Genetic predisposition to breath-hold diving-induced hemoptysis: Preliminary study

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ABSTRACT

Introduction: Breath-hold diving-induced hemoptysis (BH-DIH) has been reported in about 25% breath-hold divers (BHD) and is characterized by dyspnea, coughing, hemoptysis and chest pain. We investigated whether eNOS G894T, eNOS T786C and ACE insertion/deletion I/D genetic variants, are possible BH-DIH risk factors.

Methods: 108 experienced healthy instructor BHDs with the same minimum requirements (102 male, six female; mean age 43.90±7.49) were studied. We looked for different eNOS G894T, eNOS T786C and ACE insertion/deletion genetic variants between BH-DIH-positive and BH-DIH-negative subjects to identify the variants most frequently associated with BH-DIH.

Results: At least one BH-DIH episode was reported by 22.2% of subjects, while 77.7% never reported BH-DIH. The majority of BH-DIH-positive subjects showed eNOS G894T (p=0.001) and eNOS-T786C (p=0.001) genotype "TT" (high-risk profile). Prevalence of BH-DIH was higher in subjects with eNOS G894T TT genotype (50%) than in subjects with GT (9.5%, p<0.001) and GG (24%, (p=0.0002) genotype (low-risk profile). Similar results were observed for eNOS T786C: BH-DIH prevalence was higher in the TT genotype (41.2%) group than in the CT (15.4%, p<0.001) and CC genotype (9.1%, p<0.001) groups. BH-DIH prevalence was significantly higher in subjects showing ACE ID genotype (34.5%) than II (0%, p<0.001) and DD (10.5%, p=0.0002). Of the ACE "II" genotype group, 100% never developed BH-DIH.

Discussion: eNOS-G894T, eNOS-T786C and ACE influence NO availability and regulation of peripheral vascular tone and blood flow. Different genetic variants of eNOS-G894T, eNOS-T786C and ACE appear significantly related to the probability to develop BH-DIH (p<0.001).

INTRODUCTION

Non-cardiogenic acute pulmonary edema is a syndrome that has been occasionally observed in healthy subjects engaged in swimming [1,2,3], scuba diving [1,4] or, more rarely, in other strenuous physical activity [5,6]. On the contrary, acute respiratory symptoms (ARS) have been reported in a significant proportion (nearly 25%) of breath-hold divers (BHD) after repetitive deep breath-hold diving [7]. ARS is characterized by dyspnea combined with other acute respiratory symptoms [8,9] such as coughing, sensation of chest constriction and blood-striated expectorate (hemoptysis) [7].

Hemoptysis is a typical sign of this disorder and is significantly suggestive of an underlying situation of pulmonary edema [10-11]; however, since we could obtain imaging evidence of pulmonary edema only in about 30% of the investigated cases, we called this condition

KEYWORDS: acute respiratory distress syndrome, breath-hold diving, diving, hemoptysis

breath-hold diving-induced hemoptysis (BH-DIH). Recent studies reported an increase of extravascular lung water after maximal apnea, also in asymptomatic breath-hold divers, indicating the presence of subclinical interstitial edema [10,11].

In all these cases, an increase in pulmonary capillary pressure has been considered as the main factor inducing fluid extravasation from pulmonary capillaries and, in more severe cases, to disrupt the thin blood/gas barrier [6,12].

Acute pulmonary edema is also reported in healthy subjects exposed to high altitude (HAPE) [13,14], due to a regional pulmonary overperfusion secondary to an uneven arteriolar hypoxic vasoconstriction. Several observations seem to indicate the existence of a genetic predisposition toward HAPE [15-19].

On one hand, it is well known that populations living at very high altitudes for thousands of years have specific a variant of endothelial nitric oxide synthase (eNOS) that allows them to tolerate environmental hypoxia [20]. On the other hand, HAPE-prone subjects often show specific variants of several genes involved in vascular reactivity control and regional blood flow regulation [21], especially eNOS [19-20] and angiotensin-converting enzyme (ACE) [15-16-17-18].

