

Tissue Engineering: development of biomaterials and cellular therapies in clinical practice.

Fernando Guedes

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Tissue Engineering: development of biomaterials and cellular therapies in clinical practice.

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



Tissue Engineering: development of biomaterials and cellular therapies in clinical practice

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Tissue Engineering: development of biomaterials and cellular therapies in clinical practice

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DECLARATION

In accordance with the disposed in “artigo 17º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto, publicado em Diário da República, 2a série, nº 204 de 23 de outubro de 2018”, the author of this thesis declares to have actively participated in elaboration and execution of experimental work that led to the results presented, as well as in its interpretation and in the writing of the respective manuscripts.

List of Publications

All authors of the articles were aware of their use in the present thesis and did not raise any objection.

Articles in international Peer-Reviewed Scientific Journals

- **Guedes, F., Maurício, A. C., & Bugalho, A.** (2020). Central airway stenosis: opening the path. *Archivos de bronconeumologia*, 56(11), 695-696
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- **Guedes F., Branquinho M.V., Biscaia S., Alvites R., Sousa C., Lopes B., Sousa P., Rama A., Amorim I., Faria F., Patrício T., Alves N., Bugalho A. & Maurício A. C. (2022).** Gamma irradiation processing on 3D PCL devices - a preliminary biocompatibility assessment. *International Journal of Molecular Sciences* (Submitted).

Other Publications Complementary and Developed in Clinical Practice

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- **Oliveira, T., & Guedes, F. (2018).** Extralobar Pulmonary Sequestration with an Aneurysmatic Arterial Supply. *Archivos de Bronconeumologia*, 55(6), 329-329.
- **Rodrigues, L. V., Gomes, R., Samouco, G., Guedes, F., & Ferreira, L. (2019).** Current utilization of pleuroscopy in mainland Portugal. *Pulmonology*, 25(1), 51-53.
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Book Chapters

- **Guedes F., Abreu T., Caminha J.C.** (2022). Broncoscopia em Cuidados Intensivos. *Tratado de Medicina Intensiva da AMIB – Associação Brasileira de Medicina Intensiva e SPCI – Sociedade Portuguesa de Cuidados Intensivos*. Editora Atheneu (Accepted for publication)
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U. PORTO



CDRSP CENTRO PARA
O DESENVOLVIMENTO RÁPIDO
E SUSTENTADO DE PRODUTO

Resumo

A obstrução da via aérea central (CAO) representa uma condição que afeta adultos e crianças. A sua incidência não é conhecida com precisão, mas é consensual que se encontra subvalorizada, pois clinicamente pode apresentar inicialmente apenas sintomas incipientes e comuns a outras patologias do foro respiratório.

No *Capítulo I* abordamos essa problemática num trabalho em que procuramos rever aprofundadamente a patologia em toda a sua extensão, à luz do estado da arte, experiência pessoal e perspetiva futura. Clinicamente representa um desafio e conduz os pacientes a morbilidade e mortalidade relevantes. A opção cirúrgica é considerada o tratamento *gold standard* para situações, de patologia benigna, mas frequentemente está contraindicada, não só pela impossibilidade técnica, mas também pelas comorbilidades associadas numa população envelhecida e com doenças crónicas. Nesse sentido, a broncologia de intervenção desempenha um papel importante e um dos procedimentos complexos passa tratamento da CAO. Diversas técnicas apresentam-se como soluções válidas, designadamente dilatação mecânica, LASER, aplicação de fármacos ou próteses. No entanto, estas opções não representam tratamentos definitivos possuindo um papel paliativo em muitas situações. Em busca de um tratamento mais definitivo e eficaz surge a Medicina Regenerativa e Engenharia de Tecidos como alternativa válida para um futuro que espreita o presente.

No final de 2019 o trabalho na Unidade de Broncologia de Intervenção e no mundo em geral sofreu um profundo abalo com o aparecimento da pandemia COVID-19. Os procedimentos da broncologia de intervenção, necessitaram de uma reorganização marcada para salvaguardar a segurança dos doentes e profissionais. É neste contexto que é realizado o primeiro estudo, apresentado no *Capítulo II*, como necessidade de dar resposta às incertezas colocadas. Nesse documento propomos alterações administrativas, ambientais, organizacionais e de proteção individual, a serem aplicadas durante os procedimentos realizados nas Unidade de Broncologia de Intervenção.

Certos do impacto qualitativo e quantitativo que a pandemia teve nas nossas rotinas de trabalho nas Unidades de Broncologia de Intervenção de Portugal e da

forma como afetou os pacientes, nomeadamente os com patologia oncológica pulmonar, surge o segundo estudo, no *Capítulo II*. O objetivo foi não só preencher uma lacuna no conhecimento da prática da Broncologia de Intervenção no nosso país antes e no ano inicial da pandemia, mas também perceber a forma como afetou a problemática da obstrução da via aérea central, o nosso “problema clínico” de fundo. Os pacientes oncológicos viram os seus diagnósticos e tratamentos sofrerem atrasos que, segundo a nossa compreensão, poderiam ter impacto na patologia da via aérea central. Nesse estudo comprovamos uma diminuição do número global de procedimentos, num cenário em que uma recuperação ativa dos números pré pandémicos surgia como um imperativo.

Tentando responder à necessidade de darmos um passo em frente no tratamento, da patologia da via aérea central, efetuou-se o trabalho apresentado no *Capítulo III*. Neste estudo preliminar, abrimos a porta para a Medicina Regenerativa e a Engenharia de Tecidos na abordagem da via aérea central. Realizamos este estudo com a ideia de no futuro podermos fabricar uma prótese da via aérea central personalizada, num material biodegradável, biocompatível e citocompatível e com capacidade de promover o crescimento de células e tecidos circundantes lesados através de terapias celulares implantadas na prótese. Utilizou-se um polímero sintético biodegradável e aprovado pela Food and Drug Administration (FDA), a policaprolactona. Foram produzidos pequenos discos de 2mm em impressoras 3D, que foram analisados em termos de cito e biocompatibilidade, recorrendo a normas ISO específicas para dispositivos médicos. Testámos várias radiações gama para a sua esterilização, uma vez que a esterilização de dispositivos médicos é um passo crucial na sua preparação, sem comprometer as suas características mecânicas e físico-químicas. Concluiu-se que as suas propriedades biomecânicas permanecem inalteradas até à radiação de 45K Gy, sendo a radiação para esterilização mais eficaz. Promovemos ainda o *seeding* nas scaffolds 3D de policaprolactona, que simulam a estrutura e porosidade do material a utilizar na potencial prótese, com células estaminais mesenquimatosas da polpa dentária de dentes definitivos (DPSCs) e verificámos existir uma boa citocompatibilidade, independentemente da quantidade de radiação utilizada.

Este trabalho lança as bases e abre excelentes expectativas à possibilidade de num futuro próximo serem desenvolvidas próteses traqueais personalizadas, recorrendo a um biomaterial como a policaprolactona associada às DPSCs. Dessa

forma esperamos poder acrescentar um pequeno contributo no tratamento da nossa questão de fundo que é a “Obstrução da via aérea central”.

Abstract

Central airway obstruction (CAO) is a condition that affects adults and children. Its incidence is not precisely known, but it is consensual that it is underestimated because clinically it may initially present only incipient symptoms common to other respiratory pathologies.

In *Chapter 1* we address this problem in a work in which we try to thoroughly review the pathology in all its extension in the light of the state of the art, personal experience, and future perspectives. Clinically it represents a challenge and leads patients to relevant morbidity and mortality. The surgical option is considered the gold standard treatment for situations of benign pathology, but it is often contraindicated, not only because of technical impossibility, but also due to associated comorbidities in an ageing population with chronic diseases. In this sense, interventional bronchology plays an important role, mostly in the complex stenosis, in the treatment of CAO. Several techniques are presented as valid solutions, including mechanical dilatation, LASER, application of drugs or stents. However, these options do not represent definitive treatments and have a palliative role in many situations. In search of a more definitive and effective treatment, Regenerative Medicine and Tissue Engineering emerges as a valid alternative for a future that peeks into the present.

At the end of 2019 the work in the Intervention Bronchology Unit and in the world in general suffered a profound jolt with the emergence of COVID-19 pandemic. Intervention bronchology procedures, underwent in a deep reorganisation to safeguard of patients and professionals. It is in this context that the first study presented in *Chapter II* emerged as a need to respond to the uncertainties raised. In this document we propose administrative, environmental, organisational, and individual protection changes to be applied during the procedures performed in the Intervention Bronchology Unit.

Certain of the qualitative and quantitative impact that the pandemic had on our work routines in the Intervention Bronchology Units in Portugal and the way it affected patients, namely patients with pulmonary oncology pathology, the second study of *Chapter II* emerged. The aim was to fill a gap in the knowledge of Intervention Bronchology practice in our country before the pandemic and in the initial year of the pandemic, but also to understand how it affected the problem of central airway obstruction, our underlying "clinical problem". Cancer patients saw

their diagnoses and treatments delayed, which as we understand could have an impact on central airway pathology. In this study, we verified a decrease in the global number of procedures, in a scenario where an active search of the pre-pandemic numbers appeared as an imperative.

To respond to the need to take a step forward in the treatment of central airway pathology, the work presented in *Chapter III* arises. In this preliminary study, we open the door to Regenerative Medicine and Tissue Engineering in the approach to the central airway pathology. We conducted this study with the idea that in the future we can manufacture a customised central airway prosthesis in a biodegradable, biocompatible and cytocompatible material and with the ability to promote the growth of injured cells and surrounding tissues, through cells implanted in the prosthesis. A biodegradable synthetic polymer approved by the Food and Drug Administration (FDA), polycaprolactone, was used. Small 2mm discs were produced in 3D printers and analysed for cyto and biocompatibility using ISO standards specific for medical devices. We tested several gamma radiations for their sterilization, since sterilization of medical devices is a crucial step in their preparation, either to ensure sterility without compromising their mechanical and physicochemical characteristics. It was concluded that their biomechanical properties remain unchanged up to 45K Gy radiation, being the most effective radiation for sterilisation. We also promoted the seeding of 3D polycaprolactone scaffolds, which simulate the structure and porosity of the material to be used in the potential stent, with mesenchymal stem cells from the dental pulp of permanent teeth (DPSCs) and verified that there is a good cytocompatibility, regardless of the amount of radiation used.

This work establishes the basis and opens excellent expectations for the possibility that in the nearby future, personalised tracheal stents may be developed, using a biomaterial such as polycaprolactone associated with DPSCs. In this way we hope to be able to add a small contribution to the treatment of our underlying problem which is "Central Airway Obstruction".

List of Abbreviations

3D - Three-dimensional space
ALI - Acute Lung Injury
ARDS - Acute Respiratory Distress Syndrome
CHUP – Centro Hospitalar e Universitário do Porto
CAO – Central Airway Obstruction
COVID-19 - Coronavirus Disease 2019
DNA – Deoxyribonucleic Acid
DPSCs – Dental Pulp Stem Cells
ECM – Extracellular Matrix
FDA – Food and Drug Administration
HCW - Health Care Workers
hDPSCs – Human Dental Pulp Stem Cells
HSC - Hematopoietic Stem Cells
ICU – Intensive Care Unit
IP - Interventional Pulmonology
ISO - International Organization for Standardization
KGy - KiloGray
LASER - light amplification by stimulated emission of radiation
mm - millimetre
MSCs - Mesenchymal Stem Cells
Nd:YAG - Neodymium-doped Yttrium Aluminium Garnet
PCL - Polycaprolactone
PCR – Polymerase Chain Reaction
PPE – Personal Protective Equipment
RM - Regenerative Medicine
RT-PCR - Reverse Transcriptase with Polymerase Chain Reaction
RB – Rigid Bronchoscopy
SEM - Scanning Electron Microscope
TE - Tissue Engineering

WHO – World Health Organization

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Chapter I

Central Airway Obstruction

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Introductory note

Central airway obstruction (CAO) is a clinical condition that leads to airflow limitation of the trachea, main stem bronchi, bronchus intermedius or lobar bronchus. Its incidence is not clear because there is a lack of epidemiology studies and the symptoms may be identical to other low respiratory tract pathologies, which may lead to misdiagnosis. On the other hand, it is consensually underdiagnosed (1). Surgical approach is considered the most adequate treatment for most clinical situations, especially in benign aetiologies, but multiple factors concerning the underlying disease, the degree and type of CAO, and the patient's condition may discourage this option. In those cases, interventional pulmonology, airway stenting and the novel tissue engineering therapies may play an important role (2,3)

The causes of CAO are multiple and can go from congenital to acquired (4); in acquired can be benign or malign (5), infection, systemic diseases (6) or trauma (7). The clinical approach must be based on a multidisciplinary team discussion to select the best therapeutic options and cover all the possibilities. Therapy must be addressed according to the pathologic findings and to the patient's health status. Definitive management may include both medical and surgical interventions. Treatment possibilities embrace observation, drug therapy, radiation, tracheostomy, endoscopic treatment, and surgical management. A bronchoscopic approach may include dilatation, laser/heat resection and airway stenting (8,9).

In non-symptomatic or pauci-symptomatic patients, the therapeutic option may be more conservative and surveillance is recommended (10). In the decision of management, the most important differentiation to be made is between simple and complex stenosis as it determines a higher probability of success or failure of the endoscopic intervention (11). When the best therapeutic option is to deploy an airway stent, the decision should be carefully evaluated and the operator must consider the benefit of its application, by preventing airway re-occlusion, but also its disadvantages, mostly regarding medium and long-term associated complications. The use of stents is indicated in intraluminal and extraluminal CAO refractory to dilatation in both benign and malignant disease. It is indicated when dilatation procedures are not efficacy, when it is not possible have enough lumen

even after the bronchoscopic procedures, or as a bridge before surgery or while chemotherapy or radiation is performed (12,13).

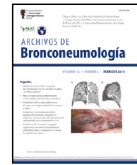
Recently, a big step was made when manufacturers proposed modifications of their products according to patient's needs, with rapid 3D printing techniques, allowing stents to be tailored for each individual. In reference centres, these stents can be in a timely manner. Meanwhile, we must be aware that a totally personalized airway stent with specific angles, widths and length would take several weeks to be prepared, which is not acceptable when rapid symptomatic relief is necessary (14). Potential benefits of the evolution of these stents include faster customization, increased freedom of design and less raw material waste. On the other side, there are still some barriers like the lack of standardization, how personalized 3D printed stents will be manufactured and regulated and the lack of partnerships between physicians and manufacturers to make the modelling software universally available

In this chapter we present two papers. The first is an editorial which works as a prelude to the second study. In both we made an overview of the problematic associated to CAO and the clinical problem that it represents. We discuss current options, their limitations and poor outcomes represented by three distinct clinical cases of my own experience as a medical doctor at Centro Hospitalar e Universitário do Porto.

We also debate the possibility of novel approaches based on Tissue Engineering (TE) and Regenerative Medicine (RM). Those therapies emerged in the last 20 years as a new approach to diseases that tending to be evolve to chronicity. The goals of RM are mainly two: to repair and regenerate damage organs and tissues; and to improve the capacity of transplantation. These possibilities can come from existing organs or through manufacturing new ones. In our case, the purpose is not to manufacture a new organ but to promote rejuvenation and regeneration of a damage one through a supporting scaffold.

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Editorial

Central Airway Stenosis: Opening the Path

Estenosis de la vía aérea central: abriendo el camino

Central airway stenosis (CAS) is a pathological condition which leads to airflow limitation of the trachea, main stem *bronchi*, *bronchus intermedius* or a *lobar bronchus*. It is a common and serious clinical problem that affects both adults and children and is widely believed to be under-diagnosed.

Several factors concerning the underlying disease and the patient's health status will define an individualized diagnostic and therapeutic approach.¹

Multiple classification systems have been proposed during the last 30 years, most of them focused on surgical approaches and in outcomes based on treatable anatomic premises, excluding extensions beyond the trachea and the possibility of endoluminal treatment.²⁻⁴ In 2007, Freitag et al. published the first classification system designed to grade tracheobronchial stenosis from a Pulmonologist's perspective.⁵ In 2010, Murgu and Colt suggested a different classification, based on both qualitative and quantitative criteria.⁶

It seems necessary to standardize nomenclatures and find descriptors that include the degree of stenosis, patient's functional status, histologic and morphologic types, extension, localization and mechanisms of obstruction.

CAS can arise from congenital or acquired conditions. It may result from intrinsic stenosis or extrinsic compression, causing a fixed obstruction; or by cartilage or pars membranous flaccidity, leading to a dynamic obstruction. Numerous clinical conditions are identified, but nowadays malignant disease outnumbers benign diseases, especially due to the increase in primary lung cancer, as well as metastatic pulmonary disease. Historically literature reported that 20–30% of patients with lung cancer will develop CAS. A most recent study shows a prevalence of 13% at lung cancer diagnosis, with a further 5% of patients developing CAS within a year.⁷

The presentation symptoms can be insidious and may be obscured for a long time resulting in a delayed diagnosis and worst outcome. They will depend on the location, extend and degree of obstruction, speed of progression and underlying disease. Early manifestations may be mistaken for other diseases such as asthma or COPD. Main symptoms are usually dyspnea and stridor, occurring in 54% of patients as initial complain.⁸ They are more prominent during exercise but may progress to dyspnea at rest. The first occurs when airway lumen is reduced to 8 mm and the second when it reaches less than 5 mm. Cough, wheezing,

diminished sputum clearance and recurrent infections will emerge when it reaches this severity.

Clinical investigations for the underlying diagnosis include computed tomography, with 97% sensitivity for extra thoracic compression⁹; dynamic CT is effective for the diagnosis of tracheobronchomalacia and excessive dynamic airway collapse. Flow-volume loops may provide information regarding the level and degree of obstruction but a plateau is only reached after severe lumen impairment.¹⁰ These exams have not yet substituted flexible bronchoscopy as the primary procedure for diagnostic work-up and pre-interventional assessment since it provides a direct view of the airway.

In the therapeutic decision, according to Galluccio et al., the most important differentiation is between simple and complex stenosis because it determines the success or failure of the endoscopic intervention.¹¹ Surgical management remains the preferred approach but there are situations in which, whether due to patient's limitation or the nature of the pathology, interventional bronchoscopy, with techniques such as laser, cryotherapy, mechanical dilation or debridement, play a preponderant role.

Airway stents are placed to maintain airway patency. They are indicated in both intraluminal and extraluminal major CAS and can be used in both benign and malignant disease. The decision to place an airway stent is balanced between capacity of prevention the airway reocclusion and long-term complications associated.

When it comes to the choice of the stent, several considerations must be taken in account, as a plethora of many different stents from various materials, sizes and shapes are available. Silicone stents are cheaper, easily retrievable and can be repositioned as much times as necessary. On the other hand, they need rigid bronchoscopy to be handled, are more suitable to migrate and interfere with mucociliary clearance. Nitinol stents can be placed by flexible bronchoscopy and are usually more adaptable to an irregular surface. Based on 2005 Food and Drug Administration warning, metallic stents are not recommended in benign airway strictures because of the reported extensive granulation tissue and stent fracture. The ideal stent should be simple to insert, easily removable, capable to resist compression, allow clearance of secretions, easy to nail to avoid migration and would match the shape of stenotic airway (but is does not exist yet).

A recent evolutionary step was made when manufacturers offered modifications of their products according to patient's needs, with

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rapid prototyping 3D printing techniques, allowing stents to be tailored to the individual's airway. In reference centers, these stents can be printed in days, or even hours, but a totally personalized airway stent with specific angles, widths and length would take several weeks to be prepared, which is not acceptable when rapid symptomatic relief is necessary.¹²

Bioabsorbable airway stents, made of different biomaterials, are under investigation and have been deployed in humans. Polydioxanone is the one that has been most used and the longest reported time free of intervention was 44 months.¹³ Other compounds are under analysis like polylactic acid, polyglycolic acid, polycaprolactone, polyurethane or polyamide.^{12,14,15}

In our group, we are using an experimental polycaprolactone (PCL)/hydrogel polymer stent coated with umbilical cord mesenchymal stem (UCMSC) cells and dental pulp stem cells (DPSC) mesenchymal stem cells. Preliminary results of *in vitro* MSC's static seeding and dynamic seeding are very promising, and we are now starting animal testing.

Although it is expected an exciting new generation of personalized airway stents along with stem cell therapy, this reality is far to be achieved. If on one hand 3D printing technology could become accessible for physicians, on the other hand there are no certified manufacturers nor a direct access between parts. Maybe a creation of a software easy-to-use between hospitals and manufacturers could provide that answer. Several legal problems will emerge such as how to get authorization from governments or what official entities should regulate this process.

Meanwhile, this dialog must go on.

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RESEARCH

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Central airway obstruction: is it time to move forward?

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Abstract

Introduction: Central airway obstruction (CAO) represents a pathological condition that can lead to airflow limitation of the trachea, main stem *bronchi*, *bronchus intermedius* or *lobar bronchus*.

