Morphometric investigation of Pulmonary Barotrauma: a study in forensic autopsies by SCUBA-diving

End-of-Term Research Project

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“If I have seen further it is by standing on the shoulders of giants.”

Isaac Newton

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1. Abreviatones

SCUBA: self-contained underwater breathing apparatus

AGE: arterial gas embolism

PBt: pulmonary barotrauma

PBt/AGE: arterial gas embolism following pulmonary barotrauma

IMLCFC: Institut de Medicina Legal i Ciències Forenses de Catalunya

PMCT: post-mortem computerized tomography

MRI: magnetic resonance imaging

P: pressure

V: volume

m²: square meters

n: emphysematous areas’ number

µm: micrometers

mm: milimeters

g: grams

PFA: paraformaldehyde

PB: phosphate buffer

SEPAF: Sociedad Española de Patología Forense
2. Abstract

**Background:** SCUBA-diving is one of the most popular and safely water sports activities in our country. Approximately 7 million divers are active worldwide. Although recreational diving is overall safe, diving accidents are potentially serious and even fatal. While there may be an initial presumption that all water-related deaths are accidental drownings, other possibilities must be considered, such as arterial gas embolism (AGE), decompression sickness, natural pathology, and trauma. PBlt/AGE has been described as the cause of death in SCUBA diving in 13-24% of cases. Currently, the medical-forensic criteria for the diagnosis of PBlt/AGE are based mainly on macroscopic autopsy and on imaging diagnostic techniques. Microscopic studies of pulmonary histopathology of PBlt have not contributed specific changes to date. Certain authors describe pulmonary emphysema as a lesion characteristic of pulmonary barotrauma and experimental animal model studies, where pulmonary barotrauma has been artificially reproduced, have described microscopic changes in alveolar light with emphysema, atelectasis and haemorrhages. Complementary tests in cases of PBlt/AGE are based mainly on conventional histopathological studies, not allowing an adequate assessment of the dimension of air spaces. Consequently, we propose the morphometric analysis of lung tissue to provide objective quantitative data of air spaces and their alterations in order to achieve more reliable data in the face of diagnose PBlt/AGE.

**Objectives:** 1) to describe frequency of acute pulmonary microscopic emphysema observed in lung tissue samples from forensic autopsies of PBlt/AGE in context of SCUBA-diving as compared to those in forensic autopsies of drowning. 2) To analize differences in variables defining acute pulmonary microscopic emphysema observed in lung tissue samples from forensic autopsies of PBlt/AGE in context of SCUBA-diving as compared to those in forensic autopsies of drowning.

**Design:** the design of the study is cross-sectional and descriptive to be performed in Girona from 2018 to 2019.

**Population:** the study population are people who died as a result of submersion diagnosed of drowning or PBlt/AGE in a context of SCUBA-diving by a macroscopic autopsy. The collection have consisted on taking lung tissue samples of all autopsied corpses diagnosed of drowning and PBlt/AGE in the province of Girona between 2014 and 2017. Sample have been stratified in two groups, according to the cause of death.

**Methods:** Pulmonary histological preparations selected according to the type of death will be used, defining two groups (a group diagnosed of PBlt/AGE and the other group diagnosed of drowning). For each histological preparation 5 fields will be selected. Each field will be photographed with a high resolution microscopic camera coupled to a triochial microscope. The captured images will be analyzed (to see the presence/absence of microscopic emphysema) and the following variables will be measured: total alveolar area, average alveolar diameter, alveolar density and average thickness of the alveolar wall. The morphometric analysis will be carried out by means of image analysis software. This project has been approved by the Comité de Ética de Investigación Clínica del Hospital Universitari de Bellvitge.

**Keywords:** SCUBA-diving, Barotrauma, Arterial Gas Embolism, Emphysema, Morphometry, Autopsy.
3. Introduction

3.1. EPIDEMIOLOGY

SCUBA (self-contained underwater breathing apparatus) diving is one of the most popular and safely water sports activities in our country.(1) The most common methods used in diving activities are: SCUBA-diving, rebreather (recirculating underwater breathing apparatus) diving, and surface-supplied breathing gas.

Approximately 7 million divers are active worldwide and 500,000 more are training every year(2). Although recreational diving is overall safe, diving accidents are potentially serious and even fatal(3). Despite all the precautions taken by divers, fatalities related to this activity are reported every year all over the world(1). Drowning remains a serious public health concern claiming an estimated 362,000 lives per year worldwide across all socioeconomic classifications and has remained under close observation by the World Health Organization and its signatories (4). While there may be an initial presumption that all water-related deaths are accidental drownings, other possibilities must be considered in the investigation of these types of deaths, such as arterial gas embolism (AGE), decompression sickness, natural pathology, and trauma (1,5,6).

PBt / AGE (arterial gas embolism following pulmonary barotrauma) has been described as the cause of death in SCUBA diving in 13-24% of cases (7), being documented in the medical texts of diving pathology (8,9).

3.2. BIOPHYSICAL BASES

Apart from other physical laws that are applicable to SCUBA diving, we will focus on regulating gas mixtures (10).
HYDROSTATIC GENERAL LAW

It refers to the pressure \( P \) exerted by a fluid (gas or liquid) depending on the height of a column of this fluid. A column of water of 10 meters high, corresponds to a pressure of an atmosphere, approximately, denoting hydrostatic or manometric pressure. This column of water supports the air pressure of the atmosphere, so the pressure must be added to the hydrostatic pressure, thus obtaining the total pressure.