Even if they are obvious, the etiological and pathogenic difference between HAPE and BH-DIH, the similarity in pooling of blood in some part of the lungs seems to be the important factors in generating this phenomenon and allows us to hypothesize a similarity in genetic predisposition.

eNOS is a group of enzymes that catalyses the synthesis of nitric oxide (NO), the molecule involved in the regulation of peripheral vascular tone and blood flow [22].

As NO is known to be a powerful vasodilator that lowers pulmonary vascular resistance [23], reduced NO levels may induce a relative vasoconstriction in the pulmonary circulatory system, possibly involved in HAPE pathogenesis.

Regional NO levels are conditioned by numerous factors such as molecule signaling and protein interactions [24], sheer stress and PaO_2 [25] availability of substrate and co-factors, hypoxia [26] and, finally, by the genetic variants that encode for eNOS [19-20] (genetic polymorphism).

One of the most investigated polymorphisms responsible for the half-life of eNOS, is the G894T [20]. This polymorphism corresponds to a change between a (guanine) G to a (thymine) T in position 894 of the nucleotide sequence of genes that results in an amino-acidic substitution between glutamic acid (GAG) and aspartic acid (GAT) in position 298 of the aminoacidic sequence of protein (Figure 1).

Glutamic and aspartic acids are two very similar amino acids, but eNOS containing aspartic acid in the 298 position is less active than eNOS containing glutamic acid at the same position [27].

Another polymorphism of eNOS often investigated for cardiovascular diseases and resistant hypertension is T786C [22-28]. This polymorphism is situated in a region of the gene known as "promoter," a zone present in all genes, which allows the initiation of the transcription of a particular gene. Different polymorphisms in this part of DNA cause different expressions of the gene. The normal gene sequence presents a thymine (T) in position T786C, while the mutant gene sequence presents cytosine (C) in the same position. Several authors have shown a clear relationship between this mutant sequence and coronary or hypertensive diseases [28-30].

Others studies have shown a relationship between HAPE and the insertion/deletion (I/D) polymorphism of angiotensin-converting enzyme gene (ACE). ACE is a part of the renin-angiotensin system (RAS) that plays an important endocrine role in the regulation of systemic blood pressure but it is also implicated in the regulation of regional blood flow and of pulmonary vascular tone [15-18].

These data support the existence of specific relationships between HAPE and genetic polymorphism of some genes involved in the regulations of systemic and peripheral vascular resistance, and particularly in the regulation of pulmonary hemodynamics.

The aim of this study is to investigate the genetic variants of eNOS G894T, T786C and ACE I/D, as possible inherent risk factors for BH-DIH.

MATERIALS AND METHODS

Subjects

A total of 108 experienced healthy BHD (102 male, six female; mean age 43.90 ± 7.49) were studied. A standardized questionnaire was developed to investigate any history of one or more of the following symptoms after breath-hold diving: coughing, feeling of thoracic constraint, and hemoptysis associated with various degrees of dyspnea as confirmation of pulmonary involvement. Documentations as requested for cases came to medical attention of researchers only.