Main body: It is a common clinical situation consensually considered under-diagnosed. Management of patients with CAO can be difficult and deciding on the best treatment approach represents a medical challenge. This work intends to review CAO classifications, causes, treatments and its therapeutic limitations, approaching benign and malign presentations. Three illustrative cases are further presented, supporting the clinical problem under review.

Conclusion: Management of CAO still remains a challenge. The available options are not always effective nor free from complications. A new generation of costume-tailored airway stents, associated with stem cell-based therapy, could be an option in specific clinical situations.

Keywords: Airway stents, Central airway obstruction, Interventional bronchology, Regenerative medicine

Introduction

Central airway obstruction (CAO) is defined as a pathological condition that leads to airflow limitation of the trachea, main stem *bronchi*, *bronchus intermedius* or *lobar bronchus*. It represents an important clinical situation that affects both adults and children. The exact incidence is, to the authors knowledge, unknown, but is widely accepted by the medical community that this disease is underdiagnosed [1]. While a surgical approach is considered the *gold-standard* treatment for most clinical situations, especially in benign etiologies, multiple factors concerning the underlying disease, the degree and type of CAO, and the patient's condition may discourage this option. Interventional bronchoscopy emerged more than 120 years ago with Gustav Killian's works in

bronchoscopy, and has been validated as a compelling treatment with immediate effects in CAO for the last 40 years [2, 3]. Regarding CAO, the scientific and medical communities currently face several limitations that arise from the anatomical, structural, and physiological characteristics of the trachea and main bronchi. The normal adult trachea is usually a rigid 11–14 cm tube supported anteriorly by C-shaped cartilaginous rings and posteriorly by smooth muscle, and this structure as a whole has some, although restricted, mobility during the respiratory process [4]. In older patients or those with hyperinflation, the posterior membranous wall may collapse during expiration. Any variation on the trachea diameter during the normal respiration cycle or any endobronchial changes, such as injury or secretions, may impair the ventilatory dynamic and affect the Reynolds number (transition from laminar to turbulent flow with increase resistance), thus resulting in the need for higher-pressure difference to maintain a normal flow rate [5, 6]. This will be reflected in the development of symptoms, especially

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in patients with a marked reduction in the cross-sectional area.

CAO is caused by several disorders and the first step for its approach is a clear etiological evaluation (Table 1).

Congenital or acquired CAO may result from intrinsic stenosis or extrinsic compression, leading to fixed obstruction, or caused by cartilage or *pars membranous* flaccidity, with dynamic obstruction. In the early twentieth century, infection and trauma were the main causes of CAO. In the 60 s, with the advent of intensive care units and the common practice of prolonged orotracheal intubations, post-intubation tracheal stenosis (PITS) and post-tracheotomy (PSST) tracheal stenosis incidence increased. Malignant causes of CAO, which have been reported in up to 20–30% of lung cancers in older studies [7], do not reflect contemporary lung cancer epidemiology. These rates are now lower, with the progressive change in lung cancer incidence and prevalence, favouring peripheral adenocarcinoma development [8]. A recent retrospect analysis of 342 baseline CT scans of lung cancer revealed 13% of patients having CAO [9].

Since CAO has multiple etiologies and different expressions, a clear definition and classification is of utmost

relevance, as to estimate prognosis and determine the best therapeutic options [7]. A classification system based on histologic findings, pathological mechanisms, dynamics, functional impairment severity of airway narrowing and extend of airway abnormalities is crucial. The multidisciplinary involvement has led to the development of several systems with distinct criteria. Regarding laringotracheal stenosis, they were first classified by Cotton in 1984 based on the cross-sectional area of the stenosis [10]. In 1987, Grundfast et al. using the same criteria, upgraded the system looking for the predictive outcome [11]. In 1992 McCaffrey proposed a classification based on the vertical extend of the stenosis and predicting decannulation centred on anatomic location and extend [12]. Grillo et al. in 1993 reviewed the anatomic location and the possibility of anastomosis. In 1994, the Myer-Cotton Airway Grading System for subglottic stenosis was created and became routinely used to predict interventional success [13]. The four degrees of this system consider an obstruction of 0–50% for grade I, 51–70% for grade II, 71–99% for grade III, and complete luminal obstruction for grade IV [14]. The proposal of Lano et al. in 1998 was based on subsites involved. All

Table 1 The main causes of tracheobronchial stenosis according to different etiologies

Acquired	Tumour	Systemic diseases
	Cystic carcinomas Bronchial carcinoid tumours Primary squamous cell carcinoma of the trachea Adenocarcinoma of the trachea Primary lung cancer Metastasis	Granulomatosis with polyangiitis Amyloidosis Sarcoidosis Systemic erythematosus lupus Relapsing polychondritis Tracheobronchopatia osteochondroplastica
	Trauma	Tracheobronchomalacia
	Previous instrumentalization Post-intubation Post-tracheostomy Foreign body Thermal Caustic Radiation Anastomotic	Extrinsic compression Abnormalities of the aortic arch Thymic neoplasm Lymphoma Mediastinal or hilar increased lymph nodes Advanced esophageal cancer Diffuse goiter Postpneumonectomy syndrome Hematomas, abscess, empyema
	Infection	Other causes
	Tuberculosis Syphilis Typhoid fever Diphtheria Fungal infection	Idiopathic Gastroesophageal reflux
Congenital	Membranous (fibrous tissue, granulation tissue) Cartilaginous (cartilage deformity) Combined	

those studies were focused on surgical approaches and outcomes based mainly on treatable anatomic premises, excluding extensions beyond the trachea and the possibility of endobronchial treatment. In 2007, Freitag et al. published a classification system dividing stenosis into structural and dynamic types, in addition to categorizing its degree and location (Fig. 1A). This classification was designed for grading tracheal stenosis from a pulmonologist's perspective, but the degree of severity was not justified clinically. On the other hand, a histological classification was not included [15].

In 2010, Murgu and Colt made a different proposal, where a qualitative and quantitative classification was considered [16]. The gaps observed in Freitag et al. classification were also filled (Fig. 1B). The idea of creating an ideal and reproducible classification for CAO has not yet been fully completed and consensus has not yet been reached. In the future, ongoing studies will standardize nomenclatures and find descriptors that include the degree of stenosis and patient's functional status, histologic and morphologic types, extension, localization, and mechanism of obstruction.

The European Laryngological Society (ELS) published a consensus paper in 2015 that offered a new classification system of benign laryngo-tracheal stenosis (LTS) in adults and children [17]. It included a routine five-step endoscopic evaluation of patients, to assess the grade of stenosis (Cotton–Myer grade I–IV), number of subsites involved (“a–d” for one to four of supraglottic, glottic, subglottic, and tracheal segments involved), and an additional plus sign (“+”) for patients with severe airway or medical comorbidities. The focus of this classification was to access the surgical outcome of patients with LTS.

Etiology

Congenital CAO

In the adult population, congenital disease itself as a cause of CAO is a very rare condition [18]. When identified, it may result from posterior fusion of the tracheal rings. In children, it is frequently associated with congenital cardiovascular abnormalities, the most common being the compression of anterior tracheal wall for an anomalous bifurcation of the innominate artery [19].

Acquired CAO

Tracheobronchial trauma/iatrogenic

Acquired non-malignant CAO is most frequently caused by iatrogenic tracheal trauma after prolonged intubation or tracheostomy tube placement. It is estimated that the incidence of PITS ranges from 6 to 21% and PTS 0.6 to 21% [20, 21]. PITS occurs mostly in the anatomical area of the inflated cuff and is related to pressure induced ischemic injury of the mucosa and cartilage. On the other

hand, the inflammation caused by a foreign body may result in granulation tissue followed by fibrosis. Web-ring stenosis is the most common form [22]. With low-pressure cuffs and maintaining a pressure under 30 mmHg, it was possible to decrease the incidence of post-intubation stenosis to 1% and of post-tracheotomy to 10–15% [23]. The duration of intubation also impacts the frequency and severity. PSST may be above the stoma, at the level of the stoma, at cuff site or at the tip of the cannula. It results from a process of tissue repairing and formation of granulation tissue around the stoma. The most common type is granulation tissue formation at the stoma site. Stenosis due to cartilage fracture, tracheomalacia and web-like can also be seen [24]. Factors associated with PITS and PSST are the level of stoma, previous tracheal procedure, low blood pressure, severe respiratory failure, sepsis, concomitant autoimmune disease, sleep apnea syndrome, obesity, diabetes, high-dose corticosteroid therapy previous radiation and intubation > 48 h [25]. Post-transplantation bronchial stenosis occurs in 5–30% of patients and results from all repair process of anastomosis [26]. These anastomosis are vulnerable for several reasons: rejection may occur, and immunosuppression often results in infection and/or ischemia, especially in the first 4 weeks, which is the necessary time for bronchial arteries to re-establish full perfusion. Other causes of tracheobronchial stenosis include lobectomy, and sleeve and tracheal resections [27].

Trauma may also arise from thermal and chemical injuries or treatment with radiotherapy, causing localized stenosis.

Foreign body aspiration is more common in children and elderly and can lead to acute CAO. If the foreign body is not large enough to significantly occlude the lumen, granulation may emerge and cause obstruction. If the foreign body has an organic nature, a chemical bronchitis can occur [28].

Infection

Tuberculosis is the most common cause of post-infection stenosis in adults, mainly in high-prevalence geographic regions. It is usually caused by endobronchial involvement or lymph node fistulation through adjacent bronchi. Early diagnosis and treatment with anti-tuberculosis medications typically prevents this situation [29]. Laryngotracheobronchitis (croup) is an acute viral respiratory disease which occurs usually in children under 6 years old and is rare in adults. Acute bacterial tracheitis due to *S. Aureus* sometimes can occur as over-infection after a viral episode and may cause CAO [30]. Laryngeal scleroma is a chronic and progressive granulomatous infection caused by *Klebsiella rhinoscleromatis*, a gram-negative bacteria endemic in Africa, Asia and South

America, and may cause airway obstruction [31]. Other conditions like diphtheria, syphilis and fungal infections, although rare, are recognized causes of CAO [32].

Inflammatory and auto-immune diseases

Granulomatosis with polyangiitis is a systemic disease characterized by necrotizing vasculitis and granulomas. The airway is involved in 15–55% of cases and can be the only manifestation in 25% of patients [33, 34]. Granulomatosis with polyangiitis may lead to subglottic, tracheal, or bronchial stenosis, and nasal sinus is frequently involved. Laboratory findings may include positive anti-neutrophil cytoplasmic autoantibodies (c-ANCA) but usually there is no link between inflammatory activity and positive c-ANCA. The mucosa's biopsy is the gold standard method for diagnosis and usually reveals granulomatous inflammation and vasculitis in early disease, while fibrosis ensues later. Unfortunately it is positive in only 5–15% of cases [35]. Systemic immunosuppressive therapy and steroids are the mainstay treatment, but endoscopic management may be required.

Tracheobronchial amyloidosis results from the pathological deposition of amyloid in the proximity of the tracheal, bronchial gland acini and blood vessel walls. It may be present as an isolated manifestation or as part of a systemic disease. Tracheal involvement can range from diffuse lesions to masses. Pulmonary manifestations as persistent pleural effusions and parenchymal nodules can be present. Diffuse wall thickening, irregular narrowing of the lumen and calcifications may be seen during bronchoscopy. Diagnosis is determined by biopsy of the lesions showing positive red Congo staining with submucosal extracellular deposits of amyloid protein. There is no effective treatment and radiation therapy in addition to interventional bronchoscopy techniques and surgery should be considered [36].

Sarcoidosis is a multisystem disorder characterized by noncaseating granulomatous inflammation. Although any organ can be involved, the disease most commonly affects the lungs and intrathoracic lymph nodes. The airway can be affected even in the absence of parenchymal involvement. Bronchial wall thickening caused by granulomas and interstitial fibrous tissue may result in smooth or irregular luminal narrowing. The compression due to mediastinal lymphadenopathy is rare. Bronchoscopic findings include cobblestone appearance of the mucosa originated by granuloma, ranging from single to multiple stenosis sites [37]. The diagnosis of sarcoidosis is established based on a compatible clinical picture, evidence of noncaseating granulomas on biopsy, and exclusion of other granulomatous disorders. Treatment with immunosuppressive therapy may be necessary in severe cases.

Bronchoscopic procedures are required in some situations [38].

Relapsing polychondritis (RP) is a multisystemic autoimmune disease characterized by recurrent inflammatory episodes affecting cartilaginous structures. These structures include ears, nose, peripheral joints, larynx, and tracheobronchial tree. It is more likely between 40 and 50 years and is often associated with another pathologic conditions as systemic vasculitis or connective tissue disorders [30]. The evolution of the disease alternates between an active inflammatory phase and non-acute phase. During disease course, about half patients will exhibit airway involvement including subglottic stenosis, tracheobronchomalacia and tracheobronchial stenosis. Thorax computer tomography (CT) and bronchoscopy reveal major airway collapse, thickening of the trachea and proximal bronchi, thickened calcified cartilaginous rings and tracheal nodularity. The posterior tracheal membrane is normally spared [35]. Usually, specific clinical criteria and bronchoscopic evaluation of airway are enough to diagnose of RP. If a biopsy is performed, inflammatory changes in the cartilage are normally present. Medical management of RP consist in control the inflammatory process with steroids combined with methotexate, azatriopine or cyclophosphamide. Some studies propose the use of immunomodulatory therapies as etanercept, infliximab or rituximab [39, 40]. Interventional bronchoscopy procedures such as dilating, and stenting may help in specific cases [41].

Tracheobronchopatia osteochondroplastica (TO) is a rare benign disease with unknown origin that affects the cartilage rings of the trachea and, in lesser extent, the main bronchi. It occurs over the age of 50 years, and it is characterized by cartilaginous or osseous nodules projecting into the airway lumen, causing serious deformity. In 10% of the cases, the tracheal lumen can be reduced up to 50%. CT scans and bronchoscopy show densely calcified cartilage nodules. If the appearance is typical, no biopsy is needed. TO is a slow benign disease and rarely complicates. In cases of more serious degrees of stenosis, endoscopic laser ablation or stent application may be required [41].

Tracheobronchomalacia

Tracheobronchomalacia (TBM) is a condition characterized by diffuse or localized loss of rigidity of the trachea and mainstem bronchi, leading to airway collapse. If the affected portion is intrathoracic, the airway obstruction will be accentuated during expiration. More rarely, the affected portion is extra-thoracic and is more accentuated during inspiration. Congenital TBM is described more frequently in children and indicates a defect of the tracheal wall. The disorder can persist into adult life

and is designated idiopathic giant trachea or Mounier-Khun syndrome. TBM may also develop along with other congenital conditions such as cystic fibrosis, Marfan syndrome, Ehlers-Danlos syndrome or congenital trachea-esophageal fistula [42]. Acquired TBM is associated with a variety of conditions. Prolonged endotracheal intubation and tracheostomy trauma are the most frequent, but head and neck surgery, trauma, radiotherapy or inflammatory conditions must also be considered [43, 44]. The diagnosis is based on dynamic Three-dimensional CT images and direct bronchoscopic visualization to confirm significant narrowing of airway lumen during forced breathing manoeuvres [45]. Applying continuous positive airway pressure has been proposed as a possible treatment [44]. In patients with diffuse TBM, a silicone stent placement should be considered in preparation for a more definitive treatment such as tracheoplasty. Majed et al. propose a self-expandable metallic stents (SEMS) trial as a bridge to surgery [46]. Other surgical approach consists in restoring the C shape of the cartilages which is lost in TBM attaching longitudinal strips of splinting material along the membranous walls of the intrathoracic trachea [47].

Tumours

Both malignant and non-malignant tumours may cause CAO, either from direct extension of a locally advanced disease or from extrinsic compression. Primary tracheo-bronchial tumours are infrequent and include adenoid cystic carcinomas, bronchial carcinoid tumours, primary squamous cell carcinoma of the trachea and adenocarcinoma of the trachea [48]. Much frequent are metastases from primary lung cancers. Metastasis from breast, renal, thyroid, ovarian, uterine, testicular carcinomas and melanoma may also occur. It is estimated that 20–30% of patients with primary lung cancer will develop CAO; of those, 35% will die in consequence of asphyxia, hemoptysis and post-obstructive pneumonia [49]. Anatomically, malignant CAO is divided into 3 groups: intra-luminal, extrinsic compression and mixed. The purpose of this classification is based on the type of interventional assessment. The interventional bronchoscopy with palliative intent should be considered in these patients which may benefit from measures to relieve dyspnea [2, 50]. Recurrent papillomatosis, resulting from infection of the human papilloma virus, produces lesions with polypoid appearance which can affect larynx, trachea, and bronchi. Endoscopic interventions are an important component, as recurrence is common [51].

Extrinsic compression

Most frequently extrinsic compression in children arises from abnormalities of the aortic arch and are defined as

vascular rings. Double aortic arch and right sided aortic arch with aberrant left subclavian artery are the most common. Compression of the trachea by large aortic or innominate artery aneurysms may occur. Mediastinal masses rarely cause serious airflow limitations. Approximately 40% of mediastinal masses are malignant and 25% are cystic [35]. Thymic neoplasms and lymphoma, both Hodgkin's and non-Hodgkin's, are also common, but neurogenic tumours and teratomas must be considered.

An analogous but rare situation can be caused by the tumefaction of mediastinal lymph nodes due to metastatic or infectious diseases. Involvement of the trachea and left side bronchus by advanced esophageal cancer is common and is associated with a poor prognosis. Retrosternal extension of diffuse goiter may cause extrathoracic or intrathoracic airway obstruction [52]. Laryngoceles, saccular cysts and parathyroid cysts may be also implicated in CAO [53]. Airway narrowing due to osteophytes has been reported [54]. Postpneumonectomy syndrome occurs due to a compression of the left main bronchus between the aortic arch and left pulmonary artery, following a right pneumonectomy [55]. Significant CAO can occur from other infectious or malignant diseases like hematomas, abscess formation, empyema, or other expanding lesions. In most cases the treatment should address the underlying cause, but urgent intervention may be needed in case of respiratory failure. Most of the times this includes airway stent deployment to restore and maintain luminal patency [56, 57].

Other causes

Idiopathic tracheal stenosis is a rare inflammatory process that develops frequently in the subglottis or in the upper third of the trachea. It occurs mainly in women, suggesting an important role of estrogens [58]. Other authors suggest that it may be associated with gastroesophageal reflux [59].

Clinical presentation

CAO is much less common compared to other lower airway diseases. However, the symptoms may be identical, which may lead to misdiagnosis. Because these symptoms can be insidious CAO may be obscured for a long time resulting in a delayed diagnosis and worst outcome. Clinical presentation will depend on the location, extension and degree of obstruction, speed of progression and underlying disease. Other conditions, such as the patient healthiness, will have an important role in progression and outcome. The main symptoms are dyspnea and stridor occurring in 54% of patients as initial complaint [45]. These manifestations are more prominent during exercise but may progress to dyspnea at rest. The first usually occurs when the lumen is reduced to 8 mm

and the second when it reaches less than 5 mm. Cough, wheezing, diminished sputum clearance and recurrent infection will emerge when it reaches this level of severity. Unlike wheezing, stridor occurs predominantly during expiration and is emphasized by hyperventilation. If the obstruction is mainly thoracic, both inspiratory and expiratory stridor will appear. Hoarseness can be heard and could be a signal of laryngeal involvement. Less common is an initial presentation with acute respiratory distress.

Diagnostic evaluation

When CAO is suspected, clinical investigations to look for the underlying diagnosis include, in the first instance, thorax CT and blood tests (inflammatory markers, autoimmune diseases), lung function tests, other imaging exams [X-ray, CT, magnetic resonance imaging (MRI)] and bronchoscopy. Blood tests are frequently normal or with nonspecific changes, but inflammatory markers, white cell blood count or auto-immune screen can identify underlying diseases [35]. As the symptoms, physiological and measurable abnormalities in lung function tests frequently do not become evident until the airway lumen is < 8 mm, which corresponds to an obstruction of more than 80%. In these cases, a variable extra-thoracic obstruction shows flapping of the inspiratory loop, intrathoracic obstruction shows flapping of expiratory loop and fixed obstructions reveal flapping in both. On the other hand, if the cause of CAO is a lung tumour, an abnormal lung function test could not be adequately valorised because of the prevalence of lung cancer in chronic obstructive pulmonary disease (COPD) [60], whereas the lung function tests are usually anomalous. Unless the cause of CAO is an extra-thoracic compression caused by a vascular abnormality or tumour, X-ray usually gives few and unspecific information. The thoracic CT with tracheal protocols is the procedure of choice for imaging the upper airway, with a sensitivity of 97% [35]. With standard chest protocols, tracheal disease is easily underestimated. CT allows to identify the precise anatomical location of the lesion, its characteristics and extension, including the visualization of patency of the distal airway. Using an axial view, it is possible to evaluate the degree of stenosis. Extremely thin slices allow 3-D reconstructions, especially useful to provide pre-management volumetric analyses. Dynamic CT is effective for the diagnosis of TBM. MRI has usually limited value although it can be used to access the surrounding mediastinum, mostly the vascular structures [35]. A direct view of the airway is provided by both flexible and rigid bronchoscopy. They allow to directly observe the extension of airway narrowing, the characteristics of the mucosa, and the specific location. Samplings can be collected for microbiological

and cytologic analyses. Because of the risk of precipitating acute and complete obstruction, flexible bronchoscopy should be performed judiciously and by trained physicians with appropriate equipment [61].

