxidant supplements (Deb et al., 2016).

Simultaneous measurement of a panel of biomarkers can provide a comprehensive assessment of oxidative stress effects on vascular function. In this study, protein biomarkers were chosen in order to cover key features of vascular function; i.e., inflammation, endothelial function, and fibrinolysis. CRP is a biomarker of inflammation and endothelial function (Ridker et al., 1997); its increase is associated with impairment of endothelium-dependent vasodilatation (Verma et al., 2003). IL-6 increases the adhesiveness of the endothelial cells for lymphocytes by up-regulating ICAM-1 for a pro-inflammatory reaction (Watson et al., 1996).

Elevated fibrinogen is a predictor of cardiovascular disease risk (Kannel et al., 1987), while PAI-1 is a biomarker of impaired fibrinolysis. In a study of simulated saturation diving in rats, PAI-1 was shown to increase markedly during the first hours after decompression on the levels of gene expression and plasma proteins (Eftedal et al., 2012).

Elevated circulating levels of LDL and triglycerides are risk factors for atherosclerosis and cardiovascular disease. Their effects are countered by HDL, which acts as an antioxidant and anti-inflammatory molecule promoting endothelial repair (Tabet and Rye, 2009). Elevated creatinine is associated with oxidative stress and inflammation (Stenvinkel et al., 1999). In this study, blood lipids and creatinine were all within normal ranges before and after saturation.

Taken together, the unchanged levels of the biomarkers chosen to assess inflammation, endothelial function, and fibrinolysis suggest that any potential effects of saturation diving on vascular function were resorbed; the diver's bodies had returned to their pre-saturation state by the end of decompression. The absence of antioxidant vitamin effects also indicates that the divers retained their vascular homeostasis through their own endogenous antioxidant defenses, as is also supported by the observed upregulation of SOD1, CAT, and GSS. Endogenous antioxidants that primarily or exclusively act in the mitochondria, e.g., SOD2, were downregulated or showed variable results. However, both the copy number and function of mitochondria may be affected by hyperoxia (Ma et al., 2018), which is outside of the scope of this study.

There are limitations to this study. First, the collection of blood took place during real-life diving operations, and was conducted so as not to disturb the work. As the divers were not available for tests from their time of entry until they exited the pressure chambers, sample collections were only performed pre- and post-saturation. At that time acclimatization to the hyperbaric heliox atmosphere appear to have restored their redox balances, thus curbing any potential effects of the antioxidant supplements. Considering that antioxidant supplements have been found to reverse liver dysfunction during the initial days of experimental saturation diving (Ikeda et al., 2004), we cannot rule out that the antioxidants in our study had effects in the early phases of saturation. Second, the vitamin group was almost twice the size of the control group, resulting in the latter not being optimally powered for the plasma protein analyses. However, as the gene expression profiling revealed no differences between the two groups, we consider it unlikely that a larger sample size would alter the outcome of the biomarker analyses.

In conclusion, acclimatization to professional saturation diving was associated with extensive downregulation of genes involved in oxygen transport, upregulation of endogenous antioxidants, immune activity and inflammatory signaling. Daily antioxidant vitamin C and E intake in recommended doses had no effect on the outcome of either genetic activity or vascular function biomarkers. However, this study did not address responses that might occur during the saturation phase. Future research to determine whether antioxidant supplements might protect professional divers' redox balances during underwater work would require sample collections to be performed in saturation.

AUTHOR CONTRIBUTIONS

FK, IE, and AM designed the study. AH and AF conducted the statistical analyses. ØL obtained the necessary consents. FK managed the blood and data sampling, initiated the

manuscript, and all the co-authors contributed to the writing and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00937/full#supplementary-material

TABLE S1 | Complete lists of differentially expressed transcripts and their corresponding genes; and top biological pathway associations for genes that were differentially expressed after saturation diving.

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Conflict of Interest Statement: FK and ØL were employed by TechnipFMC in Norway.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

Hemoglobin and erythropoietin after commercial saturation diving

Fatima Zohra Kiboub, Costantino Balestra, Øyvind Loennechen and Ingrid Eftedal

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Hemoglobin and Erythropoietin After Commercial Saturation Diving

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Saturation divers are exposed to elevated partial pressure of oxygen (ppO_2) in their hyperbaric work environment. Experimental studies indicate that oxygen transport is altered, and we have previously reported a drop in hematocrit and extensive downregulation of genes involved in blood oxygen transport capacity after decompression from professional saturation diving. Here we investigate the initial period of hematological adjustment back to normobaric air after professional saturation diving. Erythropoietin (EPO) and hemoglobin (Hb) were measured in blood from 13 divers at two time-points after saturation assignments lasting up to 4 weeks; first immediately after decompression and again 24 h later. Pre-dive levels defined baselines. The ppO_2 varied from 40 kPa in the saturation chambers during storage, 50 to 80 kPa during bell excursions, and gradually reduced to 21 kPa during decompression to surface pressure. EPO was similar to baseline immediately after saturation diving ($P = 0.4$), and markedly increased within the next 24 h (99%, $P < 0.0002$). Hb levels remained slightly reduced at both time-points (4% immediately after; $P = 0.02$, 8% 24 h after; $P < 0.001$). The results imply that the hematological acclimatization back to normobaric air was ongoing, but not completed, during the first 24 h after professional saturation diving.