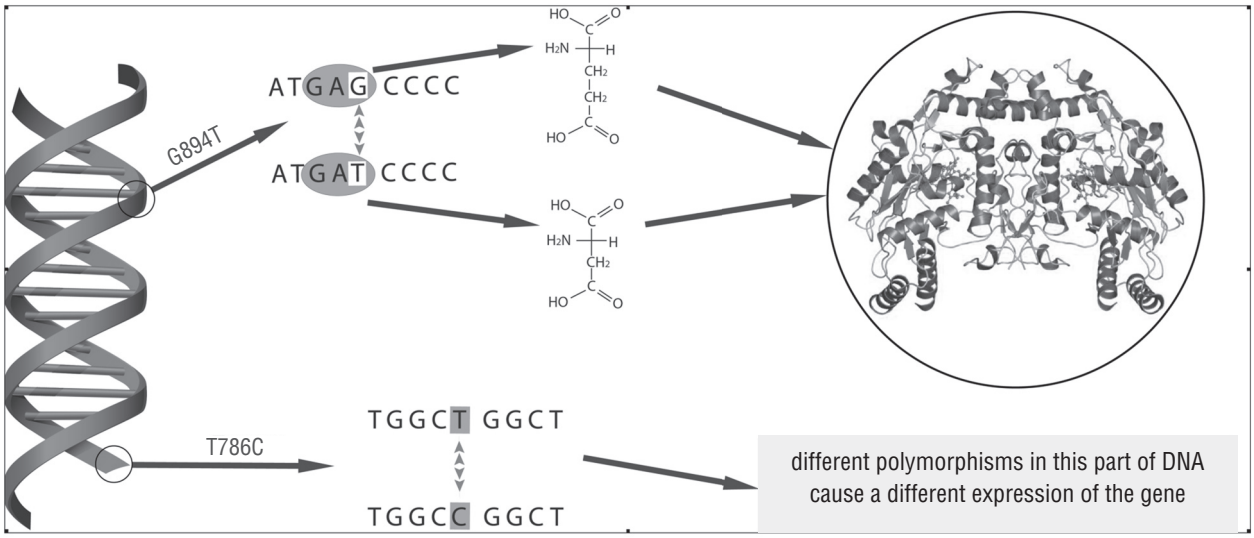

Figure 1: Investigated polymorphisms

In eNOS G894T polymorphism (guanine) G or (thymine) T changes the end of the specific codon. eNOS T786C polymorphism is situated in a region of the gene known as "promoter." Both of them cause different expressions of the gene.

Using the questionnaire we divided the sample between subjects who had suffered at last one episode of BH-DIH (BH-DIH-p) and subjects who never reported any specific symptom (BH-DIH-n).

We also requested information about standard anthropometric data such as height, weight and BMI, which was calculated. Differences between BH-DIH-p or BH-DIH-n and anthropometric data were investigated, also we looked for any difference between BH-DIP-n and BH-DIP-p in maximum achieved depth and years of BHD experience.

All subjects were affiliated with Apnea Academy, with instructor-level training. This was a select group of skilled free-divers, all with high-standard common levels of specific preparation.

All instructors met the minimum requirements to be admitted to the Apnea Academy instructor level:

- minimum depth in constant weight: -30 meters;
- minimum minutes of static BHD (at surface): 4 (four) minutes;
- Minimum dynamic BHD in swimming pool (distance): 75 meters.

All investigated subjects undergo regular training at least twice a week and practice sea- or fresh-water free-diving at least three times each month.

Sample collection and genetic marker selection

Total DNA was isolated from epithelial oral cells using two buccal swabs for each individual. All participants signed an informed consent and gave permission for the analysis.

DNA extraction was performed using the Charge-Switch Kit (Invitrogen) and followed manufacturer's instructions, using both buccal swabs and a final re-suspension in 100 microliters of elution buffer.

The eNOS rs1799983 (G894T) and rs2070744 (T786C) polymorphisms were analyzed using a real-time polymerase chain reaction (real-time PCR) technique. Specific primers and probes for the single nucleotide polymorphism (SNP) rs1799983 were designed according to the TaqMan genotyping assay by Applied Biosystems, while SNP rs2070744 was analyzed using primers and probes designed according to the Kaspar genotype assay by KBIOscience [B]. Both SNPs were analyzed on ABI 7900 following manufacturer's instructions.

Genetic determination of ACE insertion/deletion polymorphisms was performed by polymerase-chain-reaction (PCR) amplification using these primers:

- forward- CTGG AGA CCA CTC CCA TCC TTT CT;
- reverse- GAT GTG GCC ATA ACA TTC GTC AGA T [A] and separation of the PCR products by electrophoresis on a 3% agarose gel.

A negative control (containing no DNA) and a positive control were included in every amplification set up, to monitor cross contamination and reaction success.