Although many different causes of CAO and a relatively well-defined protocol approach, its management is not straightforward nor consensual and the long-time outcomes are far from satisfactory in some cases, as shown in Table 2

Discussion on CAO management and the role of tracheal stents

CAO cases must have a multidisciplinary team discussion to cover all possible treatments and to select the best therapeutic options. Therapy must be addressed according to the pathologic findings and to the patient's health status. Definitive management may include both medical and surgical interventions. Possibilities embraces observation, drug therapy, radiation, tracheostomy, endoscopic treatment, and surgical management. Endoscopic treatment includes dilatation, laser resection and airway stenting [62]. As described before, several classification systems can be used to predict the success of the intervention, although further studies are needed to confirm its usefulness. In non-symptomatic or minimal symptomatic patients with stable and non-progressive stenosis, close observation is considered an adequate approach [63]. In the decision of management, according to Gallucci et al., the most important differentiation to be made is between simple and complex stenosis as it determines the success or failure of the endoscopic intervention [64]. Complex tracheal stenosis is associated with extensive scarring (> 10 mm) and varying degrees of cartilage involvement, circumferential contraction scarring, or tracheal stenosis associated to *malacia* or inflammation. Tracheal sleeve resection is considered to be the first-option and definitive treatment in complex tracheal stenosis [65]. Nevertheless, bronchoscopic management in selected patients is reported to have a high success rate [66, 67]. Thus, a short segment, membranous, benign, concentric and not involving cartilage stenosis can be considered for endoscopic treatment as first option [30]. It is also an option for patients with surgical ineligibility due to the stenosis characteristics, infection, or poor medical status. Complex stenosis may require stent placement. Endoscopic intervention can also work as a bridge for surgical treatment [64]. In the first clinical case, the patient had a complex stenosis. Although surgery could not be performed because of comorbidities, the decision based on the difficult mechanical ventilation, easily reaching high plateau pressures with low tidal volumes, was taken with the purpose of being only temporary. Prior to this scenario, the patient manifested mild

Table 2 Examples of CAO from different etiologies

	Patient 1 (Fig. 2)	Patient 2 (Fig. 3)	Patient 3 (Fig. 4)
Identification	♀ 81 years old	♀ 66 years old	♂ 61 years old
Etiology of CAO	Post orotracheal intubation	Extrinsic compression caused by a thoracic aorta aneurism	Tumoral extrinsic compression
Medical history	Chronical renal failure Pulmonary hypertension LICU hospitalization 2 years before	HIV+ Pulmonary tuberculosis 30 years before	Smoker with 75 pack-year history
Clinical manifestations	Progressive dyspnea	Progressive dyspnea Pleuritic chest pain	Pleuritic pain Dyspnea Hoarseness
Diagnostic evaluation	Expiratory stridor Lung functions tests normal Thoracic CT scan showed mid-tracheal stenosis > 50% Flexible bronchoscopy confirmed diagnosis	Thoracic CT scan showed thoracic aorta aneurism compressing tracheal and main left bronchus Flexible bronchoscopy confirmed diagnosis	Thoracic CT scan showed voluminous adenopatic conglomerate compressing trachea causing a narrowing of > 80% of trachea and LMB Flexible bronchoscopy showed mucosal invasion and confirmed stenosis Bronchial biopsies revealed small cell lung cancer
Discussion and management	No conditions for surgery Dilatation with rigid bronchoscope unsuccessful Introduction of a silicon Dumon Stent	A silicon Dumon stent was placed in LML as a bridge to endovascular correction of the aneurism	Radiation and chemotherapy were pursued with disappointing results Y silicon Dumon stent was placed as a palliative intention
Clinical evolution	Immediate relief of dyspnea and improvement of QOL Complicated after 1 month with stent migration and infection	Difficult extubation after endovascular correction Recurrent mucus plugs with need of repeated bronchoscopies Complicated with voluminous fistula from LMB to mediastinum	Recurrent mucus plugs with need of repeated bronchoscopies A SEMS was placed instead of Dumon stent trying to avoid the repeated mucous obstruction
Outcome	Died with multi-organic shock from pulmonary origin	Died with infectious complications	Died due to progression of the underlying disease

symptoms for 2 years, and at the time, the option was the maintenance under close observation but without intervention. Interventional bronchoscopy options include the use of both rigid and flexible bronchoscopy. Both have advantages in different scenarios and should be seen as complementary procedures [62, 68]. Guidelines do not favour rigid over flexible bronchoscopy, although the procedure with rigid bronchoscopy is considered safer. Through the flexible bronchoscope thermal and cryotherapy, balloon dilatations, micro-debridement and stenting can be performed. Several thermal techniques can be used [61]: (a) electrocautery, one of the most available and cheap, achieves an immediate outcome but requires a skilled user, also requires contact and FiO_2 should be decreased; (b) argon-plasma coagulation is a non-contact technique capable to burn tissue and control bleeding. It has a penetration depth of 2–3 mm, which makes it a safe but longstanding technique; (c) LASER has supplanted other thermal techniques because of its minimum energy delivery and cut precision. CO_2 laser is more precise but has a limited coagulation effect compared with Nd:Yag laser [69]. In contrast to heat therapies, contact cryotherapy has been also described as useful in

CAO. It is exclusively used for endobronchial lesions and may require repeated procedures. Rigid bronchoscope debulking of lesions and scar tissue is also simple, effective and accomplished by suction under direct telescopic guidance [62, 68].

Several drugs used to prevent the complications associated to endoluminal wound healing have been assessed in the last decades and are still under investigation. The most used as co-adjuvant are corticosteroids [70] and mitomycin-c. A literature review in 2010 concluded that mitomycin-c may postpone but not prevent the recurrence of stenosis [71].

For patients with primary lung cancer which develop CAO, besides all interventional bronchoscopy techniques mention above, in some cases, additional stenting is required [68]. Airway stents are tube-shaped devices that are inserted into the airway to maintain patency. Stent placement is indicated in both intraluminal and extraluminal significant CAO and can be used in benign and malignant disease. Placing an airway stent is a difficult decision, and the operator must consider the benefit of its application, by preventing the airway re-occlusion, and also its disadvantages, mostly regarded with long-term

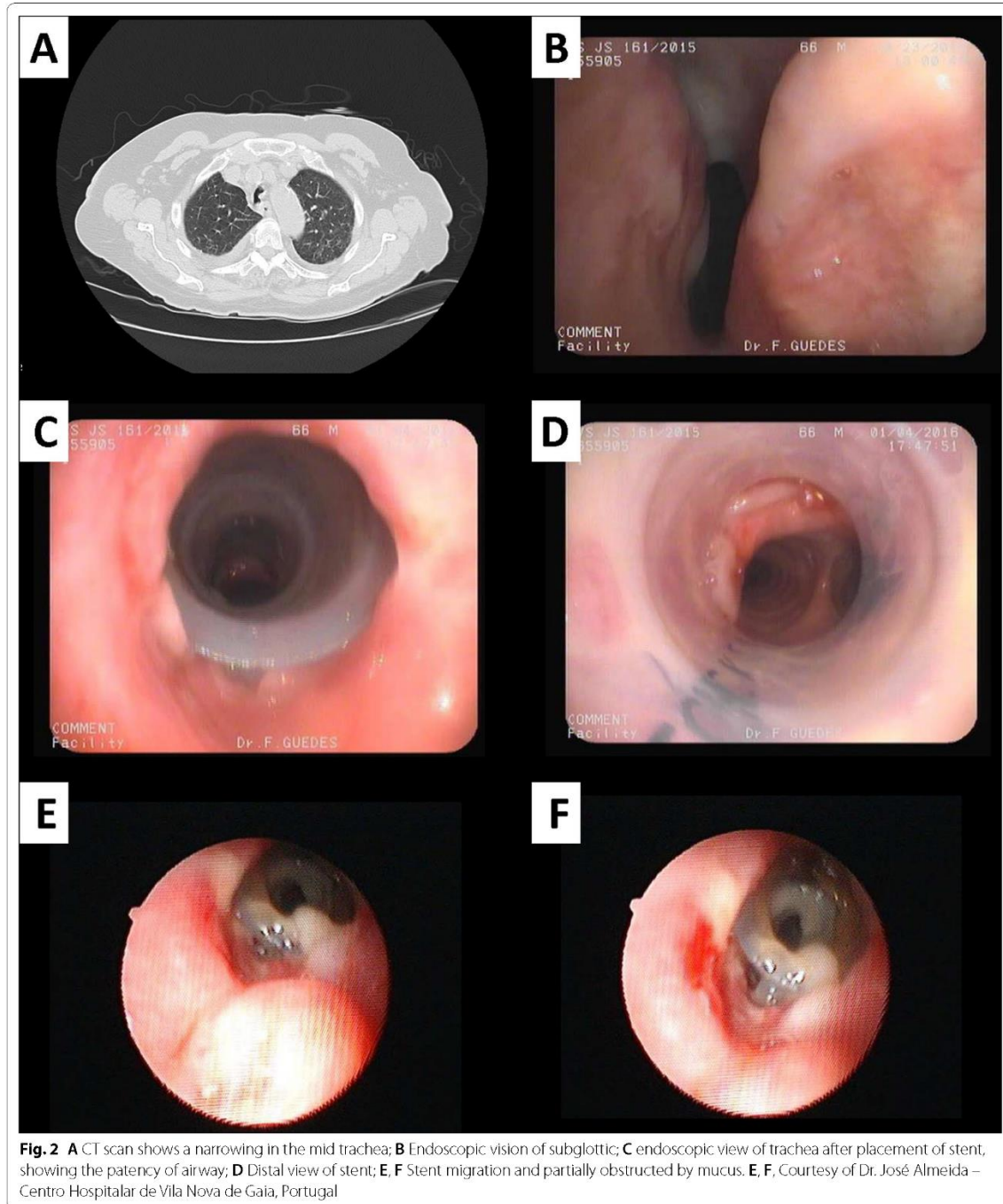


Fig. 2 **A** CT scan shows a narrowing in the mid trachea; **B** Endoscopic vision of subglottic; **C** endoscopic view of trachea after placement of stent, showing the patency of airway; **D** Distal view of stent; **E, F** Stent migration and partially obstructed by mucus. **E, F**, Courtesy of Dr. José Almeida – Centro Hospitalar de Vila Nova de Gaia, Portugal

associated complications [72, 73]. There are two main types of airway stents concerning the material they are made of: tubular stents made of silicone and SEMS.

Metallic stents come with or without a silastic or polyurethane covering, which are used to minimize tumour overgrowth and tissue granulation [74]. The development

of granulation tissue is one of the common occurrences in uncovered or partially covered stents, and its coverage was developed to limit this disadvantage. Indeed, in covered stents, granulation tissue tends to only be located at the extremities and its progression into the stent is rare [75]. Both types of stents can be placed through rigid bronchoscopy, although SEMS could also be placed with flexible bronchoscopy. As a foreign body, there are complications associated to endotracheal stents (Table 3).

Metallic stents, especially non-covered, should be considered permanent because of extensive granulation tissue and epithelialization. The longer the stent remains, the more difficult is its removal. Silicone stents are more easily replaced or removed [76]. Based on 2005 Food and Drug Administration warning, metallic stents are not recommended in benign airway strictures because of the reported extensive granulation tissue and stent fracture. Considering these recommendations, they should not be placed in patients where removal is considered in the future [32, 77]. In the 3 cases presented, disrupt of mucociliary clearance and mucus plugging were detected as clinical impairments. Infection aroused in 2 cases and migration in the first clinical case. Fistulation is a serious problem, and happens because of friction between stent and mucosa [78]. In TBM, stent insertion should be only applied in patients with symptomatic and severe obstruction (>60%) and non-responsive to positive pressure ventilation. QOL and functional status are improved in short term in 70% and dyspnea in 90% of patients after silicone stenting [79, 80]. For patients with severe, diffuse and symptomatic TBM who had repeated stent migration, external stent fixation, tracheostomy or Montgomery T-tubes are a good option to improve symptoms or as a bridge to a more definitive treatment [79]. As the ideal stent is still not available, physicians should consider the full assortment of silicone and SEMS. The main advantages of silicon stents rely on their easy removal and repositioning, less granulation tissue formation and absence of tumour ingrowth. On the other hand, rigid bronchoscopy is needed for insertion, and they are more suitable to migrate. SEMS are easy to insert, as it can be performed by flexible bronchoscopy. Following, they are more stable and more effective in extreme extrinsic compression. The main disadvantages are the impossibility of repositioning or removal and tumour ingrowth [50], especially in the case of uncovered metallic stents. The ideal stent should be simple to insert, easily removed, capable to resist compression and to allow

clearance of secretions, as well as easy to nail and avoid migration. New custom-made and bioabsorbable airway stents made of different biomaterials are under investigation and have been placed in humans. Polydioxanone is the one that has been most often used and for the longest period, with report of a patient successfully treated and free of intervention for 44 months [81, 82]. Other biomaterials are under investigation, as the polylactic acid, polyglycolic acid, polycaprolactone and polyamide [83, 84]

Outcomes

In malignant diseases, there is no controlled trial evidence regarding tracheal or bronchial intervention due to ethical concerns. However, it is consistently demonstrated in the literature that interventional bronchoscopy procedures with stenting improves symptoms of dyspnea, QOL and lung function [2, 85, 86]. Long term favourable outcomes are achieved with a combination of laser and gentle dilatation in benign tracheal stenosis [32, 64, 87]. Although the use of stents in benign clinical conditions is not consensual, a considerable number of supportive literature describes promising results [88]. According to Monnier et al., using the Myer-Cotton grading system, the success of interventions with CO₂ laser is 92% in grade I, 46% in grade II and 13% in grade III [17]. Lim SY and colleagues, concerning PIS, demonstrated that the time relapsed between stenosis and first intervention is important, with positive outcomes of 90% for those with intervention within 6 months and 61% for those with longer intervention period [89]. According to Galluccio et al. the use of silicone stents as part of endoscopic intervention, achieved airway patency of 96% in simple stenosis and 69% in complex stenosis [64]. When stents are used in TBM, the complication rates are higher when the disease occurs with loss of cartilaginous support [64].

The role of tracheal tissue engineering in CAO

Airway tissue engineering is a field of Regenerative Medicine with the goal of developing biological substitutes that can restore, maintain, or improve tissue functions. When stenting is not possible, there are other options (Table 4) with pros and cons that range from autografting and allografting to the use of prosthetic materials. Prosthetic options have some limitations such as development of inflammation and incomplete host tissue integration. In the case of grafts, autografts are good options due to the absence of rejection and inflammation after application, however the availability of donor tissue is a

(See figure on next page.)

Fig. 3 Sequence of happenings observed in thoracic CT scan and in bronchoscopy: **A** thoracic aorta aneurism with tracheal contact; **B** 3-D reconstruction of thoracic aorta aneurism; **C** carina with signs of extrinsic compression; **D** stent in left main bronchus; **E** total occlusion of stent with mucus; **F** patency of stent post laborious cleaning; **G, F** evidence of fistulization from LMB to mediastinum, contacting directly to aneurismatic sac

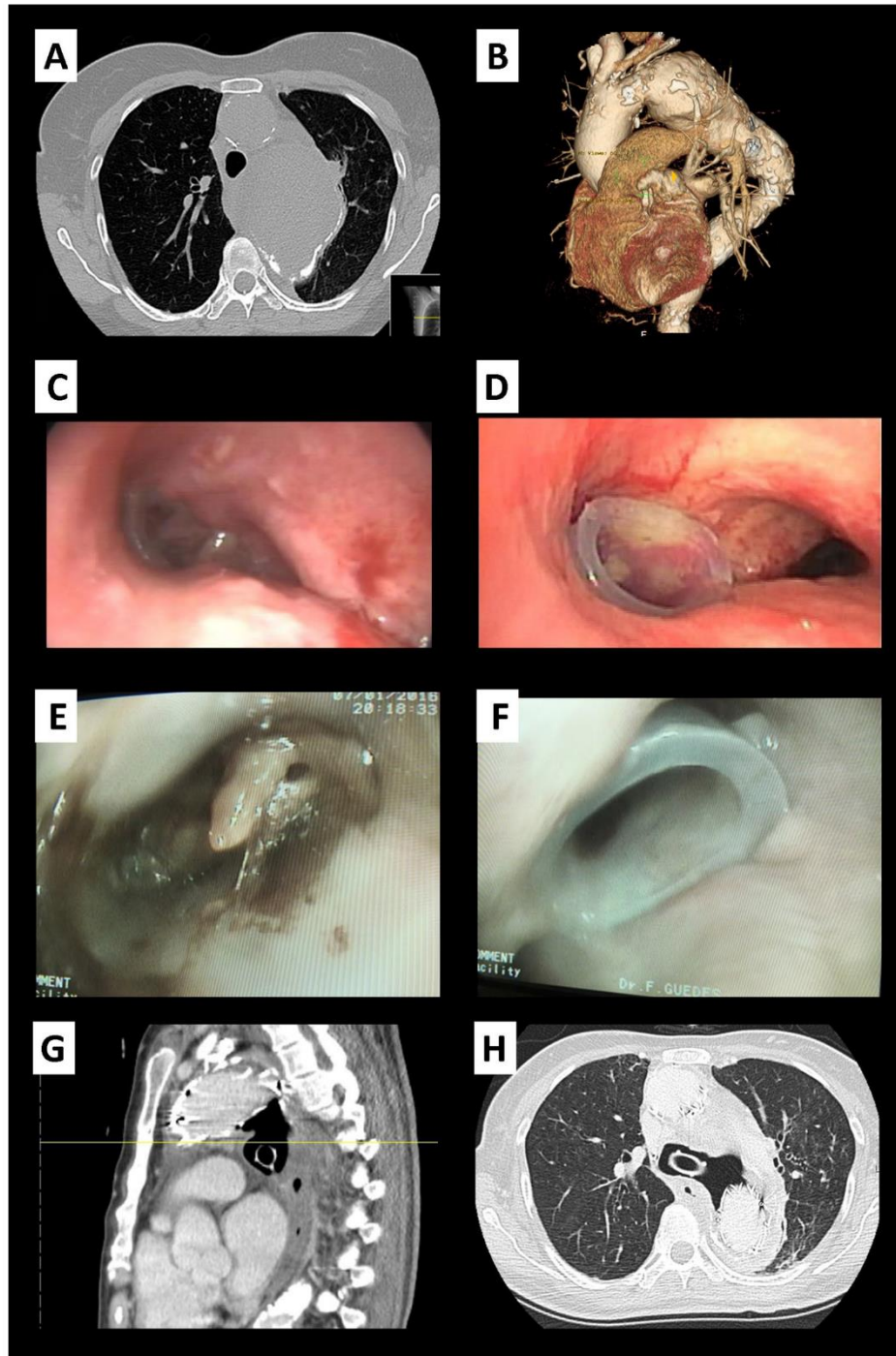


Fig. 3 (See legend on previous page.)