Keywords: hyperoxia, hypoxia, hematology, normobaric oxygen paradox, saturation diving

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INTRODUCTION

Saturation diving is considered a safe method for human subsea interventions for extended periods of time. Commercial saturation divers live onboard dive support vessels in chambers compressed to a certain storage depth. They use pressurized diving bells to take them to the seabed/work location maximum 13 msw shallower or deeper than their storage depth; where they conduct different tasks from construction to maintenance. Several experimental studies into physiological acclimatization and possible harmful effects of saturation diving reported reduction in the production of reticulocytes, erythrocytes, serum erythropoietin, and hemoglobin; and cases of anemia (Nakabayashi et al., 1991; Thorsen et al., 2001; Hofsø et al., 2005; Revelli et al., 2013). However, few investigations have been performed during real commercial saturation diving offshore. Indeed, the difference in conditions between dry and wet dives (Weathersby et al., 1990), including the use of hot-water suits (Hope et al., 2005) and the activity level during the dive; contribute to the stress levels faced during the dives (Møllerløkken et al., 2011). In a previous study of commercial saturation divers on the Norwegian Continental Shelf, the divers' hematocrit levels were measured (Kiboub et al., 2018). Data showed they were reduced post-surfacing, a possible sign

of physiological acclimatization to an elevated partial pressure of oxygen (ppO_2) during saturation. Therefore, we decided to investigate the changes in hemoglobin (Hb) and erythropoietin (EPO) during the initial 24 h after decompression from a long commercial saturation dive, to see the divers' readjustment when they move from a hyperoxic hyperbaric helium-oxygen (heliox) atmosphere to normobaric air. We hypothesized that the levels of Hb and EPO should be decreased immediately post-surfacing and start to increase during the first 24-h post-saturation with some delay for Hb increase after EPO increase.

MATERIALS AND METHODS

Research Ethics

This study was organized from Norway, and performed on occupational saturation divers during work assignments in the United Kingdom, with TechnipFMC as a Diving Contractor. The study protocol was approved by the local Regional Committee for Medical and Health Research Ethics (REK), approval reference number 2015/351; and by TechnipFMC's Diving and Health services in Norway and United Kingdom divisions. The data and sample collections were conducted according to the Declaration of Helsinki principles for ethical human experimentation. All participants gave informed, written consent before inclusion into the study, and held valid health certificates for working as saturation divers on the United Kingdom Continental Shelf. The study group consisted of males who were cleared for diving by the Diving Contractor's hyperbaric nurse after the mandatory pre-dive medical examination. All were non-smokers, and without infections at the time of their pre-dive examination.

Dive Procedure

The diving operations took place during September to November 2016 off the northeastern coast of Scotland, and were conducted according to the Diving Contractor's procedures. The divers' storage depths varied between 80 and 90 meters of seawater (msw), depending on the work location. A saturation period included compression, bottom time with bell runs, and decompression. During compression, the chamber was pressurized at 1 msw/min with 20 min stop at 10 msw for chamber leak checks before proceeding to the storage depth at which the divers were held. At storage depth, the divers had a 1 h stabilization period before bell runs commenced. Throughout the period at storage depth, the living chamber's heliox atmosphere kept a ppO_2 of 40 kPa. Each bell run lasts for up to 8 h with <6 h in-water per bell run. During bell diving, the ppO_2 was maintained between 50 and 80 kPa. Between the third and fourth hour of in-water dive time, the divers had to return to the diving bell for a 20-min restitution break for rehydration. The work tasks conducted on the seabed included, but were not limited to, pipelines tie-ins, handling of rigging and equipment and installation of flexible jumpers and umbilical. During decompression, a ppO_2 of 50 kPa was kept up to 12.7 msw; then the ppO_2 was gradually decreased while the oxygen content in the heliox was kept between 21 and 23% until surfacing. The ascent rate was 1.5 msw/h until arriving to 15 msw; from thence to surface, the ascent rate was 0.6 msw/h. The decompression was stopped for 5 h after every 19 h of ascent, for the divers to stabilize.

Four divers stayed in saturation for 25 days (d) and 15 h, $n = 5$ stayed 27 days and 6 h and $n = 4$ stayed 27 days and 21 h. The total duration of saturation depended on the operations; with a maximum of 28 days as per the Diving Contractor's requirements. After surfacing, the divers stayed on the vessel for at least 12 h for "bends watch." They were instructed to rest and avoid physical exertion during the first 24 h.

Blood Sampling

Blood samples were collected by standard antecubital venipuncture; in VACUETTE® Z Serum Sep Clot Activator 5 ml gel tubes (Greiner Bio-One, Radnor, PA, United States), prior to saturation during the pre-dive medical examinations; at the end of decompression during the post-dive medical examinations, always within 2 h before or after saturation diving. The final sample was collected 24 h after the end of the decompression. Sampling was conducted between 08:00 and 24:00 h, depending on the time of day when the divers emerged from the chamber.