Genotypes investigated

We looked for any difference in BH-DIH-positive (BH-DIH-p) and BH-DIH-negative (BH-DIH-n) between subjects with different genetic variants in the investigated polymorphisms (eNOS G894T, eNOS T786C and ACE insertion/deletion) to identify the variants most frequently associated with the disorder ("high-risk profile") and variants less frequently associated with the disorder ("low-risk profile").

We also looked for possible interactions between polymorphisms in subjects showing a majority of "high-risk profile" (three out of three or two out of three) and those showing mostly "low-risk profile" (three out of three or two out of three).

Additionally, and to confirm the importance of polymorphism interaction, we looked separately for any difference between subjects with all "high-risk profile" or all "low-risk profile" (three out of three) and those showing only a majority of "high-risk profile" or "low-risk profile" (two out of three).

In a subgroup of subjects (59.2%) we also investigated: interleukin-1 beta (IL-1β rs16944), interleukin 1 receptor antagonist (IL-1RN rs419598), glutathione S-transferase mu-1 (GSTM-1), glutathione S-transferase theta-1 (GSTT-1), superoxide dismutase 2 (SOD-2 rs4880), implicated in inflammatory and oxidative stress responses, respectively.

Statistical analysis

The data collected by the questionnaire have been produced in terms of percentage of subjects who answered positively to the questions concerning some single and/or combined events of cough, thoracic constraint and hemoptysis associated with various degrees of dyspnea as confirmation of pulmonary involvement.

The possible associations between the hypothetical risk factor and the occurrence of acute post-dive respiratory symptoms have been evaluated by means of the chi-square test.

Differences between BH-DIH-p or BH-DIH-n and anthropometric data were investigated using the Mann-Whitney U test after the normality test (Kolmogorov-Smirnov).

A probability lower than 5% was assumed as the threshold to reject the null hypothesis ($p<0.05$).

RESULTS

108 subjects (102 male, six female; mean age 43.90± 7.49) were investigated: 24 subjects (22.2%) reported at least one episode of BH-DIH, while 84 of them (77.7%) had no clinical history of BH-DIH.

Only 31 subjects (28,7%) were radiologically assessed, and in all these the presence of pulmonary edema was confirmed.

The mean height was 177.09 cm ± 7.2, and the mean weight was 76.54 kg. cm ± 9.4, BMI calculated was 24.1. We did not find any significant difference between BH-DIH-p and BH-DIH-n for age, height, weight and BMI p=0.12 (BH-DIH-n mean 43.69 ± 7.62 BH-DIH-p mean 45.96 ± 5.58); p=0.57 (BH-DIH-n mean 177.6 cm ± 7.46 BH-DIH-p mean 179.1 cm ± 6.06); p=0.59 (BH-DIH-n mean 76.49 kg. ± 9.89 BH-DIH-p mean 76.71 kg. ± 7.86); p=0.15 (BH-DIH-n mean 24.28 ± 1.99 BH-DIH-p mean 23.76 ± 1.64); respectively.

We did not find any significant difference between BH-DIH-p and BH-DIH-n for maximum achieved depth p=0.29 (BH-DIH-n mean 42.37 ± 9.49 BH-DIH-p mean 45.75 ± 14.40) and years of BHD experience p=0.30 (BH-DIH-n mean 14.57 ± 5.36 BH-DIH-p mean 12.95 ± 4.86).

The distribution of BH-DIH-p subjects across the genotypes of eNOS G894T was significantly different ($p<0.001$) (Figure 2). In particular, the prevalence of BH-DIH was significantly higher in subjects with TT genotype (50%) than in subjects with GT genotype (9.5 %, $p<0.001$) and GG (24%, p=0.0002).

Similar results were also observed for eNOS T786C ($p<0.001$): BH-DIH prevalence was significantly higher in the group with TT genotype (41.2%) than in subjects with CT (15.4%, $p<0.001$) and CC genotypes (9.1%, $p<0.001$) (Figure 3).

The distribution of BH-DIH-p subjects also resulted significantly differently across the genotypes of ACE I/D ($p<0.001$) (Figure 4). In particular, BH-DIH prevalence resulted significantly higher in subjects with ID genotype (34.5%) as compared to II (0%) ($p<0.001$) and DD (10.5%) (p=0.007). Since no subject with the "II" genotype had BH-DIH, this could be considered the "low-risk profile" for ACE polymorphism.