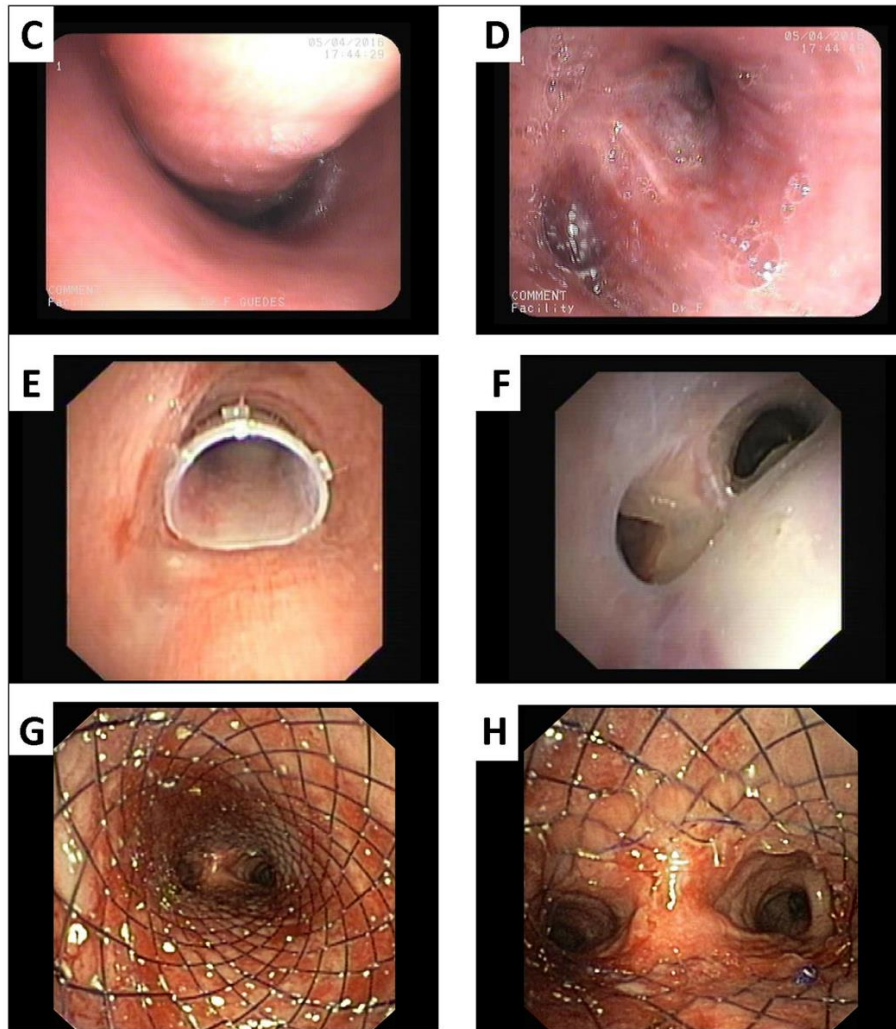


Fig. 4 **A, B** Thoracic CT scan showing voluminous adenopathic conglomerate in mediastinum compressing trachea and both main bronchi; **C** Endoscopic view of extrinsic compression of trachea; and **D** Left main bronchi; **E** Proximal view stent in trachea; **F** Inside Y stent showing left and right portion of stent

limitation. Furthermore, allografts must be associated with immunosuppressive drugs. To overcome these constraints, alternatives associated with tissue engineering have been developed in recent years. In these approaches, therapies are based on the use of three essential elements: cells, growth factors and scaffolds in distinct combined approaches [90]. Different cell populations have already been extensively applied in this field. Tissue-engineered tracheal bioprosthesis coated with chondrocytes and polymers were used and implanted in vivo, and

Mesenchymal stem cells (MSCs)-derived chondrocytes have also been considered as alternative cell sources, particularly in promoting tracheal cartilaginous tissue regeneration [91]. These cells allow biomechanically stable seeded scaffolds, but chondrocytes become infeasible after implantation. Epithelial cells were explored as an alternative, and scaffolds seeded with these cells demonstrated increased viability and promoted better tracheal regeneration in vivo [92]. To maximize the advantages of each cell type, a combination of epithelial

Table 3 Complications associated with silicon and metallic stents

Potential complications of endotracheal stents	Silicon stent	Uncovered metallic stent	Covered metallic stent
Stent migration	++	–	–
Disruption of mucociliary clearance	++	–	+
Mucous plugging	++	–	+
Recurrent infection	+	+	+
Cough	+	+	+
Granulation tissue formation	+	++	+
Stent Fracture	–	++	+
Fistula formation	+	++	+

++ Very usual, + Usual, – Unusual

Table 4 Advantages and disadvantages of Tissue Engineering techniques currently being explored for tracheal reconstruction

	Advantages	Disadvantages
Prosthetic material	Physical and functional replacement of the trachea	Risk of inflammation Incomplete host tissue integration
Autografts	Absence of rejection Absence of inflammation	Donor tissue availability
Allografts	Availability of donor tissue	Risk of intense inflammation Risk of organic rejection Dependence on immunosuppressive drugs
Cell-based therapies	Can be used in combination with prostheses, scaffolds, and growth factors Promotion of in vivo tracheal regeneration Specific cell differentiation	Inconsistent results The ideal cell type to be used has not yet been identified Risks of organic rejection Risks of genetic instability Risks of tumorigenic differentiation Difficulties in isolation and cell expansion
Scaffolds	Availability of materials with synthetic and natural origin Can be used in combination with cells to maximize their benefits	High manufacturing requirements Requirement of precise physical characteristics to guarantee cytocompatibility, biocompatibility, biodegradability, and absence of local and systemic toxicity
Growth factors	Can be used in combination with cells to maximize their benefits Influence cell proliferation and differentiation Ensure cellular growth and nutrition Modulation of the pro-regenerative environment at the site of injury and site of administration	Risk of adverse reactions Challenging manufacturing techniques Compromised product stability Inconsistent results

cells cocultured with articular chondrocytes was tested, and good in vitro performance, as well as positive tissue regeneration in vivo, were observed [93]. The co-culture of bone marrow derived MSCs with fibroblasts and endothelial cells of the cartilage and umbilical vein, their aggregation in spheroids and subsequent 3D printing in an architecture similar to native tracheal tissues allowed the development of layers of epithelial tissue with good viability and vascularization [94]. The culture of Induced pluripotent stem cells in an air–liquid interface system allowed their differentiation into ciliated epithelial cells and non-ciliated club cells, making them fair candidates for application in tracheal regeneration [95]. Tissue

engineered airway grafts seeded with isolated cells from umbilical cord blood, amniotic fluid or adipose tissue revealed to not be subjected to immune rejection and can provide mechanical characteristics compatible with tracheal physiology. Finally, the use of cells from fetal tissues allowed the synthesis of a cartilage rich in glycosaminoglycans, β -elastin and smooth muscle cells, with epithelial growth, smooth muscle having cilia movement and no ectopic growth or tumour development [90, 96]. The scaffolds to be used to promote regeneration of tracheal tissue must have some specific characteristics, namely: design that mimics the anatomical shape of the trachea or defects to be filled; mechanical strength and flexibility

identical to native tracheal tissue to prevent collapse; porosity that allows good vascularization and cell proliferations. In addition, the scaffold must be biocompatible, biodegradable, non-toxic and non-immunogenic, to ensure a good cytocompatibility and to not trigger organic reactions and rejections [97]. To guarantee these characteristics, the scaffold manufacturing methods as well as their material are important decisions to consider. Various natural and synthetic materials have already been used, either alone or in combination, including silk, gelatin, polyglycolic acid, collagen, polycaprolactone and decellularized matrix [90]. Growth factors influence cell proliferation and differentiation by modulating the pro-regenerative environment at the site of administration, also ensuring cell growth and nutrition. The list of growth factors already used in tracheal tissue engineering include insulin-like growth factor, transforming growth factor, granulocyte colony-stimulating factor, vascular epithelial growth factor, basic fibroblast growth factor, epithelial growth factor and platelet-derived growth factor [90]. These growth factors can be administered both exogenously and loaded on the scaffold, but while the first approach is more advantageous in the case of minor injuries, endogenous administration is most appropriate in significant tracheal damages [98]. The best combination of cells, scaffolds and growth factors has not yet been defined, but tissue engineering has the potential to allow the development of alternative methods for effective tracheal reconstruction. The goal of these technologies is the development of a fully autologous engineered trachea that allows to overcome the limitations of the current synthetic and grafts options.

Conclusion

CAO represents an important clinical impairment caused by benign or malignant diseases. Surgical management remains the preferred approach but there are situations in which, whether due to patient limitation or the nature of the pathology, intervention bronchoscopy, with its accessory techniques such as laser, cryotherapy, mechanical dilation, or debridement, play a preponderant role. When it comes to the choice of a stent, several considerations must be considered. Silicone stents are cheaper, easily retrievable and can be repositioned as much times as necessary. On the other hand, they need for rigid bronchoscopy to be handled, are more suitable to migrate and interfere with mucociliary clearance. Metallic stents can be placed by flexible bronchoscopy, are usually more adaptable to an irregular surface and allow reepithelization. The same way, the tumour ingrowth can happen which make them almost impossible to be securely removed. Although an exciting new generation of personalized airway stents along with stem

cell therapy is expected, this reality is far away from being achieved. A recent evolutionary step was made when manufacturers offered modifications of their products according to patient's needs, with rapid prototyping 3D printing techniques, allowing stents to be tailored to the individual's airway in days or even hours [99]. If on one hand 3D printing technology could become accessible for physicians, on the other hand there are no certified manufacturers nor a direct access between parts. Maybe a creation of a software easy-to-use between hospitals and manufacturers could provide that answer but there are still ethical and legal issues to overcome [100].

Abbreviations

c-ANCA: Cytoplasmic autoantibodies; CAO: Central airway obstruction; COPD: Chronic obstructive pulmonary disease; CT: Computer tomography; LMB: Main left bronchus; LTS: Laryngo-tracheal stenosis; MRI: Magnetic resonance imaging; MSCs: Mesenchymal stem cells; PITS: Post-intubation tracheal stenosis; PSST: Post-tracheotomy tracheal stenosis; QOL: Quality of life; RP: Relapsing polychondritis; SEMs: Self-expandable metallic stents; TBM: Tracheobronchomalacia; TO: Tracheobronchopatia osteochondroplastica.

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Authors' contributions

FG, MVB, ACS, RDA, AB and ACM conceived the project and the main conceptual ideas and contributed to the writing of the manuscript. FG performed the procedures of the clinical cases. MVB and RDA designed the figures. ACM and AB supervised the discussion. All authors read and approved the final manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Chapter II

Bronchoscopic Techniques in Mainland Portugal

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Introductory note

“On 31st December 2019, the WHO China Country office was informed of cases of pneumonia of unknown etiology detected in Wuhan City. On the 3rd of January 2020, a total of 44 patients with pneumonia of unknown etiology have been reported to WHO by the national authorities in China. Of the 44 cases reported, 11 are severely ill...”. This is how the World Health Organization reported to the world on its webpage on 5th January of 2020 the beginning of the great pandemic that we are still living today.

Coronavirus disease 2019 (COVID-19) is an infectious disease which emerged in China at the beginning of December 2019 and it's caused by a virus named SARS-Cov-2 (1). In Portugal, the pandemic started officially on March 2nd, when the first diagnosis was made in Centro Hospitalar e Universitário do Porto (CHUP), Oporto.

SARS-Cov-2 has a unique capacity for human-to-human transmission and international air travel facilitated the rapid dissemination on an unprecedented scale worldwide (2).

The diagnosis screening of the disease is made by Polymerase Chain Reaction (PCR) analysis with Reverse Transcriptase (RT-PCR) of nasopharyngeal swab. It is known that in patients with RT-PCR negative nasopharyngeal swab, bronchoscopy provides an increase in diagnostic sensitivity through deep respiratory tracts (3). Besides the diagnosis of SARS-Cov2 infection in some situations, such as patients with severe disease and especially those in Intensive Care Units (ICU), bronchoscopy may be necessary for diagnostic and therapeutic purposes such as atelectasis, haemoptysis, difficulties in mechanical ventilation or exclusion over infection.

Most adverse pathogeny of COVID-19 include major pulmonary inflammation, cytokine storm, Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS) and pulmonary oedema. During first months, several treatments were studied and tested, like chloroquine, hydroxychloroquine, Remdesivir or other drugs normally used to autoimmune diseases, corticosteroids, or plasma transfusion. The results never were enough consistent to any of them be considered a gold standard. Apparently preventing or attenuating the development of the cytokine cascade was the way for treating the COVID-19 serious disease (4). Cell-based therapies involving MSCs started to be used due their

immunomodulation and anti-inflammatory activity (5). MSCs modulate the effector function of immune cells, suppressing infiltrated cells and diminishing the oedema and helping to control ALI/ARDS (6). By that time, some expectations were made on treatment with MSCs.

In Portugal, our involvement in the first weeks was based on the experience of other European countries such as Italy and Spain where the pandemic started weeks before. Soon, we understood that major arrangements had to be performed in our Interventional Pulmonology units to avoid infection of healthcare workers during the procedures. In Italy, initially it was reported that 9% of COVID-19 cases were healthcare workers (HCW), while in Spain the rate of medical staff infected reached 26%, the highest in Europe. In Portugal, by 12th May 2020, 11.3% of infections occurred in HCW. Explanations for that high number of infected personnel were mostly two. First, the lack of proper personal protective equipment (PPE) at the beginning of the epidemic, when assisting both confirmed and suspected patients with COVID-19. Second, the duration of exposure to infected patients undergoing aerosol-generating procedures, such as non-invasive ventilation and bronchoscopy, directly resulting in an increased risk of transmission to HCW (7).

The IP department is considered a high-risk area, because of the procedures that are performed and with multiple staff involved. Structurally it is prepared to deal with occasional airborne infectious diseases, such as tuberculosis, but it was not prepared to systematically deal with high-risk cases that need additional resources. Thus, each IP unit had to rethink their administrative and logistic circuits in different areas, as well as the type and timing of performed procedures, to protect both HCW and patients (8). Moreover, as international health associations advocated, an infection-control program in healthcare settings had to be implemented, consisting of a three level hierarchy, including administrative, environmental and engineering controls, and personal protection equipment (9).

Given the absolutely need of creating a consensus document, the Portuguese Society of Pulmonology appointed me to chair a consensus group. As physicians, it is very important to know the panorama to decide which is the best treatment for our patients. That is how emerged the first study presented in this chapter.

Even nowadays, and although the direction of the recommendations tends to attenuate the level of care, based on current knowledge, all patients undergoing an IP procedure should be assumed as potentially infected with SARS-CoV2 as

proposed in the referred document. These recent changes in the bronchology field were expected to have a major impact on national interventional pulmonology units, so the Committee on Endoscopic Techniques of the Portuguese Pulmonology Society nominated a working group to conduct a national survey, to accurately characterize the Portuguese mainland panorama. Specifically, this survey aimed to quantify and qualify the human and technical resources of the Portuguese Pulmonology Departments, the specificities of the bronchoscopic procedures performed, and compare the reality before and after the COVID-19 pandemic. In this document, the problem of the CAO approach is also focused.



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SPECIAL ARTICLE

Recommendations for interventional pulmonology during COVID-19 outbreak: a consensus statement from the Portuguese Pulmonology Society



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Consensus statement

Abstract Coronavirus disease 2019 (COVID-19) is an emerging infectious disease caused by a novel SARS-CoV-2 pathogen. Its capacity for human-to-human transmission through respiratory droplets, coupled with a high-level of population mobility, has resulted in a rapid dissemination worldwide. Healthcare workers have been particularly exposed to the risk of infection and represent a significant proportion of COVID-19 cases in the worst affected regions of Europe.

Like other open airway procedures or aerosol-generating procedures, bronchoscopy poses a significant risk of spreading contaminated droplets, and medical workers must adapt the

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procedures to ensure safety of both patients and staff. Several recommendation documents were published at the beginning of the pandemic, but as the situation evolves, our thoughts should not only focus on the present, but should also reflect on how we are going to deal with the presence of the virus in the community until there is a vaccine or specific treatment available. It is in this sense that this document aims to guide interventional pulmonology throughout this period, providing a set of recommendations on how to perform bronchoscopy or pleural procedures safely and efficiently.

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Introduction

Coronavirus Disease 2019 (COVID-19), a new infectious disease that emerged in early December 2019 in Wuhan (China),¹ is triggered by a novel pathogen with phylogenetic similarity to what caused the severe acute respiratory syndrome (SARS) outbreak in 2003, and was called SARS-CoV-2.² Its capacity for human-to-human transmission and international air travel facilitated the rapid dissemination on an unprecedented scale to the rest of the world.^{3,4}

In Italy, the latest figures reported that 9% of COVID-19 cases were health care workers (HCW), while in Spain the rate of medical staff infected reached 26%, the highest in Europe.⁵ In Portugal, by 12th May 2020, 11.3% of infections occurred in HCW.⁶ There are at least two explanations for such a high number of infected personnel. First, the lack of proper personal protective equipment (PPE) at the beginning of the epidemic, when assisting both confirmed and suspected patients with COVID-19. Second, the duration of exposure to infected patients undergoing aerosol-generating procedures, such as non-invasive ventilation (NIV) and bronchoscopy, directly resulting in a significant increase in the risk of transmission to HCW.

The Portuguese Society of Pulmonology recently issued a set of recommendations for bronchoscopic procedures,⁷ shortly after the diagnosis of the first cases in Portugal. The document aimed to guarantee the protection of both patients and medical practitioners, and to ensure that the healthcare workforce would be conserved to fulfill their mission throughout the period. Since then, a significant amount of scientific evidence has been accumulated; so, the present document gives an update of the available literature, providing practical suggestions for pulmonologists undergoing bronchoscopy or pleural procedures in the setting of the current and post-pandemic phases.

Risk of transmission

Respiratory droplets comprise the main route of SARS-CoV-2 transmission, although airborne transmission is also possible through aerosol-generating procedures, such as bronchoscopy.⁸ One study during the H1N1 pandemic provided experimental evidence that bronchoscopic procedures increases more than 4 times the viral copy number per litre in positive air samples.⁹ While the heavy droplets rapidly settle, aerosol particles are much smaller (<5–10 µm) and

are dispersed in the air over extensive distances, posing a considerable risk of infection in enclosed spaces, specially if poorly ventilated.¹⁰

The contribution of asymptomatic carriers has also been subject of debate.^{11,12} A significant proportion of them have lung abnormalities on chest CT scans^{13,14} and a high level of viral shedding may be detected in presymptomatic patients,¹⁴ so it is likely that transmission occurs in the early stages of infection when patients are either minimally symptomatic or asymptomatic. Unrecognized patients pose a real challenge to infection control and, when not promptly handled with appropriate airborne precautions, are one of the most critical factors for SARS-CoV-2 infection spread in the healthcare setting.

Methods

The Portuguese Pulmonology Society appointed FG to chair this consensus group. Seven national IP specialists were selected based on their clinical expertise and different settings (university vs. non-university hospitals; state vs. private hospitals; pulmonologists vs. critical care specialists; ...). At the first online consensus meeting, attended by all members, a primary draft with several sections was created. This was shared online and further improved by written comments and suggestions. Then, each IP specialist was assigned a specific section presented in this document and was responsible for reviewing and evaluating the relevant available literature related to the topic. Electronic databases (Pubmed, OVID Medline and Embase, Web of Science, Cochrane Central Register of Controlled Trials) were used to search for the terms "COVID-19" OR "SARS-CoV-2" AND ("bronchoscopy" OR "interventional pulmonology" OR "thoracocentesis" OR "thoracocentesis" OR "pleural effusion" OR "pneumothorax" OR "rigid bronchoscopy" OR "thoracoscopy" OR "chest drain"). Position papers from major health organizations (US Centers for Disease Control and Prevention, European Centre for Disease Prevention and Control and World Health Organization) and important scientific societies (European Respiratory Society, European Association for Bronchology and Interventional Pulmonology, American Association for Bronchology and Interventional Pulmonology, World Association for Bronchology and Interventional Pulmonology and British Thoracic Society) were also reviewed.

In a second online conference the complete draft was evaluated by all team members and two working groups were created. They were responsible for discussing and revising different sections, and editing the text for consistency. Afterwards, the final manuscript was distributed to the consensus group members and assessed for final approval.

Adaptations of the interventional pulmonology (IP) department

Although there is still some heterogeneity in the definition and scope of “interventional pulmonology” (IP), it has become the most widely accepted term to describe the use of techniques for the diagnosis and treatment of a growing number of thoracic disorders.¹⁵

In the context of this document, the term IP is used to encompass the concepts of bronchoscopy (diagnostic or therapeutic), advanced bronchoscopy (flexible or rigid bronchoscopy and all its associated techniques), pleuroscopy (rigid or semi-flexible) and other simpler pleural techniques (such as thoracentesis, placement of thoracic drainage systems and indwelling pleural catheters). Though we acknowledge this wider definition of IP may be controversial, it covers all technical domains that most Portuguese pulmonologists need to address, and for the purpose of this document, it positions us to issue general recommendations. In the following subsections, specific scenarios of different technical specializations will be addressed in order to overcome this broader definition and to apply it better to individual settings.

The IP department is a high-risk area, given the type of procedures that are performed with airway manipulation and with multiple staff involved. Although this setting is generally designed to deal with occasional airborne infectious diseases, such as tuberculosis, it is not prepared to systematically assess high-risk cases that need additional resources, diminish productivity and effectiveness and generate a huge workload.

Thus, each IP unit must rethink their administrative and logistic circuits in different areas, as well as the type and timing of performed procedures, to protect both HCW and patients. Moreover, as international health associations advocate, an infection-control program in healthcare settings should be implemented, consisting of a three-level hierarchy, including administrative, environmental and engineering controls, and personal protection equipment (PPE).¹⁶ In the following subsections, each of the above listed hierarchic levels are briefly presented.