BOYLE-MARIOTTE LAW

It establishes the relationship between the pressure and the volume \( V \) of a gas when the temperature is constant. Gases can be easily compressed so that the volume is inversely proportional to the pressure: if the pressure increases, the volume decreases. This Law influences the amount of mixture introduced into our lungs, which affects pulmonary consumption and effort.

LAW OF DALTON

It refers to the partial pressure that each gas exerts in the whole of a mixture and its dependence on the proportion of each component. Therefore, the sum of the partial pressures of the different gases that make up the mixture will be equal to the total pressure.

LAW OF HENRY

It establishes the existing relationship of gas dissolution in liquids (in the case of the diver with autonomous scuba diving, blood and tissues). The amount of gas dissolved in a liquid is directly proportional to the pressure that this gas exerts on the liquid and inversely proportional to the temperature. It is applied to know the level of saturation of a gas in any fabric, with the levels of restoration, saturation and supersaturation depending on the amount of gas being below the limit, equal to this limit or would have exceeded it, respectively.
3.3. VOLUMETRIC EFFECTS OF PRESSURE CHANGES

As explained in the Boyle-Mariotte law, any underwater incursion entails, therefore, significant volumetric changes in closed air spaces. Different from other disorders that require a certain time to manifest and a minimum degree of hyperpressure for their effects to be appreciable, the volumetric variations occur immediately and are precisely maximum in the first increments of pressure (11).

Anatomical structures experience variations in volume in relation to pressure in the same way. The volumes contained in cavities or elastic and deformable structures adapt normally to the presovolumetric changes without producing any disturbance; but spaces and cavities limited by semi-elastic or rigid membranes of scarce consistency can suffer damage due to implosive volume reduction.

The respiratory system becomes a closed cavity, susceptible therefore to presovolumetrics variations, when the upper airways are occluded (snorkelling and breath-hold diving), but the lungs and the air passages of the diver and the scuba diver receive air at the same environmental pressure, which is exchanged in each phase of the respiratory cycle.

The most common form of recreational diving is the second situation: using SCUBA. It is an open-circuit system in which the diver exhales through a regulator that exits into the water, which creates the characteristic stream of bubbles. Breathing air is contained in a steel or aluminum cylinder mounted to the back of a vest device known as a buoyancy compensator. The diver typically wears weights to produce negative buoyancy, however, the diver can inflate the buoyancy compensator with breathing air to become neutrally buoyant and float in the water. Incorrectly inflating the buoyancy compensator can result in a too-rapid ascent or descent, resulting in pressurization injuries (12). In addition, in emergency situations the existence of panic worsens the aspiration of air and sometimes closes the glottis (13).

In situations in which the diver is forced to ascend sharply, the sudden decrease in pressure will produce an increase in intrapulmonary air volume that may easily exceed its limit of distension and expansion (12).
The excess of intrapulmonary volume causes a series of adverse phenomena. For a few moments, the thoracic cavity becomes a pressure vessel, which maintains the intrathoracic pressure above the ambient pressure. The trapped air then seeks out through all possible ways creating ducts and virtual spaces. The hyperextended lungs can exceed their limits of over-expansion and cause tears or ruptures of the parenchyma (emphysema), which in extreme cases could even lead to lung burst (14).

On other occasions, the air seeks out through natural ways, converting into real virtual spaces and conduits, opening arteriovenous and arteriocapillary communications. The air accesses extrapulmonary areas and can lead to pneumothorax, pneumomediastinum, subcutaneous emphysema and gas embolism (see Figure 1).

Figure 1: Manifestations of PBt (15)
Unlike decompression sickness (see the differential diagnosis in Figure 2), the bubbles in this case are preferentially directed to the arterial circulation, starting from small vessels to main arteries, thus reaching large arterial trunks and the brain, where they can cause occlusive phenomena, thus triggering distal ischemia and local endothelial lesion.

**Figure 2: Differential diagnosis between decompression sickness and PBt (12)**

If the injured person was at the time of embolization in an inverted position, it is likely that the coronary circulation could be also embolized (in the same way) (12).

### 3.4. PHYSIOPATHOLOGY AND HISTOMORPHOLOGICAL CHANGES OF PBt/AGE

When a SCUBA diver ascends to the surface too quickly, the gas retained in their lungs over-expands as the pressure decreases rapidly, causing a pulmonary barotrauma (PBt). If the air in the lungs is not vented as the diver rises, the alveolar walls may be ruptured by the air’s pressure. This leads to interstitial emphysema, air bursting through the respiratory membranes into the interalveolar, interlobular and interlobar septa. Bullae may appear on the pleural...
surface and, if these rupture, a pneumothorax is formed. Air may reach the mediastinum and track up the tissues to appear in the neck (subcutaneous emphysema).

Apart from a disabling pneumothorax, air can break into the lung capillaries and veins with the danger of air embolism to the left side of the heart, as it cannot move back down the pulmonary arteries. The filling of the left atrium and ventricle interrupts cardiac pump function and, if the volume is sufficient, can be fatal in itself (16).

Smaller volumes will be swept into the systemic arterial circulation and impact in the arterioles and capillaries of target organs, especially the myocardium, spinal cord and brain, causing microinfarcts, haemorrhagic necrosis and loss of function of vital tissues. This PBt is not necessarily confined to deep diving, and, in fact, the risk of PBt is highest in shallow water, because the ambient pressure doubles between 0 and 10 metres of sea water and the relative change in volume is maximal at that depth range (17).

The development of PBt is attributable to (18):

1. **Involuntary laryngospasm** on ascent caused by the water entering the airway, loss of consciousness, panic, etc;
2. Deliberate or accidental **stoppage of exhalation** while ascending, even for a short period of time (Valsalva maneuver, cough);
3. A sudden **increase in the volume of breathing** mixture supplied by the diving apparatus;
4. **Too-rapid ascent**, irrespective of the reason.

