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Article

Hyperbaric Exposure and Oxidative Stress in occupational activities (HEOxS): the study protocol

Katia Aquilano [1], Tiziana Paola Baccolo[2], Alberto M. Bersani[3], Matteo Bordi [1], Flavia Buonaurio [6], Rita Businaro[5], Chiara Cerocchi[2], Corrado Costanzo [7], Nazzareno De Angelis [8], Ornella De Pità[12], Maria Concetta D'Ovidio[2], Luigi Fattorini[9], Giuseppe Filomeni[1], Cristian Ieno[9], Daniele Lettieri Barbato [1], Elisa Maggi [5], Enrico Marchetti[2]*, Maria Rosaria Marchetti[2], Paola Melis[2], Alfredo Miccheli[5,13], Ottavia Giampaoli[6,13], Enrico Paci[2], Daniela Pigini[2], Alessandro Pinto[10], Floriana Sacco[2], Fabio Sciubba[6], Mariangela Spagnoli[2], Aurora Summa[9], Alberta Tomassini[6,13], M. Elisa Tosti [3], Giovanna Tranfo[2], Riccardo Turchi[1], Giuliana Valente[11].

[1] Nutritional Biochemistry Lab., Dept. Biology, University of Rome Tor Vergata
[2] INAIL, Department of Occupational Medicine, Epidemiology and Hygiene, Monte Porzio Catone, Rome Italy
[3] Department of Mechanical and Aerospace Engineering (DIMA), Sapienza University of Rome, Italy
[4] Department of Chemistry, Sapienza University of Rome, Italy
[5] Environmental Biology, Sapienza University of Rome, Italy
[6] Department of Chemistry, Sapienza University of Rome, Italy
[7] Centro di medicina Subacquea e Iperbarica G. S. C. Srl, Roma
[8] Research Unit of Subaquatic sector of Italian Federation of Environment and Sport
[9] Department of Physiology and Pharmacology "Vittorio Erspamer", Sapienza University of Rome, Italy
[10]Experimental Medicine Department, Medical Pathophysiology, Food Science and Endocrinology Section, Food Science and Human Nutrition Research Unit - Sapienza University of Rome, Italy
[11]Department of Surgical Sciences, Sapienza University of Rome, Italy
[12]Clinical Pathology and Immune Inflammatory Disease of the Skin, Cristo Re Hospital, Rome, Italy
[13]NMR Based Metabolomics Laboratory, Sapienza University of Rome, Italy

Correspondence: Dr. Enrico Marchetti, Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, INAIL, Via di Fontana Candida 1, 00078 Monte Porzio Catone (RM), 0694181584, e.marchetti@inail.it

Abstract

Background: Hyperbaric exposure (HE) is proven to be a stressor to several mechanisms in living cells. Even if after homeostasis restoration, harmful effects are expected, in particular a presence of free radicals. These latter are the stimulus to negative phenomenon as inflammation or cancer. In Italy, with 7500 km of sea shores, a large quantity of workers is exposed to HE during occupational

activities. A deep knowledge of HE and bodily effects is not well defined; hence a multidisciplinary assessment of risk is needed. To detect one or more indicators of HE a research group is organised, under the INAIL sponsorship. The research project focused on the oxidative stress (OxS) and this paper details on the possible protocol to estimate, with a large amount of techniques on several human liquids, the relationship between OxS and HE. Specific attention will be paid to identify confounding factors and their influence.

*Methods***:** Blood and urine will be sampled. Several lab techniques will be performed on samples, both targeted, to measure the level of well-known biomarkers, and untargeted. Regard the formers: products of oxidation of DNA and RNA in urine; inflammation and temperature cytokines and protein carbonyles in blood. Untargeted evaluation will be performed for a metabolomics analysis in urine. Confounding factors: temperature, body fat, fitness, allergies and dietary habits. These factors will be assessed, directly or indirectly, prior and after HE. The final scope of the project is to determine one or more indicators that relates to HE in hits twofold nature: depth and duration.

Conclusion: The relationship between OxS and HE is not deeply investigated and literature proposes diverging results. The project aims to define the time dependence of biomarkers related to OxS, to rise knowledge in risk assessment in workers exposed to HE.

Keywords: Hyperbaric exposure, Oxidative Stress, Cytokines, Metabolomics, Diet Habits, Fitness

Introduction

Physical agents are increasing their presence on technological work place. Those agents act both on specific organs or tissues and more generally on the whole organism acting as a stressor. In these conditions, physiological adaptations have to carry out and, among others, several metabolic processes will be activated to restore the homeostasis. So, an increase in energy supply is needed with consequent increases of O_2 utilization, producing free radicals in cells and tissues. As well known a free radical can be defined as any molecular species that contains an unpaired electron in an atomic orbital. This fact makes many radicals unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (1). In every case, intermediate molecules are generated and the organism carried out several biochemical ways to counteract the effects stressor-related, all of these belonging to the antioxidant system. The Oxidative Stress (OxS) reflects an imbalance between the systemic manifestation of Reactive Oxygen Species (ROS) as cellular response to several stressors and the system's capability to readily detoxify the reactive intermediates. Mechanisms involved to counteract the OxS event are correlated to stress characteristics and dose. Depending by these as well as by homeostatic systems efficiency, several feedback circuits are activated. Finally, it appears evident as same stressor is able to induce a wide spectrum of delayed metabolic responses depending on the large variation in those circuits' efficiency (2).