Administrative and organizational issues

Administrative and logistic measures are crucial to ensuring safety while still maintaining IP activity.¹⁷ Some general precautions include:

- All referrals and requests to the IP unit must preferably be made by telephone or digital means.
- Upon schedule and 24–48 h prior to arrival at the IP Unit, patients should be contacted by telephone and submitted to a pre-screening checklist that includes questions about 1) recent symptoms suggestive of COVID-19 (e.g. fever,

cough, chills, muscle pain, shortness of breath/difficulty breathing, headache, sore throat, loss of taste or smell); 2) contact with suspicious/confirmed SARS-CoV-2 cases; and 3) occupational exposure.

- Patients who have recent respiratory and infectious symptoms and/or chest imaging suggestive of COVID-19, should have their elective procedures postponed and rescheduled after all symptoms are resolved.
- On arrival at the IP Unit, all patients must be asked again for respiratory symptoms and have their temperature checked.
- If possible, all patients should have at least one negative RT-PCR for SARS-CoV-2 in the 24–48 h preceding the exam. In patients with a positive RT-PCR SARS-CoV-2, the decision to proceed with the intervention will be based on the urgency of the procedure (Chart 1 and Table 1).
- The IP unit should keep a record of deferred patients to reschedule their procedures according to the COVID-19 outbreak situation, as proposed in Table 2.

Environmental and engineering control

Physical space preparation

The design of strategies to minimize risks and a protocol fitting the characteristics of each specific Unit are crucial.¹⁸

- Reception, administrative, clinical and waiting areas should separate confirmed/high-risk patients from negative/low-risk ones. In addition, inpatients should be segregated from outpatients, either by time or physical location, to prevent cross infection.
- Specific circuits and written workflow plans must be prepared, covering the pre-procedural area, procedural room, post-procedural area, decontamination and reprocessing. The implementation of a flowchart with different areas and walking paths using a visual colour zone system can be useful: 1) red zone for contaminated areas; 2) yellow zone for transition areas, and 3) green zone for non-COVID-19 safe areas¹⁹ (Fig. 1A). These need to be formulated by internal elements from the IP unit with the cooperation of a multidisciplinary team of hospital members, including administration, engineers, and infection control board.
- A specific place to store and retrieve all items required for PPE should be defined inside the Unit.
- A designated area in the Unit should be selected, close to the procedural suite, for gowning and removal of all PPE, according to hospital protocol and standards, in order to reduce exposure to contaminated particles and droplets. When an anteroom is available, it may be used as an area for donning and doffing of PPE (Fig. 1B).
- Stations should be created to facilitate frequent hand hygiene and to distribute waste containers according to local infectious control recommendations. Posters and other visual aids should be placed at strategic locations around the intervention suite to act as reminders.
- Emergency procedures in COVID-19 positive patients should preferably be performed within the ICU environment, with controlled airway through cuffed endotracheal tube and assisted ventilation.

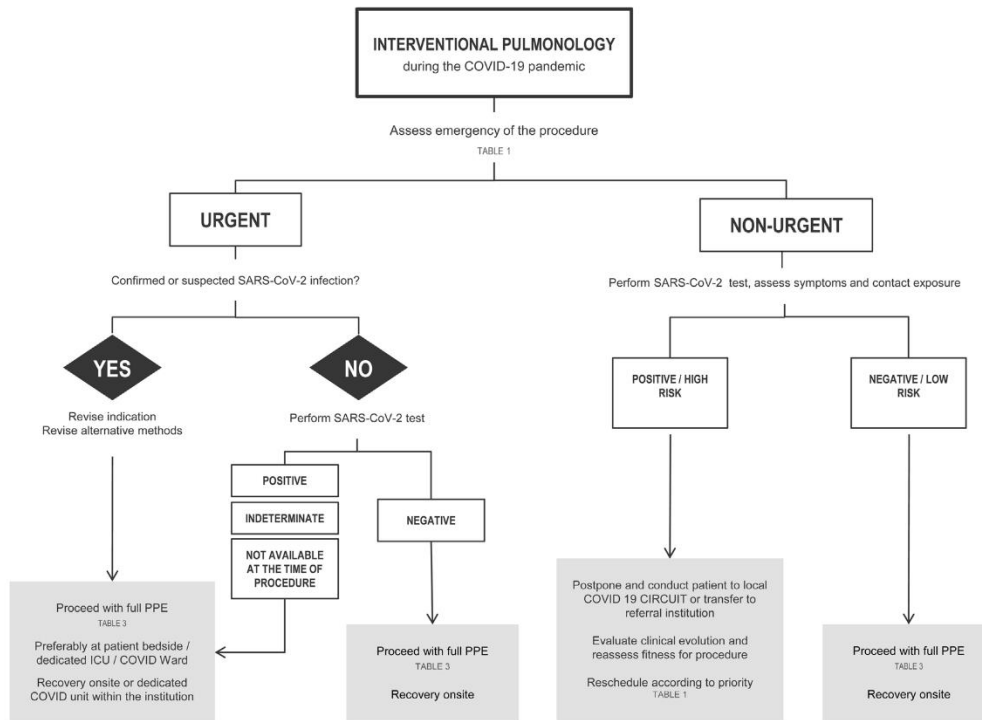


Chart 1 Proposed triage of IP procedures during the COVID-19 outbreak.

Table 1 Priorization of IP exams according to SARS-CoV-2 status and procedure urgency.

URGENT PROCEDURES		NON-URGENT PROCEDURES	
COVID-19 NEGATIVE/POSITIVE	COVID-19 NEGATIVE/POSITIVE	NON-DELAYABLE (<2-4 weeks)	DELAYABLE (≥4 weeks)
		COVID-19 NEGATIVE/LOW RISK	COVID-19 NEGATIVE/LOW RISK
COVID-19 POSITIVE/HIGH RISK	Massive hemoptysis with airway compromise	Lung cancer diagnosis and staging	Routine evaluation of tracheobronchial stenosis or endobronchial stent
	Acute foreign body aspiration	Increased mediastinal and/or hilar lymph nodes	Surveillance of lung transplant patients
COVID-19 POSITIVE/HIGH RISK	Severe symptomatic central airway obstruction	Suspected pulmonary infection in immunosuppressed hosts	Mild asymptomatic benign airway stenosis
	Suspicion of alternative (non-COVID-19) acute and severe infectious disease	Suspicion of pulmonary tuberculosis (after negative sputum)	Minor pulmonary CT abnormalities in an asymptomatic patient
COVID-19 POSITIVE/HIGH RISK	Airway management in difficult and non-delayable endotracheal intubation or complicated percutaneous tracheotomy	Interstitial lung disease in symptomatic and lung function deteriorating patients	Chronic cough evaluation (without chest CT or lung function abnormalities)
	Large and symptomatic pleural occupation (air, fluid, blood, pus)	Mild-to-moderate airway stenosis	Interstitial lung disease differential diagnosis in non-symptomatic or non-deteriorating patients
COVID-19 POSITIVE/HIGH RISK	Removal of copious secretions and mucus plugs	Mild persistent hemoptysis	Bronchoscopic lung volume reduction
	Possibility of superinfection (community acquired or nosocomial)	Pleurodesis or indwelling pleural catheter placement for recurrent and symptomatic pleural effusion	Bronchial thermoplasty
COVID-19 POSITIVE/HIGH RISK	Severe suspicious cases of COVID-19 that need to be confirmed by bronchoscopy and minimal bronchoalveolar lavage, after at least 2 negative/inconclusive nasopharyngeal RT-PCR SARS-CoV-2 tests		Minimal pleural effusion in asymptomatic patients

Table 2 Schedule of IP procedures according to the stage of COVID-19 pandemic.

COVID-19 in the community	IP Unit
Exponential increase of new cases	Urgent cases – only
Rapid increase of new cases	Urgent cases – only Elective but not delayable – evaluate case-by-case
Decrease in new cases	Urgent cases – full capacity Elective, but not delayable – full capacity Elective and delayable – resume partial capacity
Absence of new cases in the last 2 weeks	Resume all cases with full capacity

- Elective procedures should be reserved for COVID-19 negative patients (Chart 1 and Table 1). Nevertheless, these procedures should still be performed in a dedicated negative pressure room (see below, ventilation requirement) with strict isolation precautions and sufficient ventilation to avoid aerosol contamination.²⁰ If these requirements are not met in the bronchoscopy suite, then in a different venue, such as an operating theatre, isolation room or the ICU with negative pressure, if available.
- If negative pressure rooms are unavailable throughout the institution, a specific and dedicated room with adequate natural ventilation (see requirement below) may be an alternative, provided that appropriate intervals between procedures are reserved and that the suspected COVID-19 cases be programmed after all planned non-COVID daily activity, so that the unit can be carefully cleaned (following the disinfection policy) and ventilated.
- Keep the endoscopy room for procedures only (all other activities, such as planning, reporting and laboratory requisition should take place elsewhere).
- Suspected and confirmed cases of COVID-19 must be placed in an airborne infection isolation room with negative pressure before and after the procedure. Low-risk and negative patients can remain in the pre-procedural

and recovery area, if there is adequate room ventilation, protective equipment (e.g. surgical mask) and physical distance (>2 m) from other negative patients.

Ventilation

- Patient source control strategies, such as wearing a mask should be encouraged.
- Whenever feasible, it is recommended procedures are performed in a room that meets the ventilation requirements for Airborne Infection Isolation (AII), ensuring the dilution and removal of contaminated air. The preferred system is a negative pressure room with at least 12 air changes per hour (ACH) with airflow direction control (single-pass or recirculation systems with HEPA filtration). Alternatively, natural ventilation with an airflow of at least 160 L/s is an option.^{19,21}
- Enough time should be allowed to ensure that contaminated air is removed from the room before performing another procedure in the same room (depending on ACH and disinfection methods, but at least 30 min). Local adaptations must be considered according to the characteristics of the IP unit.

Cleaning and disinfecting patient care equipment and rooms

Endoscopes are considered semi-critical medical instruments according to the Spaulding classification.²² Recommendations from the Centers for Disease Control and Prevention (CDC) on reprocessing procedures should be followed. These include pre-cleaning, leak-testing, manual cleaning and visual inspection followed by disinfection/sterilization.

- A high-level manual disinfection or using an automated endoscope reprocessor is recommended.
- Proper storage and documentation are also an integral part of the reprocessing workflow.
- A pathway of contaminated equipment must be defined, as well as adequate packaging to minimize exposure (for example, a hermetic box).
- If available, disposable bronchoscopes are recommended for confirmed COVID-19 patients with clear advantages in

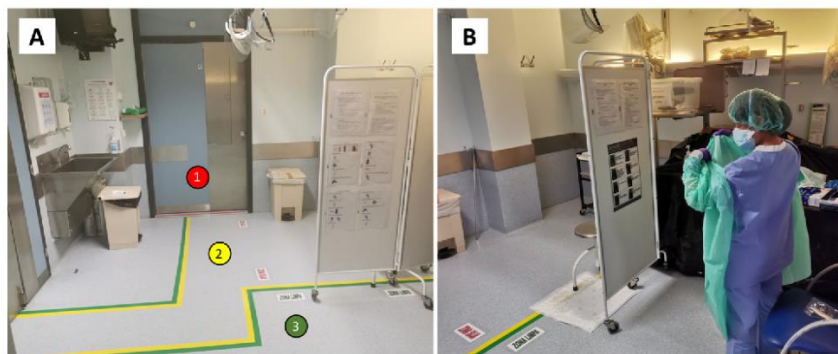


Figure 1 A. Implementation of specific circuits with colour visual zone system to distinguish contaminated (1, red zone), transition (2, yellow zone) and safe cleaned areas (3, green zone). B. Designated area for donning and doffing of PPE, where posters and other visual aids were placed strategically to act as reminders.

Table 3 Specifications for personal protective equipment during IP procedures.

PPE	Characteristics/specifications/standards	Observations
Gloves	☐ Single-use ☐ Waterproof ☐ Standard EN ISO 374-2:2014, 374-3:2014 e 374.5:2016 ☐ Double gloves: - first: long sleeved gloves - second: nitrile gloves	
Eye Protection	☐ Goggles with lateral protection ☐ Face shield	If not for single use, perform disinfection with ethanol base solution or 0.1% sodium hypochlorite
Gowns	☐ Single-use ☐ Waterproof ☐ Long sleeved ☐ Standard EN 14605:2009	Consider biological risk protection EN 14126:2004, if confirmed positive patient
Cap	☐ Single-use	Consider hood cap, if confirmed positive patient
Shoe cover	☐ Single-use	
Respiratory	☐ FFP2/N95	Perform seal check before enter the endoscopy suite.
Protection	☐ Single-use	Consider FFP3, if confirmed positive patient

PPE, personal protective equipment.

portability, post-procedural handling and cross contamination risk.²³

- Floors and surfaces of the endoscopy suite must be disinfected after each procedure.
- Intermediate level disinfectants with proven activity against enveloped viruses include 0.1% sodium hypochlorite, 62–71% ethanol, 0.5% hydrogen peroxide and quaternary ammonium compounds.^{19,24,25}

Personal protective equipment

IP procedures are considered to be consistently subjected to the highest risk of exposure. In this setting, full precautions must be taken to cover all different possible types of transmission (contact, droplet and airborne).²⁶ Personnel involved in the reprocessing procedure must also wear protective equipment consisting of eye protection, respiratory mask FP2, long sleeved gown and double gloves.^{27,28} The recommendations for the use of PPE are shown in Table 3.

Specimen transportation

Samples from the upper and lower respiratory tract, including pleural effusion, are deemed to be the most potentially infectious. Consequently, they should be handled as Category 3 pathogen and double-bagged (first the specimen must be bagged in the patient's room and then taken out of the room and placed in a separate pre-labeled specimen bag). All specimens must be manually delivered.^{28,29}

Safety rules for staff and patients

It is also important to define proper new rules for both HCW and patients circulating in the IP unit, as listed below:

Health professionals

- The IP Unit should reduce and prioritise the allocation of human resources according to the outbreak evolution and hospital needs. The minimum number of staff required to ensure a correct operation must be clearly defined.
- It is essential that all personnel follow, train and maintain competency in effective hand hygiene and every aspect of PPE (theoretical, training and simulation sessions) so that everyone is familiar with their role.
- All interactions with patients, including informed consent, should be done with appropriate PPE and frequent hand washing. The staff should not reduce the level of awareness and protection, and the idea that patients with suspected COVID-19 should be handled in the same manner as confirmed cases must be reinforced.
- A core team that includes only essential HCW should perform the procedure on SARS-CoV-2 positive patients. The most experienced staff should be responsible for the exam to reduce time and deal effectively with possible complications. Other healthcare personnel, such as residents, medical students and visitors should not be inside the unit and the examination room before, during or after the procedure.
- Of note, the scheduled exams must be done during normal working hours (avoiding an emergency basis or setting) and in an appropriate, designated room that fulfils all the standards required for care.

Patients and other personnel

- Respiratory and contact isolation should be standard and mandatory for all patients. Outpatients and inpatients should always enter the IP Unit with a suitable face mask and keep it on at all times (until the beginning and after the end of the procedure) to minimize the risk of transmission. No unnecessary personal items should be brought into the IP unit.
- Family members and caregivers should not stay in the IP waiting rooms. In case of children or patients in need of support, the Unit can allow a single relative to enter the preparation area to provide aid.
- The entry into the Unit of suppliers and medical devices sales representatives must be restricted.

Prioritization of procedures

Scheduled elective procedures should be reviewed and cancelled if potentially delayable, until local control of the outbreak is achieved. After flattening the infectious curve, many elective IP procedures will have to be performed, as they are essential to provide a definitive diagnosis and effective treatment. At this time, it is advisable to evaluate the delayed requests and to optimize the procedure planning based on clinical needs and operational capability.

A suggested rational approach for stratification of procedures is provided in Table 1, but we recognize that, in certain cases, the indication may not be straightforward, and the risk-benefit must be weighted on an individual basis by the IP team.³¹ Although rescheduling certain procedures is obvious in other cases it may not be desirable or ethical. It is important to note that these indications may change according to local epidemiological conditions and the response capabilities of the healthcare system. Several societies have recommended different levels of procedure stratification.^{26,30,31} Briefly, what is recommended is a step-wise reopening of elective IP procedures according to the national and local COVID-19 outbreak situation, depending on the number of new confirmed cases, hospital admitted cases (ward and ICU), availability of equipment and healthcare staff, time elapse and number of postponed IP cases. Some authors³⁴ have proposed a summary of the elective endoscopic procedure by phases, as shown in Table 2. Anyway, it should be noted that the evolving procedural criteria should always be communicated to other physicians who refer patients for invasive respiratory procedures and to the hospital administration.

Recommendations for bronchoscopy

Bronchoscopy under spontaneous ventilation

The following recommendations are expert opinion-based and should be adapted to local regulations and guidelines. In an optimal scenario, it is safer to perform elective bronchoscopy under general anesthesia and orotracheal intubation, clinical conditions permitting. If this is not possible, bronchoscopy can be performed under spontaneous ventilation. Some recommendations are listed below:

- Operator should be standing behind the patient's head to reduce direct exposure. Oxygen supplementation should be done without the use of humidification, either through a nasal cannula or preferably with an oxygen mask with an entrance to the bronchoscope (Fig. 2A).
- For flexible bronchoscopy, a transnasal approach should be preferred, and a surgical mask should be placed over the patient's mouth to minimize droplet emission (Fig. 2B).
- In hypoxemic patients, bronchoscopy can be performed under NIV, using a closed circuit ventilation (double circuit with viral filters in both arms) and non-ventilated masks with a dedicated bronchoscope entrance (Fig. 2C). High performance NIV ventilators with FiO₂ regulation are preferable. From the end of the procedure, NIV should be continued for 1–2 h, titrating the FiO₂ to obtain an SpO₂ of around 94–95%.
- Bronchoscopy under nasal high-flow oxygen therapy is not recommended and thus should be avoided.
- Nebulized medications should be avoided before or after the procedure.
- Proper sedation should be used to minimize cough reflex and to increase patient cooperation.
- An oral aspiration cannula should be available during the procedure (Fig. 2B).
- A transparent protective box may enhance safety by containing dispersal of droplet particles (Fig. 2D). The box is placed over the patient's head prior to bronchoscopy, with the anesthesia equipment already in place. The bronchoscope is inserted through the covered opening behind the patient (Fig. 2E).

Bronchoscopy in the intubated patient

The following recommendations are directed for patients under mechanical ventilation in an ICU setting due to respiratory failure. As reported, 5% of COVID-19 patients can develop respiratory failure and will need ventilatory support³²; moreover, associated bacterial, viral and fungal co-infection should not be neglected.³³ In critically ill patients under invasive ventilation, ventilator-associated pneumonia occurs in up to 30% and lobar collapse is frequent and multifactorial.³⁴ The same adaptations apply to elective procedures under general anesthesia, performed in the Bronchoscopy Unit or Operating Theatre.

- A cuffed endotracheal tube is preferred over supraglottic devices, such as a laryngeal mask; cuff pressure should be maintained between 25–30 cmH₂O.³⁵
- General anesthesia with muscle relaxant is recommended to reduce the aerosol production.
- FiO₂ should be adjusted to 100%.
- Volume control, pressure-limited mode is preferable and PEEP should be kept at the same level during the procedure. Adjustments can be made dynamically, with a prior assessment of the anticipated risks (e.g., lung derecruitment and desaturation, arrhythmias, pneumothorax).
- To avoid aerosol dispersion, a simple and appropriate maneuver consists of clamping the ventilation circuit just before introduction of bronchoscope, repeating the same step just before withdrawal.



Figure 2 Strategies to minimize droplets dispersal during bronchoscopy. A. The bronchoscope may be introduced through an opening made at the oxygen mask, in this case with an additional plastic sheet covering the patient's head. B. Transnasal approach, with oxygen supplementation through nasal cannula and a surgical mask placed over the patient's mouth and the oral aspiration cannula. C. Bronchoscopy can be performed under ventilatory support, using a closed circuit ventilation and non-ventilated masks with a dedicated bronchoscope entrance. D. Transparent protective box may contain droplet particles inside. E. Protective box placed over the patient's head during endobronchial ultrasound. F. Rigid bronchoscopy with rubber caps on the ports of the scope and a plastic covering.

- Bronchoscope removal and reinsertion should be avoided during the procedure.
- In hypoxemic patients, if bronchoalveolar lavage is needed for diagnostic purposes, the volume used should be reduced to a minimum. If a SARS-Cov-2 diagnosis is needed, a minimum of 2–3 mL of recovered lavage is enough.²⁶

Rigid bronchoscopy

Rigid bronchoscopy is used for diagnostic and therapeutic purposes, in procedures where flexible bronchoscopy would be deemed difficult or even impossible, like obtaining larger samples of endobronchial lesions, foreign body removal, management of central airway obstruction (including ablative techniques, like electrocautery, argon plasma coagulation, laser, cryotherapy, among others, and placement of airway stents) or massive hemoptysis.³⁶

There are different ventilation strategies used during rigid bronchoscopy, although manual jet ventilation and high frequency jet ventilation are much the most frequent.³⁷ Common to these two techniques is the fact that the proximal end of the bronchoscope is open to allow the passage of instruments, thus ventilation is achieved providing 100%

oxygen under high pressure (usually 50 psi) through an open system.^{39,40} The use of these ventilation techniques means that aerosols are released into the room, making it a high-risk procedure.