In 1985 Calder and co-workers published their findings concerning morphological markers of PBt based on 13 autopsies of divers who died due to this. Moreover, they induced experimental PBt in six corpses (19).

According to the authors, the morphological markers of PBt observed included detachment of the pleura (subpleural emphysematic blebs) and lung emphysema (macroscopic). There are not a lot of publications on the issues mentioned, and the majority of them are autopsy case reports (19–21) on
autopsy techniques and organ markers of PBt in humans, but histological testing is rarely included.

As we know from projects based on experimental models in animals, commonly rabbits, in the lungs without pneumothorax, the lung parenchyma is hyperaerated (22–24), with linear ruptures (23,24). Moreover, single or multiple subpleural emphysematic blebs are found caused by the expanding gas, which separates the pulmonary pleura from the parenchyma or disrupts the alveolar walls inducing interstitial emphysema (23,25–27) In the majority of cases, changes in aeration are accompanied by extravasation associated with parenchymal damage. Extravasated blood cells occur in the lumen of pulmonary alveoli, their thickened walls or in both structures, less commonly in the lumen of fine bronchi. In some cases, granulocytes or fibrinous exudates are detected in the region of parenchymal bursts. (23)

3.5. PHYSIOPATHOLOGY AND HISTOMORPHOLOGICAL CHANGES OF DROWNING

Drowning is the main cause of death in context of SCUBA-diving, and it is because of its physiopathology that we think that the histomorphological changes that we could see in it could be different from the emphysema of disbaric disorders.

It begins with the airway under water, leading to breath-holding; soon the person can no longer keep his or her airway clear, water entering the mouth is voluntarily spat out or swallowed. When the inspiratory drive is too high to resist, some amount of water is aspirated into the airways, and coughing occurs as a reflex response. Sometimes laryngospasm occurs, but in such cases, it is rapidly terminated by the onset of brain hypoxia. As hypoxia, hypercapnia and acidosis ensue, the victim losses consciousness (28).

The target organ of submersion injury is the lung. Injury to other systems is largely secondary to hypoxia and ischemic acidosis. Freshwater moves rapidly
across the alveolar-capillary membrane into the microcirculation. This water is considerably hypotonic relative to plasma and causes disruption of alveolar surfactant. Destruction of surfactant produces alveolar instability, atelectasis, and decreased compliance, with marked ventilation/perfusion (V/Q) mismatching.

Saltwater, which is hyperosmolar, increases the osmotic gradient and therefore draws fluid into the alveoli, diluting surfactant (surfactant washout). Protein-rich fluid then exudes rapidly into the alveoli and pulmonary interstitium. Compliance is reduced, the alveolar-capillary basement membrane is damaged directly, and shunting occurs. These processes result in acute lung injury and acute respiratory distress syndrome.

From a physiopathological perspective, death by drowning is classified as asphyxiation. Final strong respiratory efforts lead to the overinflation of lung tissue (emphysema aquosum), which is more pronounced in the peripheral region. These results in the rupture of narrow interalveolar septa, which coalesce to small blister cavities. The pulmonary alveoli are acutely dilated, while the septal capillaries are compressed and contain scant erythrocytes (see Figure 3). (29)

Figure 3: Histological correlation of emphysema aquosum in a case of death by drowning: extremely narrow to flattened interalveolar septa, occasionally stump-like at the margin of blister cavities. (29)
3.6.  MEDICAL-LEGAL APPROACH

According to the current medical-legal situation, in cases of violent death or suspected crime, the autopsy by forensic doctors will be done. Death during diving is included in the immersion or water deaths group, and is considered a violent death and even suspected of criminality because it is a quick and unexpected death (30).

The main causes of death described during diving are (8):

- Drowning
- Disbaric disorders (PBT/AGE and / or decompression sickness)
- Cardiovascular diseases
- Traumatic pathology
- Poisonings for respiratory or polluting gases

The investigation of these deaths requires a multidisciplinary approach, with the participation of police investigators specialized in underwater activities and forensic pathologists with knowledge and experience in the field of hyperbaric and underwater medicine.

In most diving-related fatalities, the identified cause of death is drowning, but this conclusion may be incomplete, being essential to identify the sequence of events that led to drowning. In full coincidence with the current European regulations (31), this investigation must include in a systematic way:

1. **Relevant death scene information** (by visiting the scene or from first responders)
2. **Revision of the clinical history** (in case of hospital deaths)
3. Development of a **technical-legal report**, that must include:
   a. A detailed external examination of the corpse
   b. An internal examination with specific autopsy techniques
   c. Complementary: radiological, histopathological, toxicological and biological exams
These autopsies represent a big challenge for any forensic pathologist; besides establishing the primary cause of death, it is essential to clarify the underlying problems, factors and sequence of events that made the victims susceptible to fatal incidents. Consequently, the investigation not only has to focus in determining the cause of death, but also in establishing its surrounding circumstances. This compels forensic pathologists to understand the physiopathology of diving and the main technical aspects of the equipment used in the immersion, and a multidisciplinary approach of the problem allows to consider the diving fatal accident as the sum of external and internal factors.

Determining external factors requires detailed and accurate information of the diving plan, the equipment used, and the diver’s profile, while determining the internal factors requires a complete autopsy. The individualized analysis of both has allowed us to reconstruct the events in each case.