According to World Health Organization "*Alterations of the endocrine, neural and immune responses to stress are involved both in aetiology and the pathophysiology of the most common health problems in modern society.*" (3). Chronic stressing stimulation are correlated to inflammatory responses that are managed by proinflammatory mediators that are described as the mostly players involved in the

progression of the diseases. Proinflammatory molecules, such as interleukin-1beta, may stimulate the expression of inducible nitric oxide synthase and cyclooxygenase, leading to the release of further inflammatory mediators. As a matter of fact, catecholamines (epinephrine, norepinephrine, and dopamine) released during the initial phase of stress promote the release of proinflammatory cytokines (4). On the other hand, catecholamines may stimulate activation of adipose tissue thermogenesis that, beside representing a protective factor against thermal stress, ameliorate inflammatory profile with important metabolic benefits (5).

Among physical agents, hyperbaric exposure (HE) is an obvious candidate as biological stressor because of the particular body pressure to cope with. As largely demonstrate, the HE could be cause of bodily negative effects. Among others, "decompression illness" (DCI) can be referred to every clinical syndrome following HE and relative normobaric return (i.e., decompression): barotrauma, acute decompression illness (described also as decompression sickness: DCS), or delayed or chronic forms of decompression illness (6) (7). In particular, DCS is caused by in-situ bubble formation from dissolved inert gas (uptake). It is known that DCS, i.e., bubble, causes hypoxia in tissues downstream the damaged area, consequently inducing a local oxidative stress (OxS). Moreover, in literature it has been reported that OxS insurgence in hyperbaric environment may be present even without DCS symptoms because both ROS are physiologically detoxified, and they cause diseases other than DCS (see redox-associated diseases) long time after the stressor onset (8).

In this regard, Obad et al. (2010) in repetitive deep dives found anti-oxidative capacity decreasing with dive, so reporting a possible cumulative and longer lasting detrimental effects (9). As reported above, OxS involves nucleic acids and protein damages. In summary, OxS and inflammation are strongly mutual depending, so that the production of ROS increases inflammation and *vice versa*, and both phenomena play a key role in the pathogenesis of the main chronic degenerative diseases, such as coronary heart disease (CHD) and other forms of cardiovascular atherosclerotic diseases (CAD), Metabolic Syndrome (MetS), diabetes mellitus type 2 (DMT2), cancers and neurodegenerative disease.

As stated, the ability to counteract the HE stressor effects depends on homeostatic mechanisms efficiency related to subject lifestyle as physical fitness, diet habits and body fat percentage or specific pathologies (hypersensitivities, allergies et al), all of these called "individual factors". The identification of individual determinants that can influence nitro-oxidative stress, and at the same time be easily evaluable and modifiable, is a necessary step to reduce the risk of exposure to HE.

Individual factors

Body fat percentage

Obesity takes place in disorders that affect mitochondrial metabolism, which promotes ROS generation and the development of OxS. The mechanism, which has been proposed, involves an effect of high triglyceride (TG) on the functioning of the mitochondrial respiratory chain, in which intracellular TG inhibits translocation of adenine nucleotides and promotes the generation of superoxide. Finally, obesity increases the mechanical load and myocardial metabolism; therefore, oxygen uptake is increased. One negative consequence of increased oxygen consumption is the production of ROS as superoxide, hydroxyl radical, and hydrogen peroxide derived from the increase

in mitochondrial respiration and, of course, from the loss of electrons produced in the electron transport chain, resulting in the formation of superoxide radical (10). In this contest, brown adipose tissue (BAT), the minor component of human adipose tissue, protects against obesity by promoting energy expenditure (11). Accordingly, obese subjects have impaired BAT activity (ref). Interestingly, adipokines secreted by active BAT (i.e., batokines) coordinate BAT activity with systemic metabolism with important anti-inflammatory benefits. Among batokines, FGF21 is of pivotal importance as it helps to decrease oxidative stress by inducing the expression of antioxidant proteins such as uncoupling protein 3 (UCP3) and superoxide dismutase-2 (SOD2) (12). FGF21 also represses NFkB acting as an anti-inflammatory stimulus (13). Upon catecholamine stimuli, also the anti-inflammatory adipokine adiponectin is abundantly secreted by BAT, thus ameliorating inflammatory profile (5).

Dietary Habits

The food intake has an immediate effect on inflammatory response, since a transient increase in blood inflammatory markers is seen within few hours after eating (14). Much information indicates that high intakes of macronutrients can promote oxidative stress and subsequently contribute to inflammation via NF-κB mediated cell signalling pathways (15). The genesis of this inflammation is closely related to lifestyle and mainly to the quality of diet and exercise. Nutritional stress also promotes oxidative stress as evident by increased lipid peroxidation products, protein carbonylation, and decreased antioxidant system and reduced glutathione (GSH) levels (11) (16). Overall, these data imply that high-carbohydrate/high-calorie/high-fat diets stimulate oxidative stress by augmenting the inflammatory response and elevating inflammatory markers. The elevation of oxidative stress is linked to chronic inflammation; other sources may also further increase the accumulation of proinflammatory cytokines in a "vicious cycle". The best dietary advice for the prevention and management of obesity and other metabolic disorders includes replacing refined carbohydrates with whole grains, increasing fruits and vegetables, substituting total and saturated fat with MUFAs, and consuming a moderate amount of calories with an ultimate goal of maintaining an ideal body weight. Overall, further studies are warranted to gain a better understanding of the types and the degree of ROS generation in relation to diet-induced metabolic disorders (15).