Investigation of possible differences in BH-DIH-p due to interactions of different genetic polymorphisms indicated that subjects with the majority of "high-risk profile" (three out of three or two out of three "high-risk" genes) had a significantly higher prevalence of BH-DIH-p. On the contrary the majority of subjects with a "low-risk profile" (three out of three or two out of

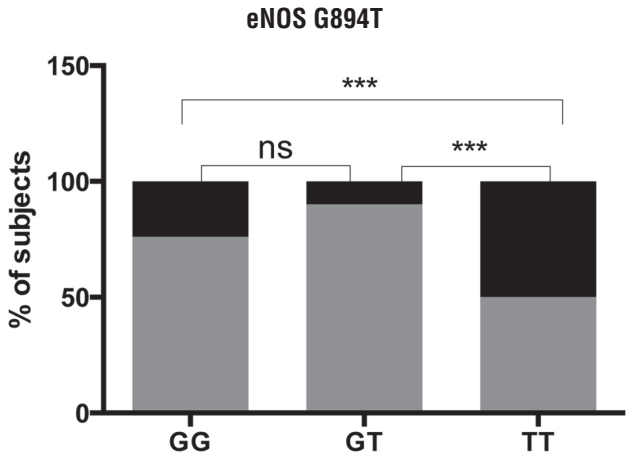

Figure 2. eNOS G894T polymorphisms

Subjects with the TT variant of this polymorphism ("high-risk profile") are more susceptible to developing BH-DIH than subjects with genotype GG or GT. BH-DIH-p was significantly higher in subjects with TT genotype (50%) then genotype GT (9.5%) and GG genotype (24%).

ns = not significant *= p<0.05 **= p<0.01 ***= p<0.001

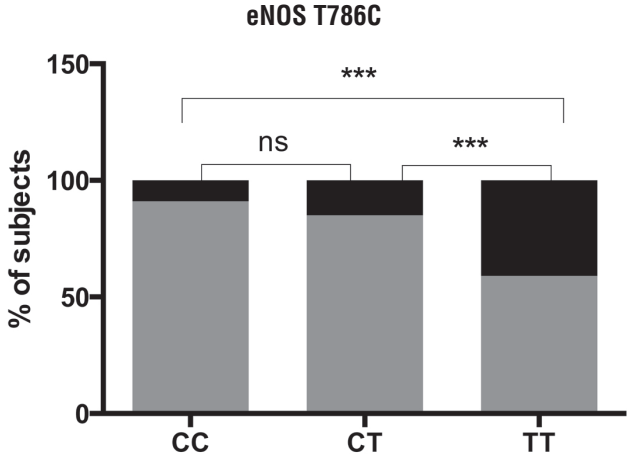

Figure 3. eNOS T786C polymorphisms

Subjects with the TT variant of this polymorphism "high-risk profile" are more susceptible to developing BH-DIH than subjects with genotype CT or CC. BH-DIH-p prevalence was significantly higher in the group with TT genotype (41.2%) than in subjects with CT (15.4%) and CC genotypes (9.1%).

ns = not significant *= p<0.05 **= p<0.01 ***= p<0.001

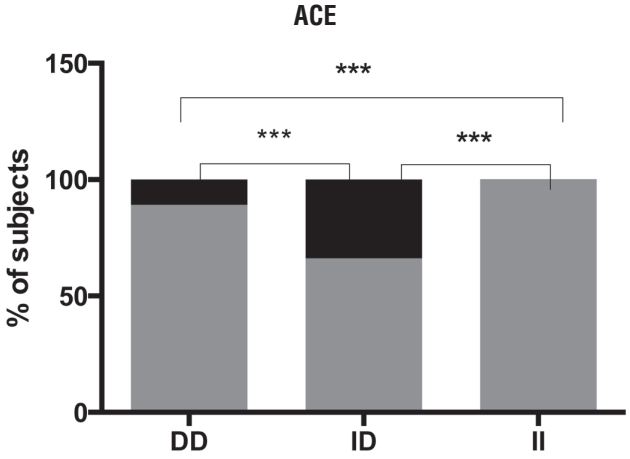

Figure 4. Insertion/deletion polymorphism of ACE

"II genotype" of ACE (homozygote for insertion) decreases the risk of HAPE, in our study all the ACE "II genotype" subjects (n:12) were BH-DIH-n "low-risk profile."