Hb was measured immediately after blood collection. A capillary pipette was used to transfer a 10 μl drop of blood from the tube and deposit it into the well of a disposable test strip attached to a portable Mission Plus Hb apparatus (ACON Laboratories, San Diego, CA, United States). The same apparatus was used for all measurements. Control strips were used regularly to check analytic performance. The manufacturer indicated that the apparatus's accuracy was ±0.4 g/dl for Hb. All Hb values were within the reported normal range for healthy males (14–18 g/dl) (Billett, 1990). The within-subject variation coefficient for Hb was 0.44 g/dl (Lacher et al., 2012).

Within 30 min of sampling, the blood tubes were centrifuged in an EBA 270 centrifuge (Hettich GmbH & Co.KG, Tuttlingen, Germany) at 4,000 RPM (1,800 G) for 10 min at room temperature to separate the serum. The serum was stored frozen at −20°C onboard the vessel before being transported in cooling cubes (VeriCor Medical Systems, Holmen, WI, United States) at 4°C to NTNU, where it was kept frozen at −80°C until analysis. Transportation from the vessel to the analytic facility took 12–18 h.

EPO measurements were made as a single-batch run on a Siemens DPC IMMULITE 2000 Immunoassay System (Siemens Healthcare GmbH, Germany) in the IEC 17025-accredited laboratory at the St Olav's University Hospital, Department of Clinical Chemistry, Trondheim, Norway. The laboratory reported analytical variation of 8.6%. With one exception, (EPO 37.7 IU/l), all EPO values were within the normal range, 4.3–29.0 IU/l, reported by the laboratory.

Statistical Analysis

Prior to the analyses, the data were assessed for normality using the Shapiro-Wilk test. Hb data was normally distributed, whereas the EPO data became so after log transformation. Grubbs' test was used to examine the data for outliers: none where identified. Percentages changes relative to pre-saturation diving

values were calculated for each parameter at 0 and 24 h post-saturation diving. A repeated measures ANOVA with a Dunnet's *post hoc* test was performed. Paired *t*-tests were conducted for group-wise comparison of data 0 h post-dive to 24 h post-dive. All statistical tests were done using a standard computer package, GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, United States). *P*-values < 0.05 were considered significant.

RESULTS

The dives were completed in accordance with the Diving Contractor's saturation diving procedures, without adverse events. Out of 20 divers originally enrolled in the study, seven were excluded as they left the vessel shortly after surfacing, before the 24 h post-dive blood samples could be obtained. One diver had a bacterial infection on his face during decompression; he was given antibiotics but not excluded from the study. See **Table 1** for a summary of the study participants' demographics, diving history, body-mass index (BMI) and resting heart rate pre- and post-saturation diving.

Hb and EPO data are shown in **Figure 1**. Pre-saturation diving values were used as a baseline for each diver to avoid the intra-subject variance. Hb fell during the dive, and continued to do so during the initial 24 h after saturation diving. Immediately after decompression, Hb was reduced by 4% relative to the pre-dive values ($P = 0.01$); and 24 h post-dive the Hb was reduced by 8% ($P < 0.0001$).

EPO was unchanged immediately after decompression relative to the pre-dive values ($P = 0.4$). However, during the 24 h post-dive, there was a marked increase in which EPO levels almost doubled (99%, $P = 0.0002$). Between 0 and 24 h post-saturation dive, there was a decrease in Hb levels ($P = 0.02$) and an increase in EPO ($P < 0.001$).

DISCUSSION

In this study, Hb levels were reduced after decompression from commercial saturation diving, and remained below their pre-dive levels 24 h later. The observed reduction of Hb immediately after decompression is in agreement with findings in a recent report (Deb et al., 2017). Whereas they found Hb no longer to be significantly reduced 24 h later, this present study observed a further decline. However, the number of subjects in both studies was limited, and our results are not necessarily conflicting. In a previous study of saturation divers, bilirubin levels were unchanged post-dive; indicating that the erythrocytes were not hemolyzed

TABLE 1 | Description of study group.

				Pre-dive		Post-dive	
	Age (years)	Total diving (years)	Saturation diving (years)	BMI (kg/m)	Heart rate at rest (beat/min)	BMI (kg/m)	Heart rate at rest (beat/min)
Mean (range)	45.8 (34.0–59.0)	21.4 (6.0–35.0)	15.8 (3.0–34.0)	26.6 (23.3–31.1)	67.3 (54.0–91.0)	26.4 (22.2–31.4)	75.4 (58.0–96.0)

n = 13, mean (range) pre- and post-dive.