Physical fitness

Prolonged endurance exercise or short-duration high intensity exercise cause an increased mitochondrial respiration with consequent increase of ROS production. The excess of ROS can affect natural cellular antioxidant defences, such as vitamin E, further contributing to muscle damage (17). These endogenous antioxidant systems are plastic and adapt in response to exercise training. Different studies, in fact, confirmed that exercise training promotes an increased in primary antioxidant enzymes in cardiac and skeletal muscle and that this adaptation increases with the exercise intensity and duration (18). In other words, the repetition of the exercise could provide an adaptive response of the muscular cell, with a consequent increase in his recovery capacity. Presumable a trained subject could show a faster recovery after the oxidative stress caused by HE.

As well known, an appropriate neuromuscular activation is need to sustain a physical workload. This implies a continuous energy provision to the muscles and both aerobic and anaerobic energy metabolisms are employed. In this respect, Bloomer et al (2005) showed that 30 minutes of aerobic

and anaerobic exercise performed by young trained men can increase certain biomarkers of oxidative stress in blood (19). Other authors reported that exercise mode, intensity, and duration, as well as the subject population tested, all can impact the extent of oxidation resulting the fitness an important factor to improve the metabolic capability in buffering ROS excess (20). Evidently, in accordance with the principle of hormesis, to allow for an up-regulation in endogenous antioxidant defences is needed a stressing solicitation as those representing by the physical exercise.

Hypersensitivity to environment

The oxidative stress may be related to allergic sensitization by proteolytic activity of several aero-allergens; this relationship being very complex and subjected to a delicate balance. Oxidative markers were associated with vitamin D and respiratory function (21) and recent literature reports a relationship between oxidative stress, total antioxidant status and total IgE levels related to allergic rhinitis (22). Exposure to HE therefore represents a risk factor with multifactorial origin (23) (24) (25) (26) (27), whose components can have synergistic and / or antagonistic effects that need to be thoroughly investigated and evaluated as a cause-effect relationship in order to produce a reasoned risk assessment criterion.

Objective

Italy has a very long coastline, about 7500 km, and the ensuing large number of amateurs or professional divers. In particular, were recognized more than 5000 individuals occupied in hyperbaric activities and about 90% are undergo to a periodical medical examination because the Italian Occupational legislation considers hyperbaric work activities with high risk level (28). At present, the risk control associated with exposure to HE is based on well-defined (tabulated) decompression protocols that only take into account the duration and the pressure level of exposure. Therefore, the present study conducted by simulating the typical professional activities carried out in HE aims to assess the weight that the aforementioned individual factors may have in the genesis of hyperbaric risk. The relevance of the expected results is in particular attributable to the possibility of conditioning these individual factors through specific and customized dietary and training protocols. Moreover, these results can be effectively used as theoretical bases for a possible adaptation of the decompression tables to the individual characteristics of the worker, pursuing the aim of minimizing, in the short and long term, the possible adverse events resulting from the work in HE.

Finally, the aim of the project is to determine, through a cross-sectional study, the influence of the individual factors on ROS and on the formation of micro bubbles in HE workers. This research project will deal exclusively with air supplied with open-loop circuits or stored in cylinders, excluding expired air reconditioners (rebreather). Present research will be performed on a sub-sample of volunteers to verify the cause-effect relationship assessing ROS risk profile while intervening on individual factors (diet and fitness).

Methods

a. Subject's recruitment and characterization

The study involves Caucasian experienced divers who are suitable to perform the hyperbaric activity, have more than 5 years of experience and are 30-70 years aged. All subjects, after have received a detailed explanation of the protocol and of the possible risks during the experiment, sign the written informed consent. Information about gender, age (years), height (m) and weight (kg) are collected and BMI, mean and standard deviation values are computed for the group characterization.

b. Experimental protocol

The protocol provides that all recruited subjects fill a self-administered questionnaire for the evaluation of risks related to hyperbaric activity. Moreover, the questionnaire collects socio-demographic, anamnestic and personal data, the food habits and the level of physical activity. Other than with the questionnaire, the functional profile evaluation is also carried out with handgrip measurements and maximal exercise test.

The body composition is evaluated by mean the skinfold measurements, the plethysmography and the Multifrequency Biolectrical Analysis (MF-BIA).

For the oxidative stress evaluation urine and blood samples are used. Concerning urine, samples are collected 30 minutes approximatively before diving, 30 minutes approximatively after diving and after about 90 minutes, 4, 8 and 12 hours. Urine samples were then transferred to the laboratory where they were stored frozen at -20°C until analysis. Concerning blood, samples are collected at 30 minutes before and about 15 minutes after diving, then at 30, 60 and 90 minutes. Sampling time will be adapted to operational conditions.