*= p<0.05 **= p<0.01 ***= p<0.001

three "low-risk" genes) was associated to a significantly higher prevalence of BH-DIH-n subjects (p<0.001; Table1). Similar results were also observed when separately looking for differences between all "high-risk profile" and all "low risk profile" subjects (three out of three "high-risk" or "low-risk" genes) (p<0.001) and between subjects with only two "high-risk profile" genes vs. subjects with only two "low-risk profile" genes (p=0.001 Table1).

We did not find any difference between BH-DIH-p regarding the other investigated polymorphisms (interleukin-1 beta (IL-1β rs16944), interleukin-1 receptor antagonist (IL1-RN rs419598), glutathione S-transferase mu-1 (GSTM-1), glutathione S-transferase theta-1 (GSTT-1) or superoxide dismutase-2 (SOD-2 rs4880).

high-risk profile	vs.	low-risk profile	description	p-value
all or mostly high	vs.	all or mostly low	three out of three or two out of three	0.001
majority high	vs.	majority low	two out of three	0.001
all high	vs.	all low	three out of three	0.001

Table 1. Subjects with combinations of mostly "high-risk profile" genes were by significant majority BH-DIH-p, while the majority of subjects with combinations of mostly "low-risk profile" were BH-DIH-n.

DISCUSSION

Our data showed that the majority of BH-DIH-p subjects have a significant increase of the predisposing genetic variants "TT" at eNOS G894T, "TT" at eNOS T786C and "ID" at ACE.

The investigated genes encode endothelial nitric oxide synthase (two different polymorphism) and the angiotensin-converting enzyme gene insertion/deletion polymorphism. Both enzymes are involved in the regulation of vascular reactivity and regional blood flow.

These data are in accordance with similar studies on high-altitude edema [15-20].

A particular point of interest in our work concerns the G894T polymorphism, where the variant T produces the specific codon (GAT) that substitutes glutamic acid with aspartic acid. The eNOS containing aspartic acid in the 298 position has 50% less activity than eNOS containing glutamic acid in the same position. Our data indicate that subjects with the TT variant of this polymorphism are more susceptible to develop BH-DIH (BH-DIH-p 50%) than subjects with genotype GG (BH-DIH-p 24%) or GT (BH-DIH-p 9.5%).

Half of the subjects with BH-DIH-p phenotype had a TT genotype (thymine-thymine association in the gene); this is significantly higher when compared to the low percentages found for other genotypes. This result shows that TT genotype is the "higher-risk profile" among those analyzed.

These data are in agreement with similar findings in individuals who are prone to develop HAPE, showing a reduced NO production and TT genotypes of G894T polymorphism [19-20].

An indirect confirmation of this is that in our BH-DIH-n subjects we found the same specific allele (G) found in populations that had lived at very high altitudes for thousands of years [20]; it is well known that these genotypes allow for increased tolerance to environmental hypoxia and reduce the possibility of developing HAPE [20].

We also found an increase in BH-DIH-p in subjects who showed genotype "TT" of eNOS T786C.

Previous studies about T786C polymorphism have reported that polymorphism C is associated with a reduction of promoter activity and correlated with coronary heart disease and hypertension. On the other hand, these data seem to suggest that polymorphism T could actually represent a favorable condition for an effective regulation of regional vascular resistances and blood flow. A possible explanation for the unexpected relationship between the TT genotype and BH-DIH could reside in the theoretical confounding effect exerted by the repeated exposure to hypoxia during BHD training. In fact, previous studies showed that the promoter effect in T786C is affected by hypoxia [22]. To the best of our knowledge, this is the first time that this polymorphism has been investigated in extreme environmental conditions.