To sum up, we can affirm that autopsy in the cases of death during diving practice should be directed to (32):

1. Confirm or exclude any evidence of **embolic disbaric disorder**, proving the presence of vital reaction: gaseous bubbles in the different organs and tissues (heart, lungs, brain, etc.).
2. Investigate typical findings of deaths in context of submersion (see **annexe 1**), given the fact that most of deaths related to diving are caused by **drowning**.
3. In cases of SCUBA-diving, identify possible blocks of **poisonings** by gas of the breathing system (nitrogen, oxygen, carbon dioxide and / or helium) or gases of external origin and contaminants (carbon monoxide).
4. Confirm or exclude the existence of associated **natural pathology**, either hidden, ignored or chronic decompensated, that has been able to cause or precipitate the death.
5. Confirm or exclude the existence of **traumatic pathology**, which directly or indirectly, has been able to contribute to the cause of death.
4. Justification

At the moment, in order to establish a PBT/AGE post-mortem diagnosis, it is essential to know the decedents dive profile and use specific autopsy techniques and/or image diagnoses. The use of plain radiography, post-mortem computerized tomography (PMCT) and magnetic resonance imaging (MRI) is recommended prior to diving autopsies.

In case these technologies are not available, some authors describe different specific autopsy techniques for the diagnosis of PBT/AGE, but these autopsy techniques are not always easy or reliable enough to prove the existence of intravascular air and the use of image diagnoses post-mortem is not common practice everywhere, justifying the need for innovation.

Complementary tests in cases of PBT are based mainly on conventional histopathological studies of lung tissue. We consider that this approach does not allow an adequate assessment of the dimension of air spaces and consequently we propose the morphometric analysis of the lung tissue to provide objective quantitative data of air spaces and their alterations in order to achieve more reliable data for the diagnosis of those that are provided by conventional histology studies. This technique has recently been applied to different types of mechanical asphyxias (drowning, strangulation, suffocation and suspension) to detect pathological dilatations of air spaces, but it has not been applied to deaths in context of PBT/AGE.

This is not a study to demonstrate that PBT/AGE can be diagnosed by microscopy, it’s a first proof of concept that tries to show that in lung tissue of deaths by PBT/AGE we can find histomorphological changes that can be quantified and are different from other type of deaths, so that this study could be a base for further investigations on the topic.
5. Bibliography


6. Hypothesis

A PBt/AGE in mortal cases of SCUBA-diving causes histomorphological changes in lung tissue that can be quantified and are different from drowning.

7. Objectives

In order to prove whether the above hypothesis can be confirmed, the following aims are planned:

1. Describe **frequency** of acute pulmonary microscopic emphysema observed in lung tissue samples from forensic autopsies of PBt/AGE in context of SCUBA-diving as compared to those in forensic autopsies of drowning.

2. Analize **differences in variables defining acute pulmonary microscopic emphysema** observed in lung tissue samples from forensic autopsies of PBt/AGE in context of SCUBA-diving as compared to those in forensic autopsies of drowning.
8. Subjects and methods

8.1. STUDY DESIGN

The design of the study is cross-sectional and descriptive.

8.2. STUDY POPULATION

Target population of the study are people who died as a result of submersion diagnosed of drowning or PBt/AGE in a context of SCUBA-diving by a macroscopic autopsy.

8.3. STUDY SAMPLE

Our study sample is based on people who died as a result of submersion diagnosed of drowning or PBt/AGE in a context of SCUBA-diving by a macroscopic autopsy (accomplishing the inclusion and exclusion criteria below) that have been processed in the Center of Forensic Pathology of Girona.

We have stratified the sample in two groups, according to the cause of death:

- **First group**: cases of SCUBA divers with necropsic macroscopic diagnosis of PBt. \( n = 7 \)
- **Second group**: cases of SCUBA divers with necropsic macroscopic diagnosis of drowning but without PBt/AGE. \( n = 10 \)
### 8.4. Inclusion and Exclusion Criteria

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
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<tbody>
<tr>
<td>Deaths diagnosed of drowning (second group) or PBt/AGE in a context of SCUBA diving (first group) by a macroscopic autopsy.</td>
<td>Age &gt; 65 years old (risk of senil emphysema)</td>
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<td>&gt; 36 hours after death (autolysis starts)</td>
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<td>Posmortem decompression</td>
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<td></td>
<td>Use of high-pressure oxygen (advanced CPR)</td>
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<td></td>
<td>Premortem neumological diseases that present with emphysema</td>
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</tbody>
</table>

We consider the macroscopic and complementary diagnose of **drowning**, if the following criteria are accomplished:

- External and internal exploration: Foaming at mouth and trâquea
- Biological test: Positive diatom test
- Chemistry test: Strontium in biventricular blood

We consider the macroscopic diagnose of **PBt/AGE** if the following four criteria are accomplished:

- History of rapid ascent followed by loss of consciousness
- Air in the left side of the heart and circle of Willis
- Low probability of post-mortem decompression artefact (PMDA) or decomposition
- Mediastinal or subcutaneous emphysema limited to the perithoracic area and/or pneumothorax
8.5. SAMPLING

**SAMPLE SIZE**

The size of the sampling has been calculated with the *Boston University Research Comittees* calculator for proportions’ and means’ comparisons.

Assuming an alpha risk of 0.05, and an statistical power of 0.8 in a bilateral contrast, 7 subjects are needed in the group of deaths by PBl/AGE and 7 subjects are needed in the group of deaths by drowning to detect statistically significant differences.

With this sample size, 7 subjects per group, the study will be powered to detect differences in mean values of the histological variables of 1,5 standard deviation or more. Therefore, this study will be exploratory (i.e. a proof of concept) in order to respond to this secondary aim.