The diving profile is the same in both dry and wet diving. Maximum depth 20 meters with a bottom time of 30 minutes, ascent speed of 10 meters per minute to the safety step with a stop of 3 minutes at less than 3 meters and ascent speed from the step to the surface of 20 seconds meter. This diving profile is a typical "quadra type", i.e. the diver spends all the time at the bottom to the maximum depth. About 45 minutes after diving the sonographic measurement are carried out to investigate the microbubbles formation. This elapsed time after diving is necessary to ensure the return of the divers to the shore in wet diving and to carry out the undressing operations, to standardize withdrawal and measurements times in the dry and wet conditions.

The study protocol is in accordance to the Declaration of Helsinki and to the Ethics Committee of the University of Rome "La Sapienza".

c. Body composition evaluation

All the recruited subjects undergo the following measurements, conforming to the procedures described in the Anthropometric Standardization Reference Manual (29): body weight was measured to the nearest 0.1 kg through a standard column body scale (SECA, Hamburg, Germany); body height (using a rigid stadiometer – SECA, Hamburg, Germany) and circumferences (wrist, arm, chest,

abdomen, waist, hips, thigh, calf; using a tape for anthropometric measurements) are measured to the nearest 1 mm; skinfolds thickness (biceps, triceps subscapular, suprailiac) are measured to the nearest 1 mm using the Harpenden skinfold callipers.

c.1 Bioelectrical Impedance Analysis (BIA): whole-body impedance components, impedance (Z), phase angle (PhA), resistance (R) and reactance (Xc) are measured with the single-frequency 50 kHz analyzer NUTRILAB (AKERN Bioresearch SRL, Pontassieve, Florence, Italy) and with the multifrequencies (5, 10, 50, 100, 250 kHz) bioelectrical impedance analyser (Human Im Touch®, DSMedica, Milan, Italy). The measurements are undertaken following standardized procedures (30). The external calibration of the instrument is checked with a calibration circuit of known impedance value. Fat Free Mass (FFM), Fat Mass (FM), Total Body Water (TBW), Extra-Cellular Water (ECW), Intra-Cellular Water (ICW), Body Cell Mass (BCM) are obtained from bioelectrical data, using the gender-specific prediction equations provided by the dedicate software (Human Im Touch®, DSMedica, Milan, Italy; release 15.00.00). The Fat Free Mass Index (FFMI) and the Fat Mass Index (FMI) are calculated through the normalization for height of FFM (kg) and FM (kg) obtained by the BIA: FFMI = FFM /height^2 (kg/m^2) and FMI = FM /height^2 (kg/m^2). Skeletal Muscle mass (SM) are estimated by Janssen equation (31) and Skeletal Muscle Mass Index are calculated by dividing SM by height (m^2); Appendicular Skeletal Muscle Mass (ASMM) are estimated by Kim equation (32). Whole-body bio-impedance vectors are analysed by the RXc graph method (33) using the software Bodygram Plus 1.2.2.8. Each subject is plotted in the tolerance ellipses (50%, 75% and 95%) of the reference population (BIVA®).

c.2 Air Displacement Plethysmography (ADP): Air displacement plethysmography (ADP) is performed by using the Bod Pod TM® Body Composition Tracking System (Cosmed, Rome, Italy). This methodology uses air displacement to estimate BF% and fat-free mass. A quality control procedure is performed prior to trial, the equipment is calibrated following the manufacturer's guidelines using a cylinder of specific volume (49.887 L). The participants wear the recommended form-fitting clothing and are weighted on a calibrated electronic scale. After sitting inside the BOD POD chamber, the participants are asked to remain still and breathe normally during measurement. Body volume is measured twice by ADP, and if there is a difference of more than 150 mL, a third measurement is taken. Thoracic gas volume is measured at the time of the BOD POD test, and this value will be integrated into the calculation of body volume following the manufacturer's recommendations. A mean value between the 2 or 3 measurements of body volume is obtained. %BF is calculated from the body density obtained by the BOD POD using the equation reported by Siri.

d. Dietary habits evaluation

Eating habits are evaluated developing a food frequency questionnaire (FFQ). A pre-test analysis of the FFQ is performed on a small sample of subjects (10 individuals) with characteristics similar to those of the sample recruited for the present study. The purpose of the pre-test is to identify and correct the possible errors of interpretation, the questions superfluous, the missing questions, the modalities of confused or inappropriate answers, etc. The interviewees are encouraged to comment

on the questions and answers, doing thus to emerge any criticalities. The ability to influence the inflammatory state and the oxidative stress is estimated based on the adherence to Mediterranean diet model. For this purpose, the items obtained by the MedDiet SCORE (34), the CARDIOPROTECTIVE MEDITERRANEAN DIET INDEX (35) and the PREDIMED SCORE (36) are included in the FFQ.

d.1. MedDiet SCORE: a diet score (range 0-55) has been developed that assesses adherence to the Mediterranean diet. For the consumption of items presumed to be close to Mediterranean dietary pattern (non-refined cereals, fruits, vegetables, legumes, olive oil, fish and potatoes) scores 0 to 5 for never, rare, frequent, very frequent, weekly and daily consumption were assigned, while for the consumption of foods presumed to be away from this pattern (red meat and products, poultry and full fat dairy products) scores on a reverse scale were assigned. Positive and negative predictive values, in relation to hypertension, hypercholesterolemia, diabetes and obesity status of the ATTICA study participants (n=3042, enrolment 2001-02 in Athens metropolitan area, aged 18-89 years) were calculated and the 10-year CHD risk based on Framingham equations was estimated, too (34).