In patients with suspected or confirmed COVID-19 diagnosis, rigid bronchoscopy should be avoided, except for urgent cases (Table 1). Clinical scenarios are mostly therapeutic, like acute foreign body aspiration, massive hemoptysis (when there is no place for embolization), severe symptomatic central airway obstruction (either benign or malignant) and migrated stents. In a clinically stable patient, upon suspicion of foreign body aspiration, one should consider non-contrast computerized tomography (CT) to confirm the presence of a foreign body before rigid bronchoscopy, to avoid unnecessary exams.^{38,39}

In some centers, the rigid scope is used to perform other techniques, like Endobronchial Ultrasound-Transbronchial Needle Aspiration (EBUS-TBNA) or transbronchial cryobiopsy; this provides comfort to the operator and safety in case of major bleeding. The authors recommend performing these diagnostic procedures through cuffed endotracheal tube to minimize the risk of exposure. However, the operator must be ready to convert to rigid bronchoscopy, if necessary.

In a patient undergoing rigid bronchoscopy, it is recommended that:

- Rigid bronchoscopy should always be performed in a negative pressure room.
- Controlled ventilation is preferred, with the rigid bronchoscope used like an endotracheal tube.
- Air leaks should be reduced using rubber caps on the ports of the rigid scope, as well as using a plastic covering (Fig. 2F) or filling the mouth with gauze.⁴⁰ While this strategy is more appealing to minimize aerosol spread, the operator may find it challenging to handle instruments through the working channel.

Recommendations for pleural techniques

Pleural effusion does not appear to be a prominent feature of COVID-19. It occurs in 5.3–5.8% of patients, according to two recent meta-analyses.^{41,42} There have been occasional reports of bilateral effusion that resolved spontaneously.⁴³ As bacterial superinfection is common in severe patients, they can also develop complicated effusions or empyema, requiring targeted treatment. There have been a few anecdotal reports of spontaneous pneumothorax and pneumomediastinum in severe COVID-19 pneumonia, requiring drainage.^{44,45} This may be more frequent in critical patients on invasive ventilation, which can lead to bronchopleural fistulae.⁴⁶ It is, therefore, plausible that pleural drainage may be necessary in some COVID-19 patients, either in the ICU or in the ward, and indications for drainage do not differ from the standard clinical guidelines. However, as with any invasive procedure in confirmed COVID-19 patients, all precautions regarding the full use of protective equipment should be taken. The procedure must be performed by trained and dedicated staff to reduce its duration and to minimize the risk of complications. In other situations, the use of ultrasound may be very helpful with COVID-19 patients.⁴⁷ Besides its wide availability, safety and low cost, it is easy to use at the bedside and allows medical staff to detect small pleural effusions and to guide pleural fluid collection and drainage, if needed. On the other hand, even patients without suspected COVID-19 can have asymptomatic infection; so, any procedure should be considered as a possible COVID-19 case and precautions should be taken. Indeed, although some procedures may be postponed, in many situations they should not be deferred, especially in suspected or confirmed cancer patients. It is crucial that cancer patients do not experience delays in diagnostic or therapeutic procedures due to the present contingency.⁴⁸

Few societies have published guidelines addressing pleural procedures during COVID-19 pandemic. The British Thoracic Society has issued guidance on pleural services provision,⁴⁹ mainly to minimize hospital visits and admissions and to ensure both patient and staff safety. Nonetheless, although we recognize lack of published evidence supporting these recommendations, this document will adopt some of them.

First of all, despite pleural procedures not being listed as Aerosol Generating Procedure (AGP) in the CDC updated recommendations,⁵⁰ they should be considered so and Level 2 PPE should be worn, as described above. Other societies

have considered potential AGP as any procedure *“likely to induce coughing, that should be performed cautiously and avoided if possible”*.⁵¹ This is particularly relevant to open procedures, such as thoracoscopy and indwelling pleural catheter insertion, and in case of pneumothorax with suspected bronchial-pleural fistula. Thus, we recommend taking the following precautions:

Pleural effusion

- On suspicion of malignant effusion, diagnostic pleural fluid aspiration should be performed, especially if the patient is a candidate for systemic therapy or if the effusion is symptomatic (Table 1).
- Consider placement of indwelling pleural catheters for recurrent malignant pleural effusions, avoiding repeated hospital drainage or admission.
- On suspicion of pleural infection, pleural fluid aspiration and analysis should be performed.
- In case of benign pleural effusions, the risk-benefit should be discussed with the attending physician; procedures can be postponed if the patient is not symptomatic.
- Thoracoscopy is not recommended as a principle; however, in the absence of alternative options, its risk-benefit must be assessed.

Pneumothorax

- Spontaneous primary pneumothorax can be managed in outpatient care if the risk assessment allows and if there is local team experience; needle aspiration and discharge should be considered if the patient is minimally symptomatic.
- When pleural drainage placement is necessary, if there is local expertise and the patient is at low risk, the use of pleural vent systems should be considered, thus allowing the patient to be managed at home.

Chest drainage placement and care

- Extra care must be taken when placing the chest tubes, in order to avoid open communication with the pleural space and the potential emission of droplets and aerosols.
- When chest tubes are placed in ventilated patients, consideration should be given to clamping the ventilator circuit before assessing the pleural cavity, so that positive pressure spreading of pleural air or fluid can be prevented.
- Whenever possible, the use of non-wired pleural drainage should be considered; it can be connected to the drainage system before insertion into the pleural cavity (closed circuit).
- When pleuroscopy is required, the use of one way valve trocars should be preferred to assess the pleural cavity and properly seal the entrance port of the pleuroscope.
- In case of prolonged air-leaks, the use of wall suction should be weighted to create a closed system.

Table 4 IP Unit checklist during COVID-19 outbreak.

PRE-PROCEDURE PLANNING	PROCEDURE EXECUTION	POST-PROCEDURE
Adapt the IP unit to include: ✓ Preprocedural area ✓ Procedural room with negative pressure or adequate ventilation¹ ✓ Postprocedural area for decontamination, reprocessing and removal of PPE Revise prioritization of all procedures (table x and y) Perform telephone pre-screening check-list (upon schedule and 24-48h before the exam)² ☐ Symptoms ☐ Contact history ☐ Occupational exposure Test for SARS-CoV-2 in the 24-48h preceding the exam Proceed according to priority / test result / risk probability (Flowchart 1, Table 1)	Gown adequate PPE (Table 3) and perform procedure at adequate endoscopy suite¹ Minimize direct exposure ✓ Stand behind the patient's head ✓ Place surgical mask over the patient's mouth ✓ Adapt oral aspiration cannula ✓ Avoid humidified O₂ and nebulized drugs ✓ Use appropriate sedation In ventilated patients: ✓ Prefer cuffed endotracheal tubes over supraglottic devices ✓ Clamp the ventilation circuit when removing or inserting the bronchoscope and immediately before assessing the pleural cavity when placing pleural drainages Avoid rigid bronchoscopy (except if indicated for urgent purposes) Avoid thoracoscopy (except if lack of alternative and after adequate risk/benefit stratification) Reduce teams and time of the procedure to minimal necessary	Collect respiratory samples (including pleural fluid) in closed circuits and double-bag for transport If available, use disposable bronchoscopes for confirmed positive / highly suspicious COVID-19 patients Reprocessing of bronchoscopes must be considered an AGP ✓ Gown adequate PPE (Table 3) ✓ Perform procedure at adequate unit¹ Disinfect floor and surfaces of the endoscopy suite after each procedure Allow adequate time (>30min) to elapse before next procedure Perform sequential removal of PPE and adequate hand hygiene

1. The preferred system is a negative pressure room with at least 12 air changes per hour with controlled of airflow direction (single pass or recirculation systems with HEPA filtration). Alternatively, natural ventilation with an airflow of at least 160 L/s is an option.

2. Pre-screening checklist should include questions regarding (1) recent symptoms suggestive of COVID-19 (namely fever, cough, shortness of breath/difficulty breathing, chills, muscle pain, headache, sore throat, loss of taste or smell), (2) contact with suspicious or confirmed SARS-CoV-2 cases, (3) occupational exposure, and (4) recent travel history to a high-incidence region

AGP: Aerosol generating procedures. PPE: personal protective equipment. IP: Interventional Pulmonology.

Concluding remarks

As with other societal consensus papers, this document was developed by a restricted panel of experts from the Portuguese Society of Pulmonology; individual clinical judgment and local resources may lead to alternative perspectives. This guidance was based on the current knowledge of COVID-19, but, as new data appears, this statement should be revised in the future to accommodate updated recommendations. At present, one of the controversial assumptions is that, every patient, even if asymptomatic, should be assumed as potentially infected with SARS-CoV-2. Therefore, it is mandatory that contact precautions and proper training on donning and doffing of PPE be provided to all HCWs involved in IP. Another key element is to plan in advance and keep each IP Unit well-organized (Table 4). Although the reduction in the number of elective procedures represents one of the central strategies to improve safety, it is crucial that patients do not suffer unnecessary delays in diagnostic or therapeutic procedures due to the current contingency. Taken together, our ultimate intention is to bring full attention to this and future outbreaks or other emerging medical situations.

Conflicts of interest

The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE

Pre- and post-COVID practice of interventional pulmonology in adults in Portugal

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Bronchoscopy;
COVID-19;
Survey;
Questionnaire;
Pulmonology

Abstract

Introduction and objectives: There is a lack of information regarding bronchoscopy practice in adults in Portugal. Our objective was to obtain an overview of the characteristics, resources, techniques and behaviors in national interventional pulmonology (IP) units, before and after SARS-CoV-2 outbreak.

Materials and Methods: An online survey was developed by an expert panel with a total of 46 questions comprising the specifications of each unit, namely physical space, equipment, staff, procedure planning, monitoring, technical differentiation, and numbers pre- and post-COVID-19. Forty-one interventional pulmonology centers were invited to participate between April and May 2021.

Results: 37 units (90.2%) responded to the survey. The majority (64.9%) have a fully dedicated space with a weekly presence of ≥ 3 chest physicians (82.1%) and support of an anesthesiologist on specific days (48.6%). There is marked heterogeneity in the IP unit's equipment, and 56.8% acquired disposable bronchoscopes after COVID-19 pandemics. Pre-bronchoscopy hemogram, platelet count and coagulation tests are regularly asked by more than 90% of the units, even when deep sedation or biopsies are not planned. In 97.3% of cases, topical anesthesia and midazolam are utilized. Propofol (21.6%) and fentanyl (29.7%) are occasionally employed in some institutions. Most units use ancillary sampling techniques to diagnose central or peripheral lesions, with radial EBUS being used for guidance of distal procedures in 37.8% of centers, linear EBUS and EUS-B-FNA for mediastinal diagnosis and/or staging in 45.9% and 27.0% of units, respectively. Cryobiopsies are used by 21.6% of respondents to diagnose diffuse lung diseases. Rigid

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bronchoscopy is performed in 37.8% of centers. There was a decrease in the number of flexible ($p < 0.001$) and rigid ($p = 0.005$) bronchoscopies and an upscale of personal protective equipment (PPE) during the COVID-19 outbreak.

Conclusions: IP units have variable bronchoscopic practices, but during the COVID-19 pandemic, they complied with most international recommendations, as elective procedures were postponed and PPE levels increased.

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Introduction

Bronchology and Interventional Pulmonology (IP) has evolved significantly in the last 20 years, with the development of technologies and practices. Despite the existence of several guidelines from international associations and institutions, most are recommendations set by experts in this field and more focused on safety and functional aspects than in robust scientific evidence, so procedures are usually not standardised.^{1–7}

Considering the existing variance of methods and the little knowledge about bronchoscopy practices, several countries undertook national surveys to characterize the availability of resources, the clinical application of techniques, and its specificities.^{8–15} In 2018, a national study was published evaluating, specifically, the use of pleuroscopy in Portuguese pulmonology departments, considering availability, professionals' experience and training, number of procedures and its characteristics.¹⁶

Recently, because of the Coronavirus Disease 2019 (COVID-19) pandemic outbreak, the Portuguese Pulmonology Society published a consensus statement aiming at the safety of both patients, medical practitioners and other health professionals during bronchoscopy and pleural procedures.¹⁷ Based on current knowledge, all patients should be assumed as potentially infected with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2); therefore, specific measures must be implemented, including organizational issues, physical space and material preparation, and personal protection equipment (PPE) of all professionals involved.¹⁷

These recent changes in the bronchology field were expected to have a major impact on national interventional pulmonology units, so the Committee on Endoscopic Techniques of the Portuguese Pulmonology Society nominated a working group to conduct a national survey, to accurately characterize the Portuguese panorama. Specifically, this survey aimed to quantify and qualify the human and technical resources of the Portuguese Pulmonology Departments, the specificities of the bronchoscopic procedures performed, and compare the reality before and after the COVID-19 pandemic.

Methods

The expert panel developed a structured questionnaire in Google Forms, and the link was e-mailed to the Bronchology Units or Pulmonology Departments of all Portuguese public hospitals and some private institutions/hospitals considering their representativeness (Supplementary Table 1). Respondents had one month to reply and send the completed form;

one reminder was sent to all, and those who did not answer were further contacted by phone. The survey occurred between 22nd April and 31st May 2021.

The questionnaire comprised 46 questions, either of quantitative or multiple option answer, considering the following sections: 1) respondent and hospital identification; 2) Interventional Pulmonology Unit characterization; 3) staff experience in bronchology; 4) nursing and 5) anesthesiology support; 6) equipment characterization; 7) planning and monitoring of the bronchoscopic procedures, including procedure specifications (e.g. complementary diagnostic tests before bronchoscopy, methods of sedation and patient monitoring); 8) endoscopist PPE pre and post the emergence of COVID-19 pandemic; 9) accessory diagnostic and therapy techniques by flexible bronchoscopy, including the number of procedures pre and post COVID-19; and 10) rigid bronchoscopy, comprising the number of procedures pre and post COVID-19.

Responses were downloaded as an Excel spreadsheet. Descriptive analysis was performed using the software SPSSv24®. The survey results were analyzed based on the total number of answers to each question and are presented as mean±standard deviation and median (range) for continuous variables, and as frequency and percentage for categorical variables. Paired samples t-student test was used to compare the means of two variables of the same sample measured in sequential years. A difference of at least 5% (two-tailed) was considered statistically significant.

Results

A total of 41 eligible Portuguese interventional pulmonology centers were invited to participate (Supplementary table 1), and 37 (90.2%) answered the entire online questionnaire. The responders included in this analysis are listed by their district in Fig. 1 and represent almost all geographic locations in the country-

General characterization of the IP units

The units were located on nine university public hospitals, 23 tertiary public hospitals, and two private hospitals. In 24 cases (64.9%), the unit space was fully dedicated to bronchoscopy/pleural procedures; in 12 (32.4%), the physical location was shared with other specialties, and in one case, the procedures were performed in a hospital operating room.

Thirty IP units (81.1%) reported the weekly presence of three or more chest physicians performing the procedures, six (16.2%) had the regular presence of two pulmonologists, and one (2.7%) relied on one doctor.

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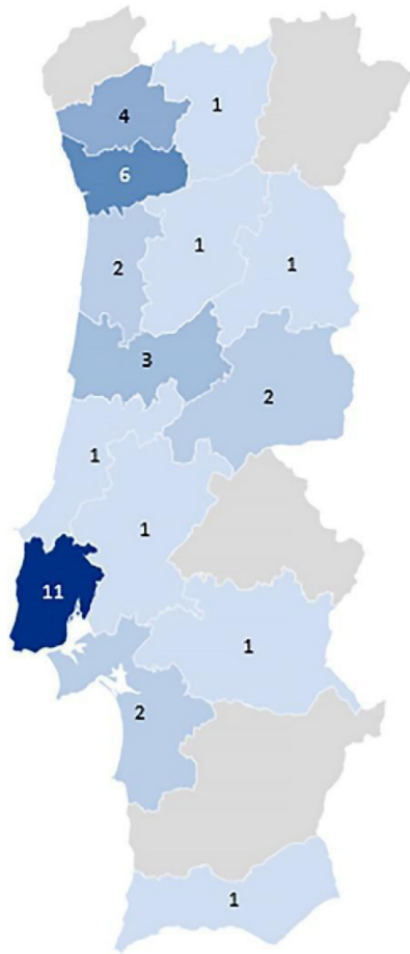


Fig. 1 Participating centers' geographical distribution.

Regarding the existence of dedicated nurses with specific IP training, three units (8.1%) stated the usual presence of one nurse, 13 (35.1%) referred two rotating nurses, ten units (27%) three nurses, and another 10 (27%), four or more nurses. One unit indicated the absence of trained nurses.

Eighteen units (48.6%) had the regular support of an anesthesiologist on specific weekdays. In 15 cases (40.5%), an anesthesiologist was present in the bronchoscopy suite-only upon request. This occurred on 1-2 days/week in 16 cases, 3-4 days/week in two cases and five days/week in other two units (totally dedicated to cancer patients). Four units (10.8%) had no assistance from an anesthesiologist.

On a regular year, the IP units reported an average of nearly 500 flexible bronchoscopies per year. However, great heterogeneity was observed with 14 centers (37.8%) registering 500 exams/year or more, 18 (48.6%) 200 to 499 exams/year and five (13.5%) less than 200 exams/year.

Overall, we were able to analyze the profile of 103 pulmonologists. Of these, 41 (39.8%) had over 15 years of experience in bronchoscopy, 23 (22.3%) had 10 to 14 years of

Table 1 IP units' equipment.

	Number of IP units	%
Video bronchoscopes	36	97.3
Optical bronchoscopes	27	73.0
Disposable bronchoscopes	21	56.8
Linear endobronchial ultrasound	16	43.2
Radial endobronchial ultrasound	14	37.8
Rigid bronchoscope	17	45.9
Thoracoscopy (semi-rigid or rigid)	23	62.2
Fluoroscopy (dedicated or shared)	10	27.0
Electrosurgery / electrocautery	11	29.7
Argon-plasma coagulation	10	27.0
Cryotherapy	10	27.0
Laser therapy	8	21.6
Electromagnetic navigation	2	5.4
Auto-fluorescence	2	5.4
Cone-beam CT	1	2.7

previous experience, 25 (24.3%) had 5 to 9 years, and 14 (13.6%) had less than 5 years of IP experience. Concerning the areas of expertise, all answering chest physicians had experience in flexible bronchoscopy, while 37 (35.9%) reported proficiency in rigid bronchoscopy and 37 (35.9%) in endobronchial ultrasound.

Pleuroscopy/medical thoracoscopy was performed by 46 (44.6%) of the respondents and was reported to be available in 20 centers.

Table 1 shows the unit's equipment. Most units (56.8%) promoted the acquisition of disposable bronchoscopes after COVID-19 pandemics. Twenty-five health care facilities (67.6%) reported using automatic high-level disinfection to reprocess their reusable bronchoscopes and accessories. In 11 hospitals (29.7%), sterilization was the reported selected method, if available, because of its greater efficacy in reducing microorganism's contamination. In one unit (2.7%), the cleaning and disinfection of the equipment was entirely manual. After reprocessing, to minimize the likelihood of recontamination, the equipment was stored in ventilated cabinets in 19 units (51.4%) and in non-ventilated facilities in 18 units (48.6%).

Patient preparation, monitoring, anesthesia and sedation

Most participants (86.5%) responded that they usually ask for at least one pre-procedural exam to evaluate each patient that is going to be submitted to a standard flexible bronchoscopy without deep sedation/anesthesia when the main purpose is the inspection of the airways and sample collection

Table 2 Pre-procedural complementary exams required when performing standard flexible bronchoscopy.

	Usually	Sometimes	Never
Standard flexible bronchoscopy, without deep sedation/anesthesia and without biopsy (n)			
Hemogram with leukogram	35	2	0
Platelet count	35	1	1
Coagulation tests	32	4	1
Serum urea and creatinine	26	9	2
Liver enzymes	12	22	3
Serum ionogram	17	16	4
Electrocardiogram	17	16	4
Echocardiogram	0	12	25
Chest x-ray	37	0	0
Chest CT	25	11	1
PET-CT scan	0	16	21
Standard flexible bronchoscopy, with deep sedation/anesthesia and/or need for biopsy (n)			
Hemogram with leukogram	36	1	0
Platelet count	36	1	0
Coagulation tests	34	3	0
Serum urea and creatinine	32	4	1
Liver enzymes	23	12	2
Serum ionogram	27	9	1
Electrocardiogram	32	4	1
Echocardiogram	2	21	14
Chest x-ray	32	5	0
Chest CT	33	4	0
PET-CT scan	0	24	13

without the need for biopsies. Similarly, most proceduralists (89.2%) require more than one pre-procedure complementary exam when sedation or biopsies are planned Table 2. presents the complementary exams asked in each situation.