**SAMPLE COLLECTION**

It has consisted on collecting lung tissue samples of all autopsied corpses diagnosed of drowning and PBl/AGE in the province of Girona between 2014 and 2017.

The postmortem macroscopic diagnosis of both groups have been done in a previous study of our investigation group, to make sure it accomplishes the previous inclusion criteria.(8)
8.6. VARIABLES

In this study, causality will not be possible to be demonstrated, only association information would be obtained. To that end, it is not feasible to define the variables as independent or dependent due to the lack of temporal sequence, and so, the variables will be designated as main and secondary outcome variables instead of dependent and independent.

MAIN VARIABLE

- **Cause of death**: It is a dichotomous (categorical) qualitative variable. It can be drowning or PBl/AGE.

SECONDARY VARIABLES

- **Microscopic emphysema**: It is a (categorical) qualitative variable. It will be expressed as presence or absence.

- **Total alveolar area**: It is a continuous quantitative variable, and it will be expressed in square meters (m²).

- **Average alveolar diameter**: It is a continuous quantitative variable, and it will be expressed in milimetres (mm).

- **Alveolar density**: It is a continuous quantitative variable, and it will be expressed in emphysematous areas’ number / square micrometers (n/µm²).

- **Average thickness of the alveolar wall**: It is a continuous quantitative variable, and it will be expressed in micrometers (µm).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Type</th>
<th>Technique</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cause of death</td>
<td>Dichotomous categorical qualitative variable</td>
<td>Macroscopic autopsy</td>
<td>Drowning or PBt/AGE</td>
</tr>
<tr>
<td>Microscopic emphysema</td>
<td>Dichotomous categorical qualitative variable</td>
<td>Conventional histopathological studies</td>
<td>Presence or absence</td>
</tr>
<tr>
<td>Total alveolar area</td>
<td>Continuous quantitative variable</td>
<td><strong>ImageJ</strong>, National Institutes of Health</td>
<td>m²</td>
</tr>
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<td><strong>ImageJ</strong>, National Institutes of Health</td>
<td>µm</td>
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Secondary qualitative variable (microscopic emphysema) analysis will be carried out using conventional histopathological studies, based mostly on direct visualization and the experience of the pathologist.

The rest of secondary variables (quantitative) analysis will be carried out using image analysis software for morphometric analysis (**ImageJ**, National Institutes of Health).
8.7. DATA COLLECTION

HISTOLOGICAL SAMPLE

During the autopsy, lung tissue samples of the 5 pulmonary lobes of 2x2 cm will be taken. The technical processing used to make the histological preparations consisted on:

1) **Fixing the samples of the different pulmonary lobes in a formalin solution**

These tissues were perfused with a solution of 4% paraformaldehyde (PFA) in phosphate buffer (PB) 0.1 M ph 7.4. It is important to adjust the final pH of the solution to ensure good tissue preservation.

2) **Dehydration by alcohol**

All tissues have been postfixied for a period of 10 hours. Before beginning to process them, they are washed at least for 12 hours in 70% ethyl alcohol.

Dehydration is started by staining the samples with a 0.5% eosin solution dissolved in 96% ethyl alcohol for 30 minutes to facilitate the subsequent location of the sample in the paraffin blocks. This dehydration is carried out with a battery of alcohol of ascending gradient and xylol:

- 2x1 hour alcohol 96%
- 3x1 hour alcohol 100%
- 3x1 hour xylol

After that, they are placed in a paraffin bath at 62º for a period of time between 4 and 6 hours.

3) **Inclusion in paraffin**

After the dehydration, preparations are placed in a paraffin bath at 62º for a period of time between 4 and 6 hours.
Samples are placed on blocks following a transversal orientation, the sections are 12 µm thick. Serial sections are placed on a slide that has a thin layer of Mayer's albumin and with distilled water. Then, they stretch with distilled water on a plate at 40-45 °C.

4) Staining of the histological sections with hematoxylin-eosin

We have done the staining following the next steps:

1. **De-parafination**: Xylol incubating 10 minutes (1 change).
   Xylol incubating 5 minutes (1 change).

2. **Hydration**: Ethanol 100 º, dipping it 5 minutes (1 change).
   Ethanol 96 º, dipping it 3 minutes (1 change).
   Ethanol 70 º, dipping it 3 minutes (1 change).
   Rinse with water, minimum 3 minutes.
   (Before starting with hematoxylin it should be filtered).

3. **Hematoxilin**: stain for 10 minutes.

4. **Differentiation**: rinse water until it is slightly transparent.

5. **Virage**: with acidic alcohol (70º) and a hydrochloric acid between 3 and 4 dips.

6. **Partial dehydration**: with ethanol (80º), 3 minutes.

7. **Eosin floxin**: stain for 5 minutes.

8. **Dehydration and differentiation**: Ethanol (80º) 3 minutes.
   Ethanol (96º) 3 minutes.
   Ethanol (100º) 5 minutes, 2 changes.

8. **Clarification**: Xylol, 2 changes (5-10 minutes each one).
   Finally, assembly with Xylol.
Presence or absence of microscopic emphysema will be analyzed using conventional histopathological studies, based mostly on direct visualization of airspace enlargement and fragmented alveolar walls.

**IMAGES’ ANALYSES**

For each histological preparation 5 microscope fields are selected (increased x 10). Each field is photographed with a high resolution microscope Zeiss brand model AXIOCAM ICc1 coupled to a triocular microscope brand Zeiss model Primo Star iLED.