d.2. CARDIOPROTECTIVE MEDITERRANEAN DIET INDEX: The short questionnaire assessed the consumption of cardioprotective elements included in the Mediterranean diet (olive oil, wine, fruits, vegetables, fish, legumes and whole-grain intake). A low consumption of meat or meat-products was also included in the composite score. The relative risk of myocardial infarction for each category of the composite score obtained (range 0-9) was computed using data from a case-control study that included 171 cases of first myocardial infarction and 171 matched controls. An increment of one point in the score was associated with an 18% reduction in the relative risk of myocardial infarction (P=0.05) (35).

d.3. PREDIMED SCORE: A brief 14-item tool was able to capture a strong monotonic inverse association between adherence to a good quality dietary pattern (Mediterranean diet) and obesity indexes in a population of adults at high cardiovascular risk (36).

e. Physical fitness evaluation

The International Physical Activity Questionnaire short form (IPAQ-SF) (https://sites.google.com/site/theipaq) is adopted to estimate the energy expenditure related to physical activities in a week. The time (minutes/weeks) spent in each considered activity is multiplied for the correspondent unitary MET metabolic equivalents (8 for vigorous activities, 4 for moderate activities, 3.3 for walking) and the total METs are computed to classify each participant as a low, moderate or high activity practitioner, according to the IPAQ-SF scoring protocol (https://sites.google.com/site/theipaq/scoring-protocol). In lab, the cardio-pulmonary incremental test is performed to assess the physiological profile. This test is realized using an cyclo-ergometer (ERS bike, Ergoline, Germany) to simulate physical activity at incremental known workload (power). During the test CO_2 and O_2 exchange and the hearth rate are measured with a metabolimeter (K5, COSMED, Italy). Exercise protocol in the test is defined by: three minutes of warm up at 60 watts,

hence the power is increased by 6 watts each 10 seconds until VO_{2max}. VO_{2max} is considered reached when VO_2 do not increase while mechanical power was still increasing and heart rate is reached the theoretical maximum. Furthermore, the muscular functional profile is indirectly evaluating whit the handgrip test. For this, using a dynamometer (HandgripDynX®, AKERN SRL, FI, Italia) the hand flexor muscles contractile force is measured. In the Handgrip test, both dominant and non-dominant side are evaluated and the best of three repetitions, with 30 seconds rest between, is considered. During the test, the subject holds the dynamometer in the hand with the arm at right angles and the elbow by the side of the body, then, avoiding any other body movements, squeezes the dynamometer with maximum isometric effort, maintaining the grip level for about 5 seconds.
In order to quantify the exercise workload during diving, adopting the measured relationship workload vs HR in the incremental test, the HR during dive is collected by means of a wearable heart rate monitor (Galileo SOL, ScubaPro, Johnson Outdoors Inc., USA).

f. Urinary Targeted biomarkers

The oxidized guanine derivatives products of oxidation of DNA (e.g., 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanine (8-oxoGua)) and RNA (e.g., 8-oxo-7,8-dihydroguanosine (8-oxoGuo)) and consequent repair products excreted into the urine can be used as biomarkers of oxidative damage in humans. (37) (38) (39). Moreover, the 5-methylcytidine (5-MetCyt) is a modified nucleoside derived from 5-methylcytosine. It belongs to the class of organic compounds known as pyrimidine nucleosides. Pyrimidine nucleosides are compounds comprising a pyrimidine base attached to a ribosyl or deoxyribosyl moiety. 5-methylcytidine is primarily located in the cytoplasm: it is an epigenetic marker of DNA and RNA damage, associated with the genetic change which is involved in the origin of cancer. It is present in multiple bio-fluids, such as faeces, urine, and blood (40) (41). Regard proteins, OxS involves the introduction of new functional groups that can alter their function and metabolism. Protein aggregation is a frequent consequence of oxidation and one of the main addition products to the phenolic ring of Tyr (Tyrosine) is 3-NO_2tyrosine, which is formed as a reaction with the NO or NO_3- and represents an oxidation product of inflammatory origin (42) (43).

f.1. Analytical Methods: The urine samples are analyzed on a Series 200 LC quaternary pump (PerkinElmer, Norwalk, CT, USA), coupled with a AB/Sciex API 4000 triple-quadrupole mass spectrometry detector equipped with a Turbo Ion Spray (TIS) probe. The 1.5 version of Analyst® software is employed for instrument control. All analytical determinations are performed by the isotopic dilution method, using isotope labelled analogues of the analytes. The concentration of 8-oxoGua, 8-oxodGuo, 8-oxoGuo, 3-NO_2Tyr and 5-MeCyt) are determined according to the analytical methods previously used in our laboratories (44) with slight modifications: a different column is used for the chromatographic separation of the first 4 analytes (Kinetex Polar C18), while the 5-MeCyt is determined using a Supelco Discovery C18. The mobile phase remains the same. Final concentration of the analytes is expressed in μg/g of creatinine, to normalize values with respect to urine dilution variability. Urinary creatinine will be determined by the Jaffè method (45), using alkaline picrate test with UV/vis detection at 490 nm.