Most flexible bronchoscopies were reported to be performed with topical anesthesia (with or without conscious sedation) using lidocaine in the form of gel (“usually” $n = 30$, 81.1%; or “sometimes” $n = 6$, 16.2%), nebulized (“usually” $n = 27$, 73%; or “sometimes” $n = 7$, 18.9%), and/or liquid in the tracheobronchial tree as “spray as you go” technique (“usually” $n = 19$, 51.4%; or “sometimes” $n = 6$, 16.2%). The most common drug used by the pulmonologists for sedation was midazolam in 97.3% of units (“usually” $n = 25$, or “sometimes” $n = 11$). Propofol and fentanyl were occasionally employed during bronchoscopy in 8 (21.6%) and 11 (29.7%) institutions, respectively.

Twenty-nine respondents (78.4%) affirmed that supplemental oxygen was routinely administered since the beginning of each flexible bronchoscopy. In contrast, the remaining eight (21.6%) participants showed a preference in administering supplemental oxygen only if required due to peripheral oxygen desaturation during the procedure.

When asked about the patient’s monitoring during flexible bronchoscopy without deep sedation, all 37 participants referred to assessing heart rate and pulse oximetry throughout the procedure. Non-invasive blood pressure was measured by 33 respondents (89.2%), and ECG monitoring was monitored by 23 participants (62.2%).

When deep sedation was planned, end-tidal CO_2 was evaluated by one participant unit (2.7%), monitor ECG was performed by 31 participants (83.8%), non-invasive blood pressure by 35 (94.6%), and all respondents referred to monitor heart rate and pulse oximetry. In both scenarios, one respondent added clinical observation, and another external pace defibrillator monitoring.

Bronchoscopy technical features

Regarding the diagnosis of central/tracheobronchial lesions by flexible bronchoscopy, biopsy forceps were used by all 37 participant units; bronchial brushings by 36 (97.3%); and needle aspiration by 31 (83.8%). Bronchial cryobiopsies were reported by seven (18.9%) participants.

Cryobiopsies were also the least reported procedure in the diagnosis of peripheral lung lesions, used by six (16.2%) respondents. Peripheral biopsy forceps and brushing were among the most used procedures for diagnosing peripheral lesions, reported by 33 (89.1%) participants in both cases. Radial EBUS and fluoroscopy were used as guidance for distal procedures in 14 (37.8%) and 10 (27.0%) centers, respectively.

In diffuse lung diseases, bronchoalveolar lavage was the preferred method, reported by all 37 centers. Transbronchial forceps biopsies were used by 33 (89.2%) of the participants, while eight centers (21.6%) reported performing transbronchial lung cryobiopsies. Fluoroscopy was used by seven centers (18.9%) to guide the adjunctive procedures for the diagnosis of diffuse lung diseases.

In what concerns the diagnosis of mediastinal lesions, non-guided transbronchial needle aspiration (TBNA) was used by 32 centers (86.5%). In contrast, EBUS-TBNA was used by 17 centers (45.9%) and EUS-B-FNA in 10 centers (27.0%).

Concerning therapeutic techniques, 34 (91.9%) centers reported using flexible bronchoscopy to remove foreign bodies. Control of hemoptysis was attempted through balloon tamponade by 30 (81.0%) centers, whereas 31 (83.8%) units used some form of thermal energy, of which LASER was the preferred, employed by 14 (37.8%) centers, followed by argon-plasma coagulation, used by 10 (27.0%) centers. Airway repermeabilization through self-expandable stents placed through flexible bronchoscopy was reported by 7 (18.9%) centers.

Other endobronchial therapeutic maneuvers such as bronchial thermoplasty and bronchoscopic lung volume reduction (with one-way valves, coils or thermal ablation) were not reported by any center.

The average number of flexible bronchoscopies reported to have been performed by all 37 participant centers in 2020 (amid the COVID-19 pandemic) was 345 (± 249). When asked about the estimated annual number of flexible bronchoscopies performed during 2019 (before the pandemic), the reported average number was 500 (± 346). There was a statistically significant decrease in the number of bronchoscopies performed due to the COVID-19 pandemic, which raised to an average of -155 ($p < 0.001$). The distribution of reported exams and the impact of the COVID-19 pandemic on the workload of flexible bronchoscopy is reported in detail in Table 3. In 2020, on average, 84% ($\pm 12.6\%$) of flexible bronchoscopy procedures were performed mainly with a diagnostic intent.

Seventeen IP units reported to have a rigid bronchoscope and 14 referred to perform the procedure. Proceduralists

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Table 3 Estimated annual number of flexible bronchoscopies in 2019 and 2020.

Number of flexible bronchoscopies	Reported number of centers (2019)	Reported number of centers (2020)
> 1000	4	1
≥ 500-999	10	8
≥ 250-499	13	14
≥ 100-249	10	10
< 100	0	4

used rigid bronchoscopy to conduct one or more adjunctive procedures: 100% mechanical debulking (“usually” $n = 12$; or “sometimes” $n = 2$); 57.1% LASER ablation (“usually” $n = 7$; or “sometimes” $n = 1$); 57.1% argon-plasma coagulation (“usually” $n = 6$; or “sometimes” $n = 2$); 50% electrosurgery (“usually” $n = 3$; or “sometimes” $n = 4$); 50% cryoablation (“usually” $n = 2$; or “sometimes” $n = 5$).

Eleven centers (29.7%) reported airway stenting deployment by rigid bronchoscopy to maintain tracheobronchial patency. Between all commercially available, the Dumon (straight or Y) and Hood stents were the most frequently used in the Portuguese IP units (Dumon $n = 11$, Hood $n = 8$, Ultraflex $n = 5$, Polyflex $n = 4$, Silmet $n = 4$, Dynamic Y-stent $n = 3$, Montgomery T-tube $n = 3$, Aerstent $n = 2$, Micro-tech $n = 1$, Noppen $n = 1$ unit). Several airway stent complications were commonly reported by most centers, being the most frequent: stent migration ($n = 11$), obstruction by secretions ($n = 10$), obstruction by granulation tissue ($n = 10$), chronic cough ($n = 5$), and infection ($n = 3$).

Of these 14 units performing rigid bronchoscopy, 11 retrieved their estimated annual number of procedures performed both in 2020 and also on a regular year (before the COVID-19 pandemic). On average, IP units performed 96 ± 99.3 (range 7-302) rigid bronchoscopies during 2019 (before the pandemic), whereas in 2020, the average number of reported rigid bronchoscopy was 70 ± 81.2 (range 0-237). When assessing the impact of the COVID-19 pandemic on rigid bronchoscopy overall workload, a statistically significant average decrease of -26 procedures was observed ($p = 0.005$).

Health professionals' protection

When asked about the current use of personal protection equipment (PPE) during interventional bronchoscopy, all 37 units confirmed choosing FFP2/N95 single-use face masks (four in combination with a surgical face mask), some sort of eye protection, single-use gown and single-use waterproof gloves Table 4. presents the standard PPE reported to be used in 2019 (before the pandemic) and afterwards.

Discussion

This survey identifies a marked heterogeneity of bronchoscopy practice and resources in adults IP units across Portugal's mainland. This was a comprehensive and extended questionnaire that was able to assess the prevalence of the practice in the country and the impact of COVID-19 at a national level. It is evident that the number and type of procedures

Table 4 Standard PPE reported before and after COVID-19 pandemic.

PPE	Reported number of centers (2019)	Reported number of centers (2020)
FFP2/N95	21	37
Surgical mask	19	4
Hair protection	20	34
Eye protection	21	37
Waterproof gown	12	34
Non-waterproof gown	22	3
Sterile gloves	18	23
Non-sterile gloves	17	18
Shoe-cover	9	25
Leg protection	1	10

performed decreased during the pandemic period; most units equipped themselves with disposable bronchoscopes, and there was a shift in the type and level of protective equipment used to increase safety for staff and patients.

Surveys in IP have been published since the 90s.¹⁰ When compared to some historical questionnaires that were able to provide the clinical picture of bronchoscopy in North America and in some European countries, much as evolved regarding practice and technical developments. The number and indications of rigid bronchoscopies have declined, new techniques have emerged (e.g., EBUS and cryobiopsies), sedation and anesthesia are now regarded as mandatory in most units and demanded by patients.

The survey response rate was very high, and the findings reveal, as expected, the national panorama, in which the majority of centers are based in Lisbon and Porto metropolitan areas and in the coastal region, where more than half of the Portuguese population lives.¹⁸

Most units (65%) are fully dedicated to bronchoscopy, more than 90% have two or more trained pulmonologists, and in 84% of cases, at least one has at least ten years of IP experience. In contrast, 46% of units have two or fewer nurses, and most of them are not fully dedicated to bronchoscopy. It is worth mentioning that there is no specific post-graduation education in IP for nurses in the country. We may speculate that the small number of nurses trained and committed to this area may impact the quality of the exams. The British Thoracic Society advised the presence of at least two qualified nurses during bronchoscopy.¹ In contrast, the European Respiratory Society and the American Thoracic Society instruct on the need for one to two dedicated nurses during interventional pulmonology procedures without anesthesia (with a third nurse required to help the anesthesiologist in the procedures performed under deep sedation / general anesthesia).² Therefore, efforts should be made to assure that nursing staff receives adequate standardized training in IP and that all units move on to comply with international recommendations and demand the regular presence of an adequate number of trained nurses. This number may vary depending on the workflow volume of the individual units. Still, it should not be less than two (with possible rotation) in low volume units, to at least three (non-rotating) in high volume units that perform advanced interventional bronchoscopy and have regular anesthesiology support.

There is great heterogeneity in technical resources and equipment between IP units, with more than half performing

only basic exams. A third uses rigid bronchoscopy, which is sufficient for the country necessities, according to the number of habitants. In 2019, 16 centers performed linear EBUS, whereas in 2014 only six did it, as reported by the Survey of Pulmonology Services, prepared as part of the review of the Pulmonology Referral Network.¹⁹ This expansion follows the natural evolution of IP units worldwide since EBUS is an important tool for mediastinal staging in lung cancer patients and in diagnosing malignant or benign lesions adjacent to the tracheobronchial tree or the oesophagus.²⁰ For the diagnosis of peripheral pulmonary nodules, 14 units have radial EBUS, ten fluoroscopy and two have electromagnetic navigation, which enables an increase in diagnostic accuracy. Regarding the COVID-19 pandemic, 56.8% of the units now have single-use bronchoscopes, which were not regularly utilized. This equipment will certainly have an increasing application in the near future due to its benefits regarding the risk of contamination, availability and preserved efficacy in most emergent clinical scenarios.²¹

Concerning the number of procedures per year, the present survey shows a clear negative impact of the COVID-19 pandemic period, with a decrease by 30% on the average number of flexible bronchoscopies and 27% on rigid bronchoscopies. This is in line with other published studies,^{22–24} where IP procedure work-load considerably decreased or was even abolished during the COVID-19 era. Elective procedures in COVID-19 negative patients were postponed and rescheduled as recommended by consensus guidelines.^{17,25}

Some kind of sedation/anesthesia is required to reduce patient's discomfort, enhance safety^{6,26} reduce procedure duration and increase diagnostic accuracy. The preferred method for bronchoscopic diagnostic procedures is conscious sedation with antianxiety and/or analgesic medications, in combination with local anesthesia, titrated by bronchoscopists and supervised by the team. Most guidelines suggest the combination of a sedative agent with an opioid since it decreases cough and pain, improves patients' tolerance, and reduces the total dose of sedative drugs.²⁷ In the present assessment, more than 97% of the IP units in the country confirmed the use of midazolam. Still, only 29.7% associated an opioid, probably due to a reduced experience with this medication and fear of complications in patients with previous respiratory diseases. These results are in-line with other real-life studies.^{11,28}

It is worth mentioning that conscious sedation has limitations, and prolonged, complex, and technically demanding bronchoscopies or therapeutic procedures usually require deeper sedation/general anesthesia with the support of an anesthesiologist, not involved in the intervention itself.²⁹ The survey identified the lack of available anesthesiologists as a potential problem, with only 5% and 48.6% of the units having the collaboration of this specialty on a daily or regular basis, respectively.

As pre-procedural requirements, nearly all responders ask routinely for a full blood count (97%), coagulation studies (92%), renal function (86%) and an electrocardiogram, even when there is no history of abnormal coagulation or cardiac risk. These numbers are much higher when compared to other published surveys. In 2002, in the United Kingdom, when performing bronchoscopy without transbronchial biopsy, physicians routinely asked for a full blood count in 57%, blood urea and electrolytes in 43% and clotting

function in 18% of cases. When a transbronchial biopsy is needed, 91% asked for a full blood count and 88% asked for clotting tests.¹² This highlights the deviation of real-life performance compared to international guidelines and current scientific literature, which states that the routine performance of coagulation studies, platelet counts, and hemoglobin levels before flexible bronchoscopy is not recommended, especially when there are no previous identifiable risk factors.^{26,30} On the other hand, a pre-procedural chest CT scan was not consistently required by 32.4% of participants. Though we acknowledge that a chest CT can be dismissed upon emergent situations (mainly emergent therapeutic bronchoscopy), the number of centers that reported inconsistent demand of this valuable exam was concerning. Again, we stress the need to standardize procedures and comply with international recommendations that underline the importance of CT in preparation for bronchoscopy.^{1,2,19} Clinical evaluation should dictate other pre-bronchoscopy investigations on an individual basis, and this should be stressed to avoid submitting the patients to unnecessary exams and increase healthcare costs.

According to the recommendations of a few guidelines,^{26,30} and good clinical practice, all Portuguese units monitor heart rate and oxygen saturation, and generally add non-invasive blood pressure and continuous ECG in patients undergoing flexible bronchoscopy, especially when deep sedation is planned. It is recognized that deficient monitoring in deep sedation may lead to unnoticed respiratory depression, changes in normal blood pressure and arrhythmia, which increase the probability of complications during bronchoscopy. This awareness was acquired especially in the last two decades, compared to the survey conducted by Smyth et al, where only 22% and 10% of physicians reported monitoring ECG and blood pressure, respectively.¹² A recent systematic review by Strohleit et al,²⁷ reinforces the idea that there is still the need for further research in this area due to the scarcity of evidence of published papers.

In the current study, most units combine biopsy, brushing and needle aspiration for the diagnosis of endobronchial lesions, to obtain the highest diagnostic yield.³¹ Compared to other published surveys that proved the underuse of TBNA,⁸ we highlight that 83.8% of centers are able to perform non-guided TBNA in central lesions and 86.5% in mediastinal lesions (including centers that also perform EBUS-TBNA). Interestingly, non-guided TBNA accuracy has been reported to benefit from previous EBUS training.³² Still, we believe that the overlap of both techniques in the same centers can be explained by the formers' wider accessibility and lower costs. Although EBUS-TBNA is used in 45.9% of units, EUS-B-FNA is only performed in 27% of centers, probably related to the lack of specific training. This reinforces the need for educational programs in this area for both residents and senior chest physicians.

In this survey, transbronchial lung cryobiopsies were performed in a scarce number of hospitals (8 out of 37). This finding perhaps reflects the novelty of this procedure and the need to complete a high volume of procedures, which has been suggested as a requirement to gain and maintain adequate proficiency in this technique.³³ Still, considering transbronchial lung cryobiopsies, we found some important deviations of the current guidelines³⁴ and expert panel

standardization recommendations³⁵ concerning the utilization of fluoroscopic control, that according to our results, was not available in one center. This practice is not in line with the safety recommendations for this procedure. Though we could not assess other in-depth details, outcomes or complications, we strongly underline the need to comply with the currently available best practice recommendations^{34,35} to avoid unnecessary risks.

Another important finding of our survey was the complete absence of offer, among all respondent centers, of two important and validated endobronchial therapies: bronchial thermoplasty (recommended for selected asthmatic patients in GINA grade 5 of symptomatic control, evidence B)³⁶ and bronchoscopic lung volume reduction strategies (recommended for selected emphysematous COPD patients with significant lung hyperinflation despite optimal medical care)³⁷. The reasons for this finding are out of the scope of this paper but may include national regulatory and reimbursement issues that deserve prompt elucidation.

They are crucial regarding infectious control measures, since it has been proved that not sufficiently protected healthcare workers attending or performing aerosol-generating procedures have a higher risk of infection.³⁸ In 2002, in the UK, a national survey reported that only 9% of physicians used four protective items: face mask, gloves, eye protection, and gown.¹² The recommendations of PPE during bronchoscopy have been upscaled since 2020 in the context of the COVID-19 pandemic and, again, most units comply with them. The awareness of safety issues of Portuguese bronchoscopists and staff attending the procedures increased noticeably after COVID-19, with 100% of centers using FFP2/N95 masks and eye protection. Recent studies have shown that most proceduralists understand the gravity of the situation and paid particular attention to the use of appropriate PPE,²² but in real clinical practice, and especially at the peak of the pandemic, some of the international recommendations were quite difficult to follow and implement.^{24,39}

In recent articles, authors report that transmission of SARS-CoV-2 seems to be low with consistent use of appropriate personal protective equipment by healthcare workers when all the measures are followed.^{40,41}

Meanwhile, on the 26th of November 2021, World Health Organization (WHO) designated the variant B.1.1.529 named Omicron as a variant of concern (VOC), with a considerable rise in cases of people testing positive for SARS-CoV-2 in South Africa in regions where the Omicron variant emerged.⁴² This variant, considered to be more contagious and associated with an increase in the risk of reinfection after a primary infection than the previous mutations, shows more than 30 mutations leading to amino-acid changes in the Spike sequence.⁴³

The question of severity and this new variant is not clear yet. However, it is suggested to be less aggressive.⁴⁴ Presently, there are too many confounding factors to compare patients with the Omicron variant with patients infected by other variants. In fact, COVID-19 severity and mortality vary enormously depending on the country, the prevalence of vaccination, the population's characteristics.

For these reasons, it is not expected that our level of care as healthcare workers will change in the near future.

There are some obvious limitations of our study and all surveys for that matter: answers rely on personal or

unit databases and, in some instances, on the memory of participants (there is no national database); lack of objectivity of some responders; differences in question interpretation; replies could not be verified; some topics were simplified and others not covered, to reduce the overall length and avoid a reduced response rate. We also acknowledge that there were questions regarding duration of practice but non-related to training or competence maintenance, and the design questionnaire was not able to assess the quality of procedures which is at least as important as the numbers, equipment or techniques attributed to each unit.

Conclusion

The present survey assessed present trends and the impact of COVID-19 in bronchoscopy practices in Portugal. It does not provide any recommendations or IP standards as with other questionnaires. It suggests that IP units in the country have a wide range of characteristics and behaviors, but during the COVID-19 pandemic, they complied with most international recommendations, as elective procedures were postponed and PPE levels increased.

Practice in the bronchoscopy field was previously guided by personal experience, determined by the peculiarities of a particular unit as new members would mimic and adopt the attitudes of older practitioners. Nowadays, there is the need to standardize practice and enhance training and competence maintenance of IP physicians within the country. The current survey helps to understand the reality and may settle the foundations to improve the IP field in Portugal, with the development of a future national database, homogenize procedures and protocols, and even organize and design better training programs.

Supplementary Material

Supplementary table 1. Pulmonology Departments invited to participate

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Declaration of interest

All authors declare no competing interests.

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Supplementary materials

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Chapter III

Tissue Engineering and Cell-based Therapies and its Applicability to Central Airway Obstruction

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Introduction

The human body has the incredible capacity to regenerate and biological systems are programmed to face injury, but this regeneration is limited and the extend and the repeated aggression may lead to the organ or system failure. Any injure beyond this critical size needs external support.

A plethora of efforts through the last years have been made and new therapeutic methodologies have been proposed and implemented, such as tissue and organ transplantation. The main problems concerning this solution are the dependence upon tissue or organ donors which can be rather scarce and comes with the risk of graft rejection due to immune response, the high biological risk of disease transmission and significant social and ethical concerns.

The role of Tissue Engineering in clinical practice

The Tissue Engineering (TE) and Regenerative Medicine (RM) arises to fulfil that empty space. Evoked in 1993 for the first time by Langer and Vacanted (1), his field of research combines the traditional concepts of medicine along with biotechnology, cellular systems and biomaterials and aims at repairing, regenerating or replacing dysfunctional cells, tissues or organs using allogenic or autologous cells, supporting scaffolding biomaterials, gene modification and molecular cues, alone or in combination (2).

Considering the problem vastly debated in chapter 1, the interest of RM in CAO has grown exponentially in recent 20 years. The basic principle behind TE of trachea is to manufacture a biocompatible scaffold that supports the growth and differentiation of the recipient's cells, to create a functioning neo-organ if the organ has exhausted its functional reserve or the implantation of a patch or a stent to promote reconstruction.