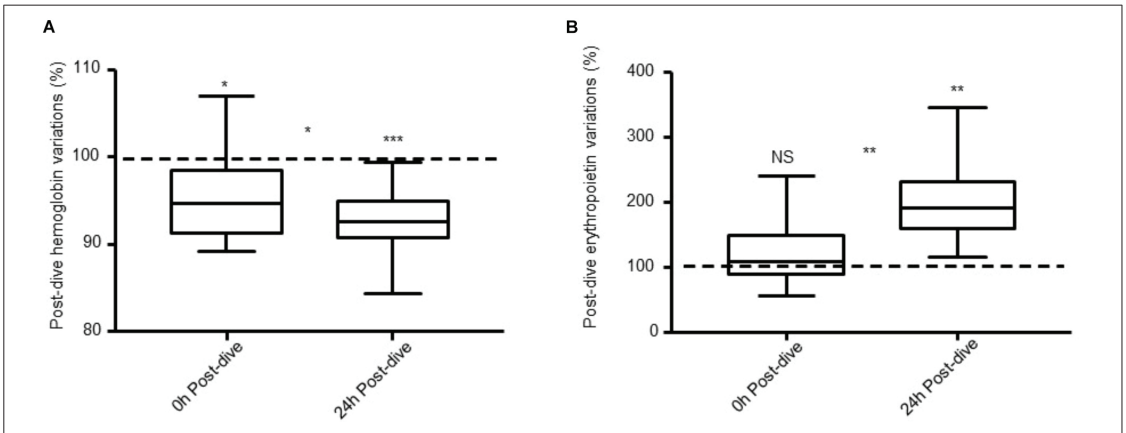

FIGURE 1 | (A) Hb and **(B)** EPO levels 0 h post-dive and 24 h post-dive expressed as percentages of pre-saturation diving levels (dotted line) in 13 saturation divers. Error bars are ± SE. $*P \leq 0.01$; $**P \leq 0.001$; $***P \leq 0.0001$ and NS, non-significant.

but rather their production was reduced, in line with the reduction of Hb production (Hofso et al., 2005). It is a challenge to maintain the divers' hydration levels during bell excursions in commercial saturation diving. In this study, measurements of hematological status were done after completion of the saturation diving assignment, and not after the bell runs. After the last bell run, it took more than 5 days to complete the decompression, during which time the divers were encouraged to keep their hydration levels high in order to facilitate efficient gas exchange as dehydration is known to interfere with plasmatic surface tension and increased bubble production during decompression (Gempp et al., 2009).

The EPO levels in this study were unchanged immediately post-saturation diving, but markedly increased during the next 24 h. During the long saturation period, the divers acclimatize to a ppO_2 of 40 kPa, which is close to twice the ppO_2 in normal air (Revelli et al., 2013). EPO down-regulation leads to a decrease in the production of erythrocytes (Krantz and Jacobson, 1970), working to reduce the toxicity caused by the increased concentration of reactive oxygen species (ROS) at high ppO_2. EPO is up-regulated when a reduction is sensed in the breathing gas oxygen content; which occurs after the divers emerge from the hyperbaric chamber and readjust to life in ambient air (Nakabayashi et al., 1991). Moving from hyperbaric hyperoxia to normobaric normoxia during the first 24 h after decompression causes a relative hypoxia. During this phase, up-regulation of EPO triggers erythrocyte production. This phenomenon, known as the normobaric oxygen paradox (NOP), was first described in healthy subjects breathing normobaric oxygen (Balestra et al., 2004); and later reported in experimental deep saturation dives (Hofso et al., 2005). The primary endogenous antioxidant glutathione (GSH) scavenges ROS in hyperoxic hyperbaric environments by oxidation. When GSH is oxidized, it forms glutathione disulphide (GSSH) which takes time to return to its reduced state. The reduction process is catalyzed by the enzyme GSH reductase using NADPH, the efficiency of which depends on the glucose conversion rate. The slow rate of the latter leads to GSH depletion and accumulation of GSSH, which inhibits the activation of the transcription factor hypoxia inducible factor-1 alpha (HIF-1α) via the hypoxia-responsive element (HRE) (Cimino et al., 2012). Binding of HIF to HRE controls the expression of genes involved in oxygen homeostasis, including EPO (Semenza et al., 1996; Masson et al., 2001). Under stable normoxic conditions, HIF-1α is inactivated by binding to Von Hippel Lindau (VHL) protein; this complex is constantly bound to ubiquitin ligase (Maxwell et al., 1999). But when returning to normobaric normoxia or in the case of our divers to a relative hypoxia; the GSH ratio increases. The extra GSH will neutralize ROS and inhibit the binding of HIF-1α to VHL; which leads to the activation of HIF-1-mediated gene transcription. This triggers the production of EPO; thus, increasing the hemoglobin and erythrocytes production (Huang et al., 1997; De Bels et al., 2012).

In a previous experiment (Revelli et al., 2013), six divers were kept in a habitat at 9 msw; breathing air at 40.5 kPa ppO_2 for 14 days. Before the experiment, EPO was 11.6 ± 3.1 IU/l. This decreased by the 14th day of saturation to 4.2 ± 1.6 IU/l, and at the end of the experiment, EPO was 4.5 ± 1.7 IU/l. At this point, one of the divers displayed mild signs of anemia. The authors discussed that the prolonged hyperoxia might have caused a clinically relevant anemia if the exposure was prolonged. In the present study's setup, despite the higher ppO_2 and longer exposure times, the results show a different trend. EPO levels trended toward a decrease at the end of the decompression before increasing, but still – with one exception – they remained within the normal range. To our knowledge, a transient increase in EPO is not associated with disease risk in healthy individuals. Also, even though total duration of the diver's stays in saturation differed, this did not affect the outcome.