The following m/z ion combinations (precursor → product) are monitored in the positive ion mode: 168.4→140.1 and 171.0→143.0 for 8-oxoGua and its internal standard ([$^{13}C^{15}N_2$] 8-oxoGua) respectively, 284.3→168.0 and 287.13→171.1 for 8-oxodGuo and its internal standard ([$^{13}C^{15}N_2$] 8-oxodGuo), 300.24→168.2 for 8-oxoGuo and 303.24→171.0 for the internal standard ([$^{13}C^{15}N_2$] 8-oxoGuo), 226.99→181.0 for 3NO_2tyrosine and 229.99→184.0 for 3NO_2tyrosine d_3, and 258.1→126.1 for 5Methylcytidine; 5-MeCyt is quantitated using cotinine d_3 as internal standard (180.3→80.10).

g. Urinary UnTargeted biomarkers

g.1. Sample preparation: 1200 μL of urine were centrifuged at 11000 g for 15 min at 4 °C to remove the cellular debris. 100 μL of a 3-trimethylsilyl-propionic-2,2,3,3-d4 acid (TSP) - D_2O solution (2 mM final concentration) as internal standard are added to 1 mL of centrifuged samples and the pH is then measured and adjusted at pH 7 by adding NaOH or HCl. Finally, 700 μL of sample are transferred in NMR precision tubes and submitted to the NMR analysis.

g.2. ^{1}H-NMR spectroscopy: All urine samples are analysed in an untargeted fashion at the NMR-based Metabolomics Laboratory (NMLab) of Sapienza University of Rome. A comprehensive strategy, including a repeatable sample preparation protocol and an efficient fingerprinting method, is conducted following the metabolomic workflow reported in Figure 1.

^{1}H-NMR spectra are acquired for each sample with a JEOL JNM-ECZ 600R spectrometer (JEOL Ltd, Tokyo, Japan) equipped with a magnet operating at 14.09 Tesla and at 600.17 MHz for ^{1}H frequency and an automated sample changer. Before measurement, each sample is kept for 180 s inside the NMR probe head to reach temperature equilibrium of 298 K and then automatic tuning, matching, lock and shimming are performed using JEOL Delta v5.3.1 software (JEOL Ltd, Tokyo, Japan). All the ^{1}H-NMR spectra are recorded after 4 dummy scans, with 64k points and 64 scans, setting spectral width to 9.03 KHz (15 ppm), with a pre-saturation pulse length of 2.00 s and a relaxation delay of 5.72 s, for an acquisition time of 5.81 s. Two dimensional (2D) homonuclear ^{1}H–^{1}H Total Correlation Spectroscopy (TOCSY) and ^{1}H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) bidimensional experiments are performed to confirm signal assignment. TOCSY experiments are acquired with a spectral width of 6009.15 Hz in both dimensions, a data matrix of 8k × 256 points, mixing time of 80 msec, repetition time of 3.00 s and 80 scans. HSQC experiments are carried out with spectral width of 9030 Hz in proton dimension and 30000 Hz in the carbon dimension, using 8k × 256 data points matrix for the proton and the carbon dimensions, respectively, with a repetition delay of 2s and 96 scans. HMBC spectra were acquired with a spectral width of 9030 Hz and 30000 Hz for the proton and carbon, respectively, with a data matrix of 8 k × 256 points, long-range constants nJC–H of 4, 8, and 12 Hz and 96 scans. The monodimensional NMR spectra are processed with ACD 1D NMR Manager software ver. 12 (ACD/Laboratories, Toronto, Canada), including Fourier transformation of free induction decay, with a line-broadening of 0.3 Hz, manually phased, baseline corrected and referenced to the chemical shift of the TSP methyl resonance at δ = 0.00 ppm. Bidimensional NMR spectra are processed by using JEOL Delta v5.3.1 software (JEOL Ltd, Tokyo, Japan). The quantification of metabolites is obtained by comparing the integrals of their diagnostic resonances with the internal standard TSP integral and normalized for their number of protons. Metabolite levels are finally expressed as μmol mmol−1 of creatinine, referred at its δ = 4.05 ppm resonance.

g.3. Statistical Analysis: The statistical multivariate analysis is submitted on the entire dataset using Unscrambler 10.5 software (CAMO, Oslo, Norway). Before performing the analysis, data matrix is centered and auto-scaled. Principal component analysis (PCA) and Partial Least Squares – discriminant analysis (PLS-DA) models are then applied. PCA is an unsupervised method used to evaluate a spontaneous grouping among the categories taken into account, according to their metabolic profile, while PLS-DA is a supervised classification method which is used to identify variables involved in the discrimination of predefined classes. Subsequently, a univariate statistical analysis is applied to the significant variables using Sigmaplot 14.0 Systat Software, Inc (San Jose, California, USA), in order to evaluate the single metabolite's changes between two classes or more. According to Shapiro-Wilk normality test positive or negative result, a parametric Student t-test or non-parametric Mann-Whitney U-test respectively are applied. If more than one factor is present, parametric analysis of variance (ANOVA) or non-parametric Kruskal-Wallis tests are applied according to respectively positive or negative normality test result. A p-value of 0.05 is the threshold for statistical significance.