The captured images will be analyzed using image analysis software (ImageJ, National Institutes of Health) and the following parameters will be measured:

- Total alveolar area
- Average alveolar diameter
- Alveolar density
- Average thickness of the alveolar wall.
9. Statistical analysis

As stated above, this study is not attainable to define independent or dependent variables. On the other hand, in order to perform the appropriate statistical analysis, it is necessary to define the variables as independent and dependent, so we will consider the cause of death as independent variable and the rest of them as dependent variables.

Statistical analysis will be performed using Statistical Package for Social Sciences (SPSS) and Microsoft Excel Windows to manage computed data.

*p value of < 0.10* will be considered statistically significant.

As the sample size is reduced, the probability of committing a type II error (not rejecting the null hypothesis when it is false, i.e. beta) will be very high (i.e. very reduced statistical power).

To increase statistical power (and, therefore, reduce beta), we could increase the sample size (not possible in our case) or increase the risk (i.e. alpha).

That’s the reason why we have chosen to increase the risk.

9.1. DESCRIPTIVE ANALYSES

We will summarize all the variables using proportions when the variable is qualitative (microscopic emphysema), and means (standard deviation) and medians (interquartilic interval IQR) when the variable is quantitative (total alveolar area, average alveolar diameter, alveolar density and average thickness of the alveolar wall).

We will stratify it basing on the cause of death (drowning and PBt / AGE).
9.2. INFERENCE ANALYSES

We will contrast the difference of proportions, means and medians between drowning and PBt/AGE (cause of death) according to the other variables (microscopic emphysema, total alveolar area, average alveolar diameter, alveolar density and average thickness of the alveolar wall) using \( \chi^2 \) (fisher's exact test when the expected frequencies are less than 5, and in case of proportions) and using \textbf{U Mann-Whitney} (in case of means and medians).

9.3. MULTIVARIATE ANALYSES

In this study, we will not do this assessment because the sample size is not enough to include covariates. When we would have a bigger sample, we would consider to correct the results according to gender and age, among others.
10. Ethical considerations

This study does not use any invasive technical procedure on a corpse since we will work only with processed histopathological samples. Histological processed samples of corpses will be used without significant judicial implications, all the proceedings are in a procedural situation of provisional or free dismissal.

This project does not use clinical data or identification of the subjects, only the histological pattern of the lung sample and the cause of death. It has been presented and approved by the Comisión de Docencia e Investigación del IMLCFC.

The IMLCFC Forensic Pathology Service does not have direct access to the relatives of the subjects in this study, so the request for consent for the use of samples is not possible and in any case it would mean an effort not reasonable as set forth in article 58 of Law 14/2007 on Biomedical Research.

That’s the reason why this project has been passed through the Comité de Ética del Hospital Universitario de Bellvitge in order to be approved and to begin to work without authorization of the families (annexe 2).

All information shared with collaborators will be encrypted or codified. Personal and clinical data protection of all participants and collaborators involved with the study is guaranteed, in accordance with the Reglamento (UE) 2016/679 General de Protección de Datos and the Ley Orgánica 15/1999 de Protección de Datos de Carácter Personal. Samples will be collected and stored in maximum quality conditions, in harmony with the quality standards held up throughout the study. Data maintenance and custody will be implemented by the IMLCFC (Divisió de Girona).

All the investigators have declared no conflict of interest.
11. Study limitations and strengths

As we have mentioned in the statistical analysis, the sample size is reduced (n=7), which increases the risk for type II errors (not rejecting the null hypothesis when it is false, i.e. beta). We have increased the alpha risk in order to reduce the probability of making this error. In any event, this will be considered as being a pilot study. Therefore, further investigations will be needed in the future.

Because of the same reason, another limitation is the impossibility to do a multivariate analysis stratifying by gender, age or other covariates.

The standardized values for the histological images that we use in our project (with the quantitative variables) have not been officially validated by any Medical society but they used in common practice in pathologic studies.

On the other hand, since there is a lack of investigation on this topic, we consider this study could be a breakthrough in terms of what we know right now about PBT/AGE. Developing new specific tests such as morphometry in these cases constitutes a challenge for a Forensic Pathology Service, but this is not possible if there is no scientific evidence of histomorphological changes in this type of death, and with this project we are trying to make a first step towards a new diagnostic method in the future.
12. Chronogram

The study began at May 2018, when we started writing a project (a summarize of what we wanted to do) to present it to the Comité de Ética del Hospital Universitario de Bellvitge (Phase 0). Plannification had been adapted to the approval of this Comittee that arrived on 20 September 2018.

October, November and December (2018):
Selection and preparation of histopathological samples according to the cause of death (Phase 1). This task has been developed in the Department of Histopathology of the IMLCFC Forensic Pathology Service.
Participating researcher members: Dr. Josep Maria Casadesús; Dr. José Manuel Tortosa; and Mrs. Aina Torres.

January, February (2019):
Digitalization of images through photography with high resolution microscopic camera and morphometric analysis of the parameters previously referenced in each one of the samples using image analysis software (Phase 2).
This task is being developed in the morphology laboratory of the Faculty of Medicine of the UdG.
Participating researcher members: Dr. Josep Maria Casadesús i Valbí; Dra. Maite Serrando Querol, Dra. Anna Carrera Burgaya, Dr. Pere Boadas-Vaello; Francisco Reina de la Torre; and Mrs. Aina Torres de la Rosa.

March (2019):
Statistical analysis and multidisciplinary results assessment (Phase 3).
This task will be developed at the Center de Patologia Forense de Girona of the IMLCFC and the Faculty of Medicine of the UdG.
Participating researcher members: Dr. Josep Maria Casadesús Valbí; D. Fernando Aguirre Lirón; Dr. Jordi Desolà Alà; Dra. Maite Serrando Querol, Dra. Anna Carrera Burgaya, Dr. Pere Boadas-Vaello; Dr. Francisco Reina de la Torre; and Mrs. Aina Torres de la Rosa.
April, May, June (2019):
Final report elaboration, publication of the results and dissemination of the study by a conference (Phase 4). This task will be developed at the Center de Patologia Forense de Girona of the IMLCFC and the Faculty of Medicine of the UdG.