Limitations

Hematological variables may be subject to circadian and other temporal variations. Studies of circadian variation in EPO have come to different conclusions (Pasqualetti and Casale, 1996; Roberts and Smith, 1996; Balestra et al., 2004; Gunga et al., 2007) and it was not determined whether circadian rhythms had an impact in the present study. Due to operational restraints, it was not possible to measure the hydration state of the divers throughout the saturation period, nor to control the timing of blood sampling as this depended on the divers' availability on-board. Therefore, variations associated with circadian rhythms or dehydration could not be ruled out.

CONCLUSION

Blood Hb was reduced immediately after decompression from commercial saturation diving, compatible with acclimatization to the hyperbaric hyperoxia the divers experienced during saturation. A marked increase of EPO levels 24 h after decompression suggested re-acclimatization back to breathing normobaric air, advocating for a physiologically compensated effect of the dive procedure.

AUTHOR CONTRIBUTIONS

FK, IE, and CB designed the study. ØL obtained the necessary consents. FK managed the blood and data sampling. CB conducted the statistical analysis. FK initiated the manuscript and all the co-authors contributed to the analysis, writing and approval of the final manuscript.

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Paper III

Commercial divers' subjective evaluation of saturation

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Commercial divers' subjective evaluation of saturation

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Abstract

Commercial saturation diving involves divers living and working in an enclosed atmosphere with elevated partial pressure of oxygen (ppO_2) for weeks. The divers must acclimatize to these conditions during compression, and for up to 28 days until decompression is completed. During decompression, the ppO_2 and ambient pressure are gradually decreased; then the divers must acclimatize again to breathing normal air in atmospheric pressure when they arrive at surface.

We investigated 51 saturation divers' subjective evaluation of the saturation and post decompression phase via questionnaires and individual interviews. The questions were about decompression headaches and fatigue; and time before recovering to a pre-saturation state.

Twenty-two (44%) of the divers who responded declared having headaches; near surface (44%) or after surfacing (56%). 71% reported post-saturation fatigue after their last saturation, 82% of them described it as typical and systematic after each saturation. Recovery was reported to normally take from 1 to 10 days.

The fatigue and headaches observed are compatible with divers' acclimatization to the changes in ppO_2 levels during saturation and decompression. They appear to be reversible post- decompression.

1. Introduction

Commercial saturation diving in the North Sea started with the emergence of the offshore oil and gas industry in 1969. The North Sea has depths from 100 to 180 meters of sea water (msw) that became the standard range of manned underwater operations. In 1980, the interest shifted to deeper diving in Norway and in Brazil. A series of contracts was awarded in Norway for validating diver interventions to 300-350 msw. During this period, outstanding developments were conducted at the Norwegian Underwater Technology Centre (NUTEC) in Bergen and several deep saturation dives were performed in the Norwegian fjords (Hope et al., 2005a).

However, at the turn of the 90's, the Norwegian media raised the issue of potential long-term health effects of deep diving. This became a national debate and in 1993, the Godøysund conference concluded that standard saturation diving in the Norwegian continental shelf should be limited. Today, NORSOK-U100 standards (Standards Norway, 2014) that regulates manned underwater operations in Norway limits standard operations to 180 msw.

The limitation of diving depths in Norway was not the end of deep diving. The expertise was transferred from Norway to Brazil by companies like Comex that were operating internationally. In Brazil, deep diving to 200-240 msw became a routine (Hope et al., 2005b). The key factors for preserving divers' health identified during the Brazilian operations were: a) selection on experience, b) selection on fitness, and c) progressive adaptation to increasing depths (Vivaqua, 2017).

A second conference held in Norway in 2005 revisited the 1993 Godøysund conclusions. This conference first accounted for the evolution of diving procedures and the success of Brazilian operations. It also permitted separating the issues of the potential neurological effects due to long exposures to elevated partial pressure of oxygen (ppO_2), the effect of circulating venous gas on the lung function (Thorsen et al., 1995), and oxygen effects. Oxygen effects were further split into oxygen pulmonary toxicity (Thorsen et al., 1990; Thorsen et al., 1993) and changes in blood hemoglobin concentration (Hofso et al., 2005). During the conference, the results of the Examination of Long Term Health Impact of diving (ELTHI) study were presented (Murray et al., 2004). The ELTHI study failed to detect any long-term health effects except for welder divers.

Saturation procedures use higher than normal levels of oxygen in the breathing gas, as illustrated in Figure 6 for a typical saturation profile. We therefore postulated that some of the symptoms reported during and after decompression from saturation were related to relative hypoxia when the diver returns to surface; breathing normobaric air with a ppO_2 of 21 kPa. The rationale for this relative hypoxia was based on the succession of two oxygen exposures. In the first step, during saturation, the divers become acclimatized to elevated levels of oxygen. This is supported by observations of a reduction in red blood cell counts. Drops in hematocrit and hemoglobin concentrations have also been measured in divers after a period of saturation, although the values remained within normal range (Thorsen et al., 2001; Deb et al., 2017; Kiboub et al., 2018a; Kiboub et al., 2018b).