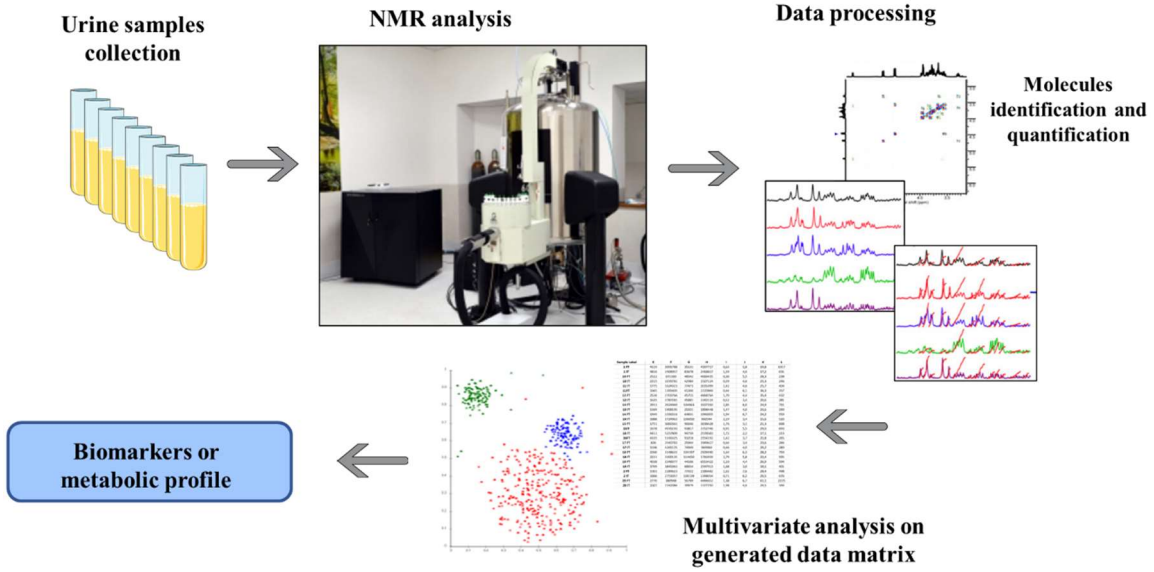

Fig.1 Data processing for metabolomics analysis.

h. Blood targeted Biomarkers

As blood markers of OxS, we will focus on protein carbonyls and nitrites, which represent stable oxidized derivatives of proteins and nitric oxide (NO), respectively. Their levels are a good index of the production of ROS and NO upon certain stimuli.

Blood samples are maintained in ice in EDTA-treated tubes and will be processed soon after the last withdrawal is taken. Then blood components (plasma, red and white blood cells) are separated by standard procedures using *EasySep™ Whole Blood* kit. Plasma was used to evaluate nitrites by Griess

assay system (Promega), serum is used to measured batokines (NRG4) levels by enzyme-linked immunosorbent assay (ELISA), whereas white blood cells are used to evaluate protein carbonyls. Briefly, cells are disrupted by 30 min of incubation on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM sodium Orto vanadate) and protease inhibitor cocktail (Sigma) followed by centrifugation at 22,300 × *g* for 20 min at 4°C. Carbonylated proteins are detected using the Oxyblot Kit (Merck-Millipore) after reaction with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25 °C. Samples are then resolved by 10% SDS-PAGE and DNP-derivatized proteins are identified by immunoblot using an anti-DNP antibody. Immunoreaction is identified using a Fluorchem Imaging system after incubation with Pierce™ Fast Western Kit, SuperSignal™ West Pico (Thermo Scientific) chemiluminescent substrate.

i. Inflammatory Blood parameters

Blood samples is collected as described above and samples added with sodium citrate are centrifuged at 1200 rpm 10 minutes at 4°C. Top layer containing plasma is recovered and stored at -80°C for subsequent measurements of plasma cytokines by ELISA. Buffy coat layer is incubated with 1ml lysis buffer (Buffer EL-ERythrocyte lysis buffer cat. n. 79217, Quiagen) for 20 min at 4°C in order to eliminate residual erythrocytes; samples are then centrifuged at 1200 rpm for 10 min at 4 °C; wash twice with PBS and then the pellet is suspended in 700 microliters Qiazol (Qiagen cat. 1023537) and processed for real-time quantitative PCR analysis.

i.1. Batokine serum levels: An aliquot of each individual blood sample is placed in BD micro container SSt tubes and then centrifuged for 90 seconds at 1000 x g. The serum is collected and stored at -80. During the test, the samples is brought to room temperature. Serum levels of adipokines is measured using the enzyme-linked immunosorbent assay (ELISA). The ELISA kit is a sandwich enzyme immunoassay for the quantitative in vitro measurement of various cytokines in human serum, plasma, tissue homogenates and other biological fluids.

i.2. Real-time quantitative PRC analysis: Total RNA is extracted from the mononuclear cells using the miRNeasy Micro kit (50) (Qiagen, Hilden, Germany) and quantified using NanoDrop One/OneC (Thermo Fisher Scientific, Waltham, MA, USA). cDNA is generated using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA, USA). Quantitative real-time PCR (qPCR) is performed for each sample in triplicate on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystem, Cheshire, UK) through the program SDS2.1.1 (Applied Biosystem, Foster City, CA, USA) using the Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). The primers for PCR amplification are designed with UCSC Genome Browser and are reported in the table 1. A comparative threshold cycle (C_T) method is used to analyze the real-time PCR data, where the amount of target, normalized to the endogenous reference of hGAPDH (ΔC_T) and relative to the calibrator of untreated control ($\Delta\Delta C_T$), is calculated by the equation $2^{-\Delta\Delta C_T}$ as described elsewhere (46).