Participating researcher members: Dr. Josep Maria Casadesús Valbí; D. Fernando Aguirre Lirón; Dr. Jordi Desolà Alà; Dra. Maite Serrando Querol, Dra. Anna Carrera Burgaya, Dr. Pere Boadas-Vaello; Dr. Francisco Reina de la Torre; and Mrs. Aina Torres de la Rosa.
<table>
<thead>
<tr>
<th>Phase</th>
<th>2018</th>
<th>2019</th>
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<tr>
<td></td>
<td>May</td>
<td>June</td>
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<td>Phase 0</td>
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<tr>
<td>Sample selection</td>
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<td>Publication of the results</td>
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<td>Dissemination of the study (conference)</td>
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# 13. Budget

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<tr>
<th>STUDY BUDGE</th>
<th>COST</th>
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<tr>
<td><strong>Staff cost</strong></td>
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<tr>
<td>Pathology Staff *1</td>
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<td>Qualified statistician (30h x 35€/h)</td>
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<td><strong>Subtotal:</strong></td>
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<td><strong>Histological materials cost</strong> (for all samples)</td>
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<tr>
<td>Formaldehyde solution 4L</td>
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<td>Alcohols for dehydration</td>
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<td>Paraffin for inclusion</td>
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<td>Hematoxilin and eosin solution</td>
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<td><em>(from Sigma-Aldrich)</em></td>
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<td><strong>Subtotal:</strong></td>
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<td><strong>Imaging analysis cost</strong></td>
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<td>ImageJ license</td>
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<tr>
<td>Digitalization *2</td>
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</tr>
<tr>
<td><strong>Subtotal:</strong></td>
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</tr>
<tr>
<td><strong>Publication cost</strong></td>
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</tr>
<tr>
<td>Publication in <em>Forensic Science, Medicine and Pathology</em> (1Q)</td>
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<td>Dissemination of the study (conference) *3</td>
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<td><strong>Subtotal:</strong></td>
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<tr>
<td><strong>Total cost:</strong></td>
<td><strong>3,145,10 €</strong></td>
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</table>
All the pathology staff that is working in the project is already part of the research team so additional personnel is not required.

The high resolution microscope *Zeiss brand model AXIOCAM ICc1* coupled to a triocular microscope brand *Zeiss model Primo Star iLED* used for the imaging digitalization is available in the Faculty of Medicine of the University of Girona and thanks to the collaboration done, its utilization for our project is free.

The dissemination of the study consists in attending the **V Congreso Nacional de la Sociedad Española de Patologia Forense (SEPAF)** for two investigators that will take place in Granada on 22th-24th May 2019.

The cost of it consist on (for each person):

- Registration: 560€ (diets included)
- Round trip train + flight (Girona-Bcn, Bcn-Granada): 140€
- Lodging: 160€

**Total for each person: 860€**
14. Feasibility

14.1. TEAM EXPERIENCE ABOUT THE TOPIC

This project is being done by the following investigators (affiliations are represented by numbers):

Josep M. Casadesús¹,²,³; Fernando Aguirre⁴; Anna Carrera²,³; Pere Boadas-Vaello²,³; Maria T. Serrando³,⁵; José M. Tortosa¹; Jordi Desola⁶; Aina Torres²; Francisco Reina²,³

- ¹ Institute of Legal Medicine and Forensic Sciences of Catalonia (IMLCFC)
- ² Research Group on Clinical Anatomy, Embryology and Neuroscience (NEOMA). Department of Medical Sciences, University of Girona (UdG)
- ³ Department of Medical Sciences, Faculty of Medicine, UdG
- ⁴ Special Group for Underwater Activities (GEAS) of the Spanish Civil Guard. Estartit (Girona).
- ⁵ ICS-IAS Girona Clinical Laboratory, Salt (Girona).
- ⁶ Hospital de Sant Joan Despí Moisés Broggi, Sant Joan Despí (Barcelona).

This group has a large experience on this topic. Their participation in previous research forensic studies during diving has resulted in a total of:

- One chapter of a book nationwide
- Two publications in nationals books
- Conferences and specialized courses
- Ten communications and specialized courses and seminars nationals
- Two communications and specialized courses and seminars internationals
- One publication indexed in an international relevant journal of the speciality (1Q)
- One publication submitted in an international relevant journal of the speciality (1Q)
14.2. MEANS AVAILABLE

We have the collaboration of the IMFC Forensic Pathology Service, the Center of Forensic Pathology of Girona and the collaboration of the Faculty of Medicine of the University of Girona.

All materials and drugs needed are already available in the both mentioned locations, requiring no additional special facilities or new equipment.

Additional personnel (except for a statistician) shall not be required because every person working on it is already on the investigation group, and the revision and publication of the report in Forensic Science, Medicine and Pathology (1Q) is free.