In a second step, by the end of the decompression, the ppO_2 decreases and the oxygen content in the chamber breathing gas must be kept below 23% to prevent the risk of fire. Depending on the diving procedures, this happens at 12-13 msw and lasts for 16-21 hours until the chamber reaches surface pressure. At the end of the decompression, this drop of breathing gas ppO_2 is perceived by the body as hypoxia (De Bels et al., 2012; Kiboub et al., 2018a).
Our objective in this study was to assess signs and symptoms of hypoxia in saturation divers surfacing from decompression in the North Sea, by implementing an evaluation questionnaire.

2. Materials and methods

2.1. Research ethics

This study involved professional divers working in the North Sea, with TechnipFMC as a diving contractor. The study protocol was approved by the Academic Ethical Committee of Brussels (B20-2009-039) and TechnipFMC Diving and Health services in Norway and UK divisions. Data collection was conducted according to the Declaration of Helsinki principles for ethical human experimentation. All participants agreed in writing on individual consents before inclusion in the study. Data collected from the questionnaires were included into an Excel database. The database complied with the 2018 European general data protection regulations; it was fully anonymized, and the anonymization was irreversible.

2.2. Worksite

The monitoring sessions were conducted onboard the Diving Support Vessel (DSV) Deep Arctic between 2015 and 2016. The study covered two operations; one in the Norwegian sector at 110 msw storage depth (121 msw working depth) and the other in the UK sector at 136 msw storage depth (155 msw working depth). In the two sectors, the divers used the same breathing gases and equipment and performed the same type of work (well intervention). However, the saturation procedures differed slightly because of local regulations.

2.3. Study group

The study group consisted of male certified commercial saturation divers. All divers who were cleared for diving by the hyperbaric nurse during the pre-dive medical examination, were eligible for participation. The divers were organized in teams of 3 men. Each team was involved in one excursion per day during their 12 hours-shift. The divers' shift time was fixed over the saturation period, and the shifts were distributed over the day as the vessel operated a two-bells system for 24 hours' coverage. Bell run time was limited to 8 hours and diver's in-water time to 6 hours; with a 30-min restitution break inside the bell in the middle of the in-water time. Table 1 describes the study group demographics, total and saturation diving experience and body-mass index (BMI).

2.4. Saturation procedures

Saturation was conducted using heliox (helium and oxygen mixture) as breathing gas. Two saturation procedures were used, NORSOK U-100 saturation procedures (Standards Norway, 2014) in Norway, and the TechnipFMC standard saturation procedures in the UK; which only differ in the final decompression.

During compression, the chamber ppO_2 was maintained between 21-45 kPa. The divers were pressurized to storage depth at a rate of 1 m/min. At storage depth, the chamber ppO_2 was kept between 38-42 kPa. During the excursions, the breathing gas ppO_2 varied from 60 to 80 kPa. During decompression, the chamber ppO_2 was kept between 46-50 kPa in Norway and between 48-52 kPa in the UK (difference of 2 kPa, or 4%). Close to surface, the chamber oxygen percentage was kept between 21-23% in both procedures.

The decompression durations were 5 days and 4 hours in the Norwegian project and 5 days 13 hours in the UK project. The decompression durations differed by 9 hours (5%). The maximum saturation durations are limited to 14-days bottom time in Norway and 28-days total time in the UK. The actual saturation durations varied according to the operational needs.

2.5. Questionnaire

The study was part of a larger project of divers' monitoring, aiming at collecting information on divers' high pressure nervous syndrome (HPNS) symptoms, fatigue, heat and cold exposure, stress, sleep and hydration. The study was based on a questionnaire focusing on the diver's subjective evaluation of oxygen acclimatization:

- Diver's experience of headaches during or after decompression,
- Diver's subjective evaluation of post-saturation fatigue,
- Time required to return to normal pre-saturation state regarding the factors above,
- Diver's strategy to cope with return to normal life.

The questionnaire included boxes to tick-off and, when relevant, a visual scale using a line of 10 cm (0 to 10) allowing a continuous evaluation. The divers were asked to complete the questionnaire within 12 hours after surfacing from decompression. Some then went into structured interviews with the investigator in a separate room in the vessel's hospital, one at a time. The objective was to provide explanations if needed and ensure that all the questions were answered. The interviewer did not influence or change the divers' answers at any stage.

2.6. Transthoracic venous gas bubble detection

Transthoracic ultrasound echography and Doppler examinations for circulating venous gas bubble detection were conducted within 2 hours of the end of decompression. A Mindray M7 echocardiograph (Mindray, Shenzhen, China), equipped with a 2.5 MHz linear array transducer was used. Each diver was at rest on a bed in supine position for 3 minutes, before his heart was examined in an apical 4 chamber view as described in (Bulwer et al., 2010). After the first examination, the subject performed a series of 3 squats prior to a second examination to detect potential bubbles released after the effort. Venous bubbles were also monitored using the Doppler. Several video sequences of 250 frames were

registered for each diver, and used for the bubbles count. The Eftedal-Brubakk bubbles grading system was to be referred to in the eventual presence of bubbles (Eftedal and Brubakk, 1997).

2.7. Statistical analysis

Different statistical tests were adopted depending on the nature of the data. Two-sided Fisher exact tests were applied with categorical data. Pearsons test was conducted to examine correlations between age and recovery time. *P* values < 0.05 were considered significant.