Embryologically the respiratory system arouses at the level of the primitive foregut. From there, it gives origin to the respiratory diverticulum that further develops into formation of trachea, bronchi, and lungs. All the process starts at approximately at 4 weeks. The endodermal part gives origin to the respiratory epithelium of the

respiratory diverticulum, while the connective tissue, cartilage and smooth muscle cells rise from mesodermal part.

Due its primary function of passive air conduction to the lungs and a relatively simple tubular anatomy, trachea was initially considered as a good starter organ (3) and historically many attempts were made with autografts, allografts and prosthetic materials (4-6).

The ideal scaffold produced by tissue engineering for respiratory system should be capable of promoting exogenous cell engraftment and endogenous cell ingrowth, proliferation and appropriate differentiation, while maintaining a patent airway. Mechanically, the main property must be the ability to withstand collapse under the physiological internal and external pressures during respiration (7). Some currents consider that the tracheal cartilage component is the key to its structural support and harvesting of cells for the generation of functional cartilage structures is a major challenge in tracheal reconstructing (8).

The function of a degradable scaffold is to act as a temporary support matrix for transplanted or host cells to restore, maintain or improve the tissue functional biomechanics.

There are two main classes of biomaterials based upon their source: natural or synthetic.

Naturally-occurring biomaterials like collagen, chitosan, hyaluronic acid, alginate, etc, are frequently used because of their biocompatibility, biodegradability and abundant availability. As they are naturally present in extracellular matrix (ECM), these biomaterials are cyto- and biocompatible and it is observed cells' positive growth response.

Synthetic biomaterials could be either modified naturally-occurring biomaterials or completely synthetic ones. Non-degradable synthetic materials are widely used in medicine as orthopaedic implants, sutures, or catheters. They are mostly polyethylene derivates, polyethers, polyacrylamides among others, etc. The class of degradable biomaterials include polyesters, polylactones, polyorthoesters, polycarbonates, polyanhydrides and polyphosphazenes. They are not only used as scaffolds but also as drug delivery systems. These materials can be tailored to defined degradation kinetics along the desired growth factor releases rates (9).

To date, two main scaffold strategies have been proposed in tracheal TE: decellularized human cadaveric donor tissue or synthetic scaffolds created *de novo* (7). The first consists in removing all cellular compounds from cadaveric tissue of a donor before the scaffold is seeded with autologous cells, to decrease the probability of it being recognized as non-self-recognized and avoiding an immune response. The limitation of this strategy are the need of a donor, the long duration of decellularization process and the risk of contamination and difficult neovascularization (7). The synthetic strategy employs the referred synthetic biomaterials (polymers) to create an entire scaffold (10). These scaffolds are easy to manufacture and sterilize, carry few ethical concerns and they can be produced in a fully customized way. Clinical applications of scaffolds from this material were described only in the last decade when the synthetic scaffolds were seeded with autologous progenitor cells and after implanted in patients. Since then, considerable efforts are being devoted to optimizing the production and biocompatibility of synthetic materials for TE (11,12)

Although all the efforts, there is no international consensus on the ideal biomaterial for supporting tracheal regeneration. The results of pre-clinical *in-vivo* studies are difficult to compare between clinical and research centres due to the methodological differences.

In our group we used as biomaterial polycaprolactone (PCL), a hydrophobic, semicrystalline, non-toxic, biodegradable polymer with good mechanical properties. Its good solubility in common solvents, low melting point (50-64°C), Food and Drug Administration approval and outstanding blend compatibility has led it as a biomaterial with particularly good potential of application in biomedical field. PCL is easily processed and it's easy to manipulate into a broad range of shapes and sizes and it has been successfully incorporated in several medical devices.

Alternative TE approaches proposes the use of patient's own cells to manufacture the tissue of interest to bypass the need for anti-rejection medications, thus avoiding that the relevant challenges that modern transplantation faces, namely the chronic toxicity and the increased risk of tumour development in patients suffering from lifelong immunosuppression (13).

The main sources of cells used in TE of the trachea are mesenchymal stem cells (MSCs), chondrocytes, hematopoietic stem cells (HSCs) and epithelial cells (14).

Mesenchymal Stem/ Stromal Cells (MSCs) present themselves as promising cellular systems to address this need. They can be obtained from a variety of tissue sources and have been investigated and demonstrated to bare noteworthy benefits to tissue regeneration *in vivo* (15, 16). A dominant part of these beneficial effects is assigned to the secretory capacity of these cells, through which they produce and release an array of bioactive molecules that impact the course of tissue healing (17).

MSCs can be isolated from bone marrow (18), adipose tissue (19), Wharton´s Jelly (20), amniotic fluid (21) and dental pulp (22), among other tissues.

The trophic factors produced by MSCs can promote angiogenesis, inhibit the formation of scar tissues, remodel ECM and stimulate the normal regenerative sequence of local tissues. Because of the lack of specific cell marker, the International Society for Cellular Therapy established the minimum criteria to be classified as an MSC: 1)adherence to plastic under standard culture conditions; 2) expression of CD105, CD 73 and CD90; 3) lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leucocyte antigen -DR; and 4) differentiation into osteoblasts, adipocytes and chondroblasts following *in vitro* culture under specific conditions (23). It is important to recognize that MSC initially were thought because of its regenerative potential and the capacity of differentiate into several cell types of the mesoderm lineage. Subsequently they were also found to display enormous effects on all cells involved in immune response with the end result of dampening inflammation and favouring tolerance (24). Notably, unstimulated MSC lack of expression of class II human leucocyte antigens and co-stimulatory molecules on their surface while displaying expression of class I leucocyte antigens in a very low level. This is why MSC use does not require an immunoablative conditioning regimen, even when using an allogenic source (25).

Our research was based on MSCs isolated from definitive teeth dental pulp. They are a popular choice for recellularization the trachea since they have the ability to differentiate in bone, fat, and cartilage (26), produce immunomodulatory factors (27), they are easy to isolate and ability to expand *in vitro* (28).

Alone, cells have growth and proliferation limitations, and trachea scaffolds may be required to allow the development of new tissues. Scaffolds provide support, allow cells' attachment, giving strength yet flexibility, help cells to proliferate, and can degrade at an appropriate rate to allow tissues to form a natural structure (29).

There are several methods for scaffold fabrication from different biomaterials. Not all the biomaterials are suitable for every fabrication method, thus they are always being modified to enable their use in each fabrication method. Conventional fabrication methods include electrospinning, phase separation, freeze drying, self-assembly, solvent casting, textile technologies, material injections and additive manufacturing (3D printing) (30).

In this chapter, we present a preliminary work where, considering the problematic of CAO, we approach the biocompatibility of a biomaterial that could be used clinically in the future. Our aim is to study the cytocompatibility and biocompatibility of 3D printed PCL based devices after sterilization with different levels of gamma radiation. Ionizing is likely the method of choice and gamma radiation represents the most extensively studied sterilization method for PCL (31, 32). Gamma radiation is highly penetrative and kills bacteria by breaking down bacterial DNA, thereby inhibiting bacterial division. On the other side, such photon-induced damage at the molecular level can cause also physical and chemical properties of the polymer. A minimum dose of 25kGy is routinely applied for sterilization of many medical devices and biological tissues. As recommended by the International Organization of Standardization (ISO), the sterilization dose must be set for each type of product depending on its characteristics and the load of microbes (31).

The choice for the biomaterial relied on the premise that, in future assays, the device will be used to promote regeneration of tracheal tissue. Biomaterial production and characterization studies, *in vitro* and *in vivo* assays were conducted as to assess on the gamma radiation effect on the device's properties, the first applying evaluating cytocompatibility with MSCs from the dental pulp tissue of human origin (hDPSCs) and the latter considering subcutaneous implantation on a rat animal model for evaluation of the biocompatibility according to ISO 10993-6: 2016 guidelines for "Biological evaluation of medical devices, Part 6: Tests for local effects after implantation". Solvent casting method was used for the preparation of PCL scaffolds and cylindrical scaffolds of PCL were produced using an additive manufacturing system.



Article

Gamma irradiation processing on 3D PCL devices - a preliminary biocompatibility assessment

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Abstract: Additive manufacturing or 3D printing applying polycaprolactone-(PCL)-based medical devices represents an important branch of tissue engineering, where the sterilization method is a key process for further safe application *in vitro* and *in vivo*. In this study, the authors intend to access the most suitable gamma radiation conditions to sterilize PCL-based scaffolds in a preliminary

biocompatibility assessment, envisioning future studies for airway obstruction conditions. Three radiation levels were considered, 25 kGy, 35 kGy and 45 kGy and evaluated as to their cyto-and-biocompatibility. All three groups presented biocompatible properties, indicating an adequate sterility condition. As for the cytocompatibility analysis, devices sterilized by 35 kGy and 45 kGy showed better results, with the 45 kGy showing overall better outcomes. This study allowed to select the most suitable sterilization condition for PCL-based scaffolds, aiming at immediate future assays, by applying 3D-customized printing techniques to specific airway obstruction lesions of the trachea.

Keywords: gamma irradiation; 3D polycaprolactone; mesenchymal stem cells; cytocompatibility; biocompatibility

1. Introduction

Several fabrication techniques have been used to create clinically applicable scaffolds with uniform pore size and control over geometry. Additive manufacturing (AM), also known as 3D printing, enables customize fabrication of 3D constructs based on computer-aided design software or images obtained from computer tomography and magnetic resonance. There are several AM techniques, among which only some techniques are widely applied in the medical industry [1]. The most widely used are droplet-based printing [2], and extrusion-based printing [3]. Tissue engineering (TE) with 3D printing is focused on two different perspectives: functional biomaterials for tissue implantation and tissue models for disease [1]. This study will focus on biomaterials based on the first option.

Scaffolds have found their place as templates for cell interaction, providing physical support to the afresh developed tissue [3]. Furthermore, scaffolds can function as delivery vehicles to incorporate essential growth factors to control and enhance tissue growth [4]. The aim of 3D bioprinting is to mimic the natural cellular architecture by depositing materials and cells to restore the normal structure and functionality of complex tissues. TE scaffolds are fabricated in two major methods, printing with cells mixed in ink or gel or seeding cells onto scaffolds post printing.

The role of airway TE, a field of regenerative medicine, is to develop biological substitutes that can restore, maintain, or improve tissue functions. As a “simple” cylinder and with a relatively main function, which is to conduct the air, trachea was initially considered as a good starter organ for TE and historically many attempts were made with autografts, allografts, and prosthetic materials [5, 6]. The ideal tissue engineered scaffold for airway would be capable of promoting exogenous cell engraftment and endogenous cell ingrowth, proliferation, and appropriate differentiation, while maintaining a patent airway.

In the context of airway TE, central airway obstruction (CAO) represents a pathological condition that leads to airflow limitation of the trachea, main stem *bronchi*, *bronchi intermedius* or *lobar bronchus*. That represents an important clinical impairment and can be caused either from benign or malignant diseases [7, 8]. The incidence is still not well known, but it tends to be underdiagnosed [9]. While the surgical procedure is considered the gold standard, several clinical conditions concerning the clinical condition of the patient, the degree and the type of CAO could compromise the feasibility of the standard therapy [10]. In those cases, interventional bronchology with its accessory techniques such as laser, cryotherapy dilatation or airway stenting could represent a good option [11, 12]. Airway stenting could be used in both benign and malign disease, but placing the stent must be a very well balanced decision because if for one hand the re-occlusion is prevented, on the other hand it has also disadvantages such as stent migration, mucous plugging, recurrent infection or fistulation [10, 13]. There are two main types of airway stenting concerning the material: silicone stents and metallic stents. new custom-made and bioabsorbable airway stents made of different biomaterials are under

investigation and have been placed in humans. Polydioxanone is the one that has been most often used and for the longest period [14]. Other biomaterials like polylactic acid, polyglycolic acid, polycaprolactone, polyurethane or polyamide are under investigation [15, 16]. Biodegradable polymers such as polycaprolactone (PCL) are increasingly used for 3D printing of scaffolds. This material offers great advantages such as biocompatibility, biodegradability, and good mechanical properties [17]. However, to be approved for human implantation, they must be in sterile conditions. Numerous techniques have been researched, all of them subject of limitations [18]. The most frequently used are ethylene oxide, beta radiation, gamma radiation, peracetic acid and hydrogen-peroxide plasma. Steam and heat sterilization are not practicable in this polymer as it has a melting point of 59–64°C. Ethylene oxide could be an option, but it is known to soften PCL and its residual vapors left in the device found to be mutagenic and carcinogenic [19]. Thus, ionizing is likely the method of choice and gamma radiation represents the most extensively studied sterilization method for PCL [20, 21]. Gamma radiation is highly penetrative and kills bacteria by breaking down bacterial DNA, thereby inhibiting bacterial division. On the other hand such photon-induced damage at the molecular level can also cause changes in the physical and chemical properties of the polymer. A minimum dose of 25 kGy is routinely applied for sterilization of many medical devices and biological tissues. As recommended by the International Organization of Standardization (ISO), the sterilization dose must be set for each type of product depending on its characteristics and the load of microbes [20].

The most important properties of a bioabsorbable scaffold are the degradation rate, mechanical strength, and ability to support cell growth. Gamma rays at 30.8 kGy significantly decrease the rate of degradation of PCL, without affecting molecular weight nor cells attachment and growth. Considering mechanical properties, yield stress increased significantly but not the stress at break. In the case of the scaffold it represents an important support rule as in the airway, this is of the upmost relevance [22]. Augustine *et al* report a low radiation dose first would lead to improved PCL mechanical properties, however, higher doses would decrease them. Thus, results for the effect of gamma radiation on the mechanical properties are ambiguous and a general trend has not yet been established.

In this preliminary work the authors intended to study the cytocompatibility and biocompatibility of 3D printed polycaprolactone (PCL) based devices [23] after different gamma radiation conditions. The choice for the biomaterial relied on the premise that, in future assays, the device to be studied to promote regeneration of tracheal tissue must have a design that mimics the anatomical shape of trachea, must have mechanical strength and flexibility like native trachea and porosity that allows good vascularization and cell proliferation. In addition, it must be biocompatible, biodegradable and non-immunogenic [24]. Furthermore, PCL allows the production of 3D devices with interconnected porous network and high reproducibility [25]. Following the biomaterial production and characterization studies, *in vitro* and *in vivo* assays were conducted as to assess on the gamma radiation effect on the device's properties, the first applying mesenchymal stem cells (MSCs) from the dental pulp tissue of human origin and the latter considering subcutaneous implantation on a rat animal model.

2. Results

2.1. Devices Characterization

FTIR spectra of the scaffolds sterilized with different conditions are depicted in Figure 1. All samples present similar results in terms of chemical structure, with the characteristic bands of pure PCL. The bands at 2865 and 2941 cm⁻¹ are related with the symmetric and asymmetric stretching of the CH₂ group. A strong absorbance at 1720 cm⁻¹ also corresponds to a structural group of PCL, more precisely C=O stretching vibration of the ester linkages. Figure 2 shows the results of the SEM examination of the PCL scaffolds

gamma irradiated up to 45 kGy. All samples present micropores in the filament surface, indicating that the sterilization at 25, 35 and 45 kGy has no effect on the morphology of the filaments.

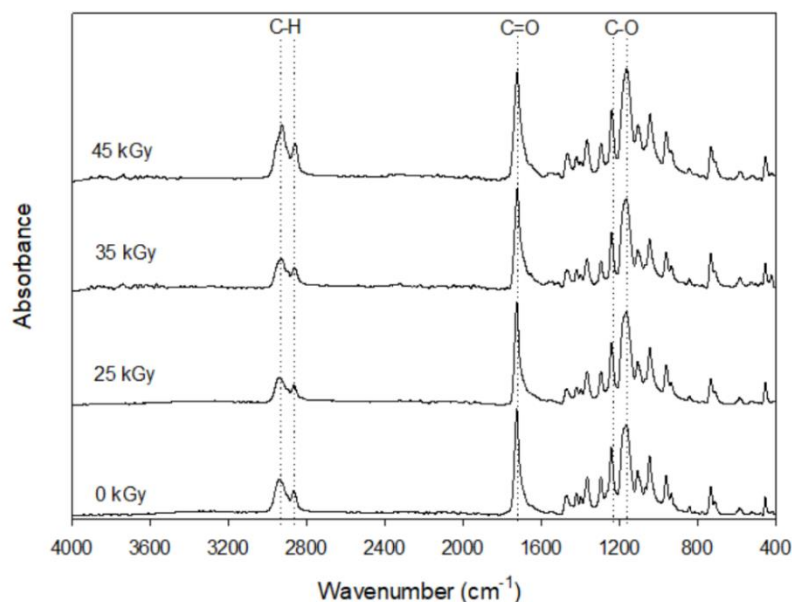


Figure 1. FTIR spectra of PCL scaffolds sterilized with different gamma radiation conditions.

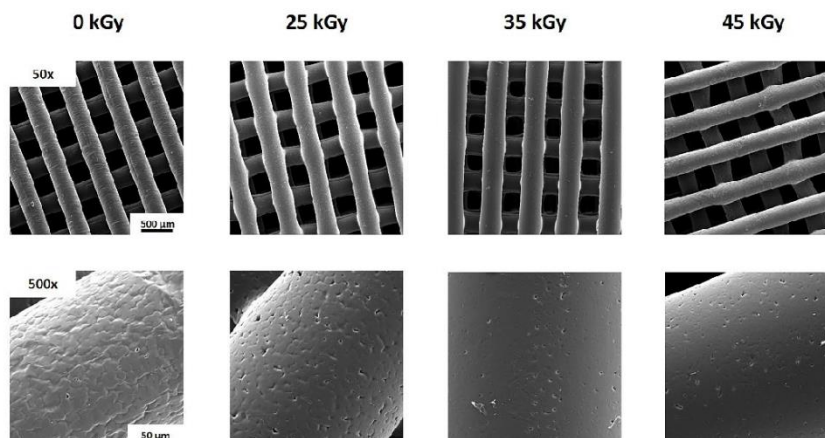


Figure 2. SEM images of gamma sterilized and unsterilized PCL scaffolds.

2.2. *In vitro* cytocompatibility assessment

A PrestoBlue™ cytocompatibility assessment was conducted on the produced scaffolds, following sterilization. Scaffolds were divided into three groups, considering 3 different levels of sterilization: 25 kGy, 35 kGy and 45kGy. A control group of the cell population was considered, by seeding cells directly to the well with no scaffold, as to

access cell normal behaviour and proliferation in culture. For each time-point, corrected absorbance values were obtained for each group and are presented in Figure 3 (upper panel) and Table 1.

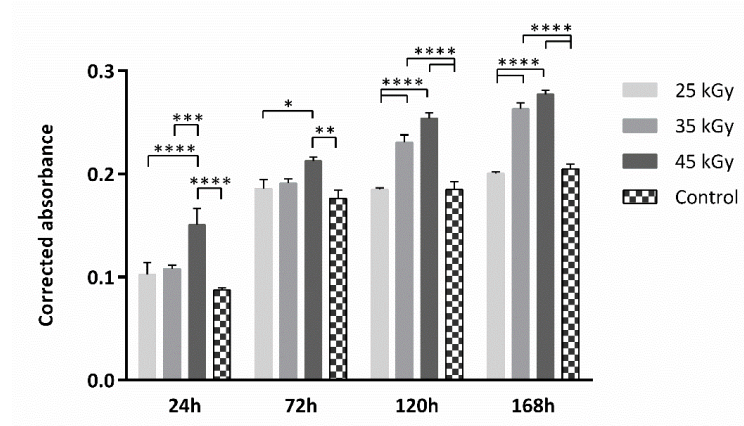


Figure 3. Presto Blue™ cytocompatibility assessment with hDPSCs. Results presented as mean ± SE. Results' significance is presented through the symbol (*), according to the p value, with one, two, three or four symbols, corresponding to $0.01 < p \leq 0.05$; $0.001 < p \leq 0.01$; $0.0001 < p \leq 0.001$; and $p \leq 0.0001$, respectively.

Table 1. Presto Blue™ cytocompatibility assessment with hDPSCs. Results presented as mean ± SE.

	25 kGy	35 kGy	45 kGy	Control
24h	0.102 ± 0.012	0.108 ± 0.003	0.151 ± 0.016	0.087 ± 0.002
72h	0.186 ± 0.009	0.191 ± 0.004	0.213 ± 0.004	0.176 ± 0.008
120h	0.185 ± 0.002	0.231 ± 0.007	0.254 ± 0.005	0.185 ± 0.007
168h	0.200 ± 0.002	0.263 ± 0.006	0.277 ± 0.004	0.205 ± 0.005

Human dental pulp stem/stromal cells (hDPSCs) were employed in this assay, following previous works [26–28]. These cells' population regenerative potential towards the osteogenic lineage has been established by Campos *et al* [29] and was selected for the purpose of this study, as the authors intend to further analyse the regenerative potential of PCL-based devices for tracheal airway-obstruction cases.

Following the Presto Blue™ cytocompatibility assessment, samples were processed for Scanning Electronic