Moreover, a significantly higher BH-DIH-p prevalence occurred in heterozygotes for ACE insertion/deletion (ID) versus both homozygotes for insertion (II) and homozygotes for deletion (DD) subjects. This is in partial agreement with specific literature that indicates that the deletion allele (D allele) is associated with HAPE and responsible for hyperresponsiveness of pulmonary vascular resistance.

In addition, and very important, ACE "II genotype" (homozygote for insertion) decreases the risk of HAPE, and in our study all the ACE "II genotype" subjects (n:12) were BH-DIH-n. This confirms a direct relationship between ACE I/D and BH-DIH.

The data about the interaction of various genotypes support the possible presence of a genetic predisposition to BH-DIH; in fact having two or three out of three "high-risk profile" subjects resulted in an association

with an increased prevalence of BH-DIH, (BH-DIH-p 56.25%). On the contrary, having two or three out of three "low-risk profile" subjects seems to be associated with a reduced susceptibility to BH-DIH (BH-DIH-n 92.1%).

The data we would like to highlight is the effect of the combination of the "high-risk profile" and "low-risk profile." It appears that the presence of two "low-risk profiles" of eNOS polymorphism would nullify the impact of the "high-risk profile" of ACE I/D polymorphism, reducing the risk of BH-DIH-p (BH-DIH-p 23% and BH-DIH-n 77%) while the presence of just one low-risk profile of eNOS phenotype does not cause the same effect (BH-DIH-p 53% and BH-DIH-n 46%).

Our data suggest that the enzymes responsible for the regulation of vasomotor tone may be involved in the pathogenesis of BH-DIH. We speculate that in susceptible individuals there is increased peripheral vasoconstriction during BHD, causing greater central translocation of blood and hence increased pulmonary capillary blood pressure, leading to capillary stress failure [6-12].

This hypothesis is in agreement with accepted theories on capillary stress failure during breath-hold diving [8] and during other sport activities [1-3,5-6]. Low eNOS availability could contribute in amplifying the effect of some etiological factors that are known to increase the prevalence of BH-DIH [7], such as: increased peripheral relative vasoconstriction during cold-water exposure, with a greater central blood shift; increased diaphragm contractions because of less metabolite washout; reaching maximum personal depth; low lung volume; and fragility of pulmonary capillaries.

Our data are also in agreement with Lindholm's works [31] describing different possible lung consequences caused by increased ambient pressure during BHD: alveolar collapse, membrane rupture in the lung, and fluid filtration into the alveolar space. Lindholm also describes BH-DIH that occurred at shallow depths in empty lungs [32]. All these possibilities could be related to the ability to modify pulmonary blood flow, which also depends on the availability of the enzymes we tested.

Diving-induced cardiovascular changes [33-35], the reduction of lung volume, the increase of intrathoracic blood content induced by BHD [36-40] and some maneuvers like forced ear equalization and diaphragmatic contractions while breath-holding at depth may act as additional contributors to increase transpulmonary capillary pressure and may thus be important co-factors in BH-DIH pathophysiology.

These conditions and maneuvers in fact contribute to increase intrathoracic pressure [41] and may be possible triggers for the described pathological events in chest mechanics, determining ultra-structural modifications of pulmonary capillary walls as described by West and Mathieu-Costello [6-12]. These changes can in turn predispose to alveolar hemorrhage.

Most likely, maneuvers that increase intrapulmonary pressure, such as diving up to one's personal breath-hold depth limit, forceful ear equalization, and prolonged diaphragmatic contractions [7], increase the probability of BH-DIH in genetically predisposed subjects.

If confirmed, our observations suggest that there may be a genetic predisposition to BH-DIH, possibly related to greater translocation of blood into the thorax during a dive, leading to higher transcapillary pressure in susceptible individuals.

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Conflict of interest

The authors have declared that no conflict of interest exists with this submission. ■

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