3. Results

The diving operations were concluded without any incidents. A total of 51 divers participated in the questionnaire survey (n = 29 in the Norwegian sector, and n = 22 in the UK sector). The average saturation duration for the divers involved in the study was 19.7 ± 6.5 days. No bubbles were detected after saturation in any of the divers, either in the ultrasound images or by Doppler. The questions' response rates and results are described in Tables 2 and 3. There was no significant relation between age and recovery time (correlation coefficient = 0.023).

4. Discussion

Although the storage and diving depths were different in the Norwegian and UK sectors, the same breathing gases and equipment were used; and the work scope was similar. The decompression durations and chamber ppO_2 were comparable; and no bubbles were detected, suggesting that the decompression stresses in the two sectors were also similar. Therefore, the data for the two saturation procedures in the Norwegian and UK sectors were merged in further discussion.

Oxygen levels in saturation

The use of oxygen in diving is a trade-off between positive and negative effects. Saturation procedures use elevated oxygen content. The principle is that a high ppO_2 in the breathing gas mixture increases the inert gas gradient and accelerates its elimination during decompression. The disadvantage is the negative effect of elevated oxygen levels on the pulmonary function, and its potential toxicity on the central nervous system (Davis et al., 1983; Manning, 2016).

The ppO_2 values used in commercial saturation diving have been empirically set. For instance, the chamber ppO_2 at storage depth, which is currently around 40 kPa, came after a chamber with an external regeneration system had a pipe rupture. The chamber dropped from 150 msw to 70 msw before the chamber operators could close the skin valves. At the time, it was though that if a chamber could drop half its depth, then the chamber ppO_2 should be twice the normal value for the divers to avoid hypoxia. And it has remained thus since.

Post-saturation headaches

Headaches were frequently reported by the end of decompression. The headache score was 32% (question Q1). When combining the groups “often” and “sometimes” in question Q4, the score became 44%, no difference was found between Q1 and Q4 ($P = 0.25$). The reported severity of the headaches differentiated two groups of divers; with one group describing headaches as light (grade 1 to 4) and the other group describing them as severe (score 6 to 10). This last group also represented divers who reported being prone to migraines, thus there could be a link between their sensibility and the severity of the post-saturation headache effects.
The onset of headaches occurred near surface (44%) and after decompression (56%), which coincided with the reduction of the chamber ppO_2 or the switch to atmospheric 21 kPa ppO_2. The headache occurrences thus appeared to be synchronized with the changes of inhaled ppO_2. This is consistent with a reactive cerebral vasodilation due to hypoxia.

Post-saturation fatigue

Most divers reported a feeling of fatigue, lasting up to 10 days after the end of decompression. It is reasonable to expect divers performing intense efforts 8 hours a day, for several consecutive weeks, to experience physical fatigue. Several divers involved in night shift dives also mentioned the time required to readjust their circadian rhythms. However, if 71% of the divers reported post-saturation fatigue after their last saturation (question Q8), they also described it as typical and systematic (82% in question Q10). There is no difference between Q8 and Q10, ($P = 0.5$). The feeling of fatigue was presented in the following way:

- The feeling of fatigue was declared to be more physical (82%) than mental (question Q11). This feeling was generally described as a limitation to effort, like becoming breathless when climbing stairs.
- The sense of fatigue remained within moderate levels on a scale from 1(light) to 10 (severe) (mean ± standard deviation (SD) = 3.4 ± 1.92 in question Q9).
- The overall well-being or mood, graded on a scale from 1 (bad) to 10 (excellent), was not affected (6.18 ± 1.56 in question Q12).
- The capacity to focus or mental alertness, graded on a scale from 1 (bad) to 10 (excellent), was generally unaffected (6.23 ± 1.91 in question Q13). However, one diver mentioned difficulty to concentrate post-saturation.

Recovery from post-saturation fatigue

All the divers indicated a recovery process and reversible symptoms. However, their post-saturation sense of fatigue could last from 1 to 10 days (4.31 ± 2.92 days in question Q14). One diver reported a recovery in 3 days by comparing his bicycling performance on a regularly used circuit. After returning home, the divers adopted different strategies to manage this feeling of fatigue. Some said they immediately caught up with life and got intensely involved in sport, social life and auxiliary business. Others reported they preferred to take a relaxing week.
An often-depicted relative paleness after decompression was observed on most of the divers. Some divers confirmed that this paleness was noticed by their families after their return home (question Q7, 95%). The divers transferring from the chamber to surface

instantly switched from breathing heliox to air. It is conceivable that their bodies may react to the relative drop in oxygen at this point. The transient isobaric counter diffusion and the counter fluxes of helium and nitrogen might have momentarily disturbed alveolar ventilation and reduced oxygen exchanges. The deprivation of sunlight may also contribute to the paleness of the divers.
A study was conducted simultaneously onboard the same vessel in 2016, where hemoglobin and erythropoietin (EPO) levels were measured pre-saturation, immediately post-saturation and 24-hours post-saturation (Kiboub et al., 2018a). An increase in EPO was registered over the initial 24-hours post-saturation. As EPO regulates erythrocyte production, a post-saturation increase in EPO may counteract the hypoxia perceived after decompression. EPO may thus contribute to the reversible nature of the symptoms observed in this study.