Thus, our cost budget includes mostly expenses due to dissemination of our study in a National Congress, with an estimated total amount as 3.145,10 €, making it financially feasible.
15. Annexes

Annex 1

MACROSCOPIC DIAGNOSTIC CRITERIA
(Characteristic but not pathognomonic)

External examination

- Foam in the mouth and nostrils
- Cooling, pale skin and pink lividity
- Maceration and skin whitening hands and feet
- Cutis anserine
- Shrinkage of the penis and scrotum
- Saponification
  (when remains > 2 months in water)

Internal examination

- Respiratory system
  - Foam in airway
  - Lung volume increased
  - Increased weight of the lungs (variable between: 1400-2500 g)
  - Subpleural Patchett bleeding
  - Transudate liquid in pleural cavity (average volume 432 ml)

- Digestive system
  - Presence of water in stomach or duodenum
    (volume greater than 500 ml)
  - Tears in the mucosa of the cardia

- Other
  - Haemorrhages in anterior neck and chest muscle groups
  - Fluidity in the blood
    (mainly left cardiac cavities)

Correlation with the circumstances of the death

Traumatic pathology ruled out as cause of death

From aquatic medium
- Nautical collision
- Aquatic animals
- From diving (baro-trauma)

MACROSCOPIC Diagnosis of SUBMERSION
MACROSCOPIC findings characteristic of submersion

- Not evident, doubtful and/or non-specific
- No correlation with the circumstances of death

CONFIRM or EXCLUDE

- Death due to submersion
- Non-traumatic predisposing factors

Taking samples (Order JUS/1291/2010 of 13 May)

- Essential: lung (5 lobes and hilus) and whole heart. In addition, and depending on the autopsy findings: brain, liver, spleen, kidney, pancreas, possible wounds, etc. Fixed with formol
- Peripheral blood (two 5 ml tubes, each with preservatives, anticoagulants, full and with no air pocket) and/or viscera (liver, kidney and lung, 50 g of each, refrigerated and without fixative liquid)
- Left and right ventricular cardiac cavity blood (2 tubes with EDTA). Distal portions of the lung lobes (100-200 g), liver and spleen (100 g each), sternum or femur and CNS (dura mater, full cerebellum, choroid plexus) Separated, refrigerated and without fixative liquid

Histopathological studies: study of lung submersion and sudden death

Toxicological studies: determination of drugs of abuse, ethanol, medicine and/or other (CO, etc.)

Biological studies: determination of strontium and/or study of diatoms

Institut de Medicina Legal de Catalunya Laboratory Service
Instituto Nacional de Toxicología y Ciencias Forenses
INFORME DEL COMITÉ DE ÉTICA DE INVESTIGACIÓN CLÍNICA
SOBRE PROYECTOS DE INVESTIGACIÓN

El Comité de Ética de Investigación Clínica del Hospital Universitari de Bellvitge, mediante el procedimiento de evaluación rápida de la documentación contemplado en los Procedimientos Normalizados de Trabajo del Comité (esta aprobación constará en el Acta 16/18 de fecha 20/09/18), tras examinar toda la documentación presentada sobre el proyecto de investigación con nuestra ref. PR321/18, titulado:

"ESTUDI MORFOMÈTRIC DEL BAROTRAUMA PULMONAR: ANÀLISIS A PARTIR DE MOSTRES PROCEDENTS D’AUTÒPSIES FORENSES PER BUSSEIG AMB ESCAFANDRE AUTÒNOM".

Presentado por el Dr. Josep Maria Casadesús i Valbí del Servicio de Patología Forense del Institut de Medicina Legal i Ciències Forenses de Catalunya, como promotor e investigador principal, ha acordado emitir INFORME FAVORABLE al mencionado proyecto.

Que la composición actual del Comité de Ética de Investigación Clínica es la siguiente:

Presidente: Dr. Francesc Esteve Urbano
Vicepresidente: Dra. Pilar Hereu Boher
Secretario: Dr. Enric Sospedra Martínez
Vocales:
- Dra. Jordi Adamuz Tomás
- Dra. Maria Berdasco Menéndez
- Dra. Concepción Cañete Ramos
- Dr. Enric Condom Mundo
- Dr. Xavier Corbella Virós
- Sra. Consol Felip Farràs
- Dr. José Luis Ferreiro Gutiérrez
- Dra. Ana María Ferrer Artola
- Dr. Josep Ricard Frago Montanu
- Dr. Xavier Fullidosa Oliveras
- Dra. Margarita García Martín
- Dr. Carles Liadó i Carbonell
- Dr. Josep Manel Llop Talaveron
- Sra. Sonia López Ortega
- Dr. Sergio Morchón Ramos
- Dr. Joan Josep Queralt Jiménez
- Dr. Ricard Ramos Izquierdo
- Dra. Gemma Rodríguez Palomar
- Dra. Nuria Sala Serra
- Dr. Petru Cristian Simon

Médico - Medicina Intensiva
Médico - Farmacología Clínica
Farmacéutico - Farmacia Hospitalaria
Enfermero – Enfermería
Bióloga - miembro no sanitario
Médico - Neumología
Médico - Anatomía Patológica
Médico - Medicina Interna
Miembro Laico - Docencia
Médico - Cardiología
Farmacéutica - miembro sanitario
Médico - Cirugía General y Digestiva
Médico - Nefrología
Médico - Oncología Médica
Médico- Urología
Farmacéutico – Farmacia Hospitalaria
Graduado Social - Atención a la Ciudadanía
Médico - Medicina Preventiva
Jurista
Médico - Cirugía Torácica
Farmacéutica – Atención Primaria
Bióloga - miembro no sanitario
Médico - Farmacología Clínica
Que este Comité cumple la legislación española vigente para este tipo de proyectos, así como las normas ICH y las Normas de Buena Práctica Clínica.

Que en dicha reunión del Comité de Ética de Investigación Clínica se cumplió el quórum preceptivo legalmente.

Lo que firmo en L'Hospitalet de Llobregat, a 20 de septiembre de 2018.

Fdo. Dr. Enric Sospedra Martínez
Secretario del CEIC