Table 1. Primers used by quantitative PCR analysis

GENE	Forward primer (5′–3′)	Reverse primer (5′–3′)
hIL-1β	GCTTATTACAGTGGCAATGAGG	GGTGGTCGGAGATTCGTAG
hTNF-α	ATCTTCTCGAACCCCGAGTGA	CGGTTCAGCCACTGGAGCT
hIL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
hIL-4	ACTGCACAGCAGTTCCACAG	CTCTGGTTGGCTTCCTTCAC
hIL10	GATGCCTTCAGCAGAGTGAA	GCAACCCAGGTAACCCTTAAA
hTGF-◎	GCAGAGCTGCGTCTGCTGAGGC	CCCGTTGATGTCCACTTGCAGTG
hGAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC

j. Immune response Blood parameters

The ImmunoCAP ISAC (Immuno Solid-phase Allergen Chip) sIgE 112, a microarray-based immunoassay, is selected for study the susceptibility versus 112 allergens classified as food allergens aeroallergens, and others.

k. Test field and diving protocol

Wet and Dry diving operations are performed following the below descriptions. The diving profile is a typical "square dive", i.e. the diver spends all the time at the bottom to the maximum depth: 20 m – 30 minutes.

k.1. Dry Test: The dry test facility is a multi-place, multi-environment Hyperbaric Chamber with working pressure of up to 6 Bars for dry diving. The diving profile is the same in both, hyperbaric chamber and wet. Maximum depth 20 meters with a bottom time of 30 minutes, ascent speed of 10 meters per minute to the safety stop of 3 minutes at 3 meters; ascent speed from the stop to the surface of 20 seconds meter. All subjects will be compressed in hyperbaric chamber, divided in two groups for experimental reasons.

k.2. Wet Test: These tests are carried out on the Bracciano lake (Surface area 56.76 km^2, elevation 160 m), inside of the municipal area of Trevignano (42°09′N 12°15′E), near Rome. On the lake beach, temporary enclose space with fitting, medical and scientific instrumentation rooms were realized (Scientific Compound). 30 meters away from here, inside of the lake, an experimental area is identified by four boats. Subjects wearing diving equipment reach the wet test field on foot. A safety boat hovers during the dive, equipped with specific first aid kit, with staff qualified for underwater rescue. On board there is a resuscitation doctor.

The wet test field is performed in a stretch of water delimited by appropriate warnings. The area will be a perimeter of 25 x 50 meters, with the long side parallel to the shore. The delimitation is

assured by an orange rope, at the end of which two buoys are fixed, surmounted by a red flag with white diagonal (following Italian sea regulation it means diver under water).
At the centre of the perimeter, there are three more buoys, parallel to the shore, 10 meters from each other, which identify the path that the divers will follow while swimming at 20 meters deep. All buoys are secured to a mooring on the bottom with relative dampers. On the central one a safety tank is attached for the decompression stop, with two regulators and a pressure gauge.

k.3. Dive Equipment: A 6 mm thick one-piece wetsuit with a separate hood. A tank with a capacity of 15 l, with air pressure at 200 atm. The tank allows the assembly of two separate regulators, for redundancy. The divers are all provided with a buoyancy compensator (B.C.), snorkel, mask and fins. An underwater computer logs the instantaneous and maximum depth reached, the profile of the dive, the decompression and the decompression stop time, in addition to the water temperature, air consumption and heart rate.

l. Numeric Data Elaboration

The experimental data obtained from the different tests is analysed in order to determine common qualitative features of the temporal evolution of the stress marker. Let us remark that, for several stress markers chosen in this project, there is not yet clear evidence about their dynamics and these studies can bring much new information in this sense.
The aim is to understand how personal characteristics of the subjects (weight, physical stress, diet, age etc.) can influence the base values and the dynamics of the markers (in particular, the time and the value of the concentration peaks, the speed of concentration growth and decrease.

Different suitable functions, for the pre-stress phase and for the post-stress one, where the different parameters will have physiological meaning, will be selected for the nonlinear regression of the experimental data. The goal is multiple: to detect qualitative dynamics common to the different subjects; to determine the most suitable regression function and the characteristic physiological parameters for each subject; to study the sensitivity of the parameters with respect to the personal characteristics of the subjects or to external stress factors (temperature, humidity, depth, diving time etc.). Statistical and mathematical tools will be implemented in a numerical software, in order to apply the appropriate regression and interpolation techniques, which will be adapted to the different data set.

Conclusions

The relationship between OxS and HE is not deeply investigated and literature proposes diverging results. The project aims to define the time dependence of biomarkers related to OxS, to rise knowledge in risk assessment in workers exposed to HE. This project doesn't have results yet and this paper describes the experimental protocol. Authors conclusion is that organizing and running a research group with many different disciplines is complex and needs time to set. Sampling time of different specimen has to be organised to allow every subgroup to analyse properly samples within their own protocols. Even distance from experimental site and laboratories has to be coped with, to avoid samples decay.

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Conflict of interest

None

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