Possible evolution of saturation procedures

Considering that most of the saturation procedures used in the offshore industry were designed empirically in the 90's, knowledge obtained via research may contribute to their improvement. The benefit would not primarily be divers' safety, since saturation diving is already relatively safe (1 decompression sickness per 1,000 exposures) (Petroleum Safety Authority Norway (PTIL), 2018) but rather the divers' well-being.

During decompression from saturation, the chamber ppO_2 is linked to the ascent rate (Balestra et al., 2014). An experimental saturation dive was performed in Norway in 2004 where a lower chamber ppO_2 protocol was used. The authors reported a case of decompression sickness and neurological deficit after this dive (Thorsen et al., 2006). The margin for changing ppO_2 is thus narrow, but there may still be room for improvement. Currently, the ppO_2 used for the diving mixtures are specified within 60 to 80 kPa. This refers to the US Navy diving manual used in the early 70's, that authorized large excursion distances. These excursion distances have since been restricted to safer values, but the ppO_2 has remained constant. It is possible that diving mixtures might be redefined according to new experience in the diving industry. The results from the present study may help mitigate some concerns of diving long-terms effects, by showing that some symptoms appear to be related to oxygen acclimatization with reversible short-term effects.

This study has some limitations. When the interviewer was present, questions were answered with 100% compliance. However, in the interviewer's absence some answers were missed. This is a known limitation; as already shown by other authors in evaluation of other aspects of saturation (Dolan et al., 2016). The data concerning time for recovery after saturation diving were based on the divers recollection, and not assessed for the dive after which the questionnaire was completed. Future studies should address the progress of post-saturation recovery concerning symptoms of readjustment to normal life.

5. Conclusions

We conclude that the diving post-saturation effects on headaches and fatigues include a dimension that is compatible with acclimatization to the higher than normal levels of oxygen experienced in saturation. This assumption is consistent with the hemoglobin concentration changes measured in similar conditions and supported by the subjective

evaluation of saturation by the divers as assessed by the questionnaire. These effects appeared reversible post-decompression.

It may be that parts of the alleged long-term effects of saturation diving, developed in 1993 at the Godøysund Conference, are in reality short-termed, reversible effects, associated with oxygen acclimatization.

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7. Conflict of interest statement

Authors Fatima Z. Kiboub and Øyvind Loennechen were employed by TechnipFMC in Norway. All other authors declare no competing interests.

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Figure 6

A typical saturation profile (blow down, storage and bell dives, decompression at constant partial pressure of oxygen -ppO_2- and at constant oxygen percentage) and the corresponding ppO_2 profile.

Table 1

Subjects (n = 51) biometric information and experience expressed in mean (range).

	Age [Years]	**Total diving [Years]**	**Saturation diving [Years]**	**BMI [kg/m^2]**
Mean (range)	46.1 (31 to 61)	21.8 (6 to 42)	15.9 (2 to 39)	26.5 (21.0 to 32.3)

Table 2

Questions related to headaches.

Questions	**Participant's number**	**Scores**	**Response Percentage**
Q1: During this last decompression, have you experienced headaches?	34	Yes = 11	32%
		No = 23	68%
Q2: During this last decompression, if you had headache, when did the symptoms declare?	11	Near surface = 6	55%
		After surfacing = 5	45%
Q3: During this last decompression, if you had headache, grade its severity on a scale from 1 (light) to 10 (severe)	10	4 (grade 1 to 2)	40%
		6 (grade 6 to 9)	60%
Q4: Usually, do you experience headache during or after decompression?	34	Never = 14	41%
		Sometimes = 10	29%
		Often = 5	15%
		Always = 5	15%
		Sometimes + often = 15	44%

Q5: Usually, if you had headache, when do the symptoms declare?	16	Near surface = 9 After surfacing = 7	44% 56%
Q6: Usually, if you had headache, how long does the headache last?	13	Few hours = 3 Half a day = 3 One day = 4 More than 1 day = 3	23% 23% 31% 23%
Q7: When back home after a saturation, do people around you say you look pale?	20	Yes = 19 No = 1	95% 5%

Table 1

Questions related to post-saturation fatigue.

Questions	**Participant's number**	**Scores**	**Response Percentage**
Q8: After this last saturation, have you experienced fatigue?	24	Yes = 17 No = 7	71% 29%
Q9: After this last saturation, if you experienced fatigue, grade its severity on a scale from 1 (light) to 10 (severe)	17	(Mean ± SD) 3.4 ± 1.92	100%
Q10: Usually, after a saturation, do you experience fatigue?	22	Yes = 18 No = 4	82% 18%
Q11: Usually, if you experienced fatigue, is it physical or mental?	19	Physical = 17 Mental = 2	89% 11%
Q12: Usually, after a saturation, grade your wellbeing or mood on a scale from 1 (bad) to 5 (normal) and 10 (excellent)	22	(Mean ± SD) 6.18 ± 1.56	100%

Q13: Usually, after a saturation, grade your alertness on a scale from 1 (bad) to 5 (normal) and 10 (excellent)	22	(Mean ± SD) 6.23 ± 1.91	100%
Q14: Usually, after a saturation, how many days does it take to return to normal?	26	(Mean ± SD) 4.31 ± 2.92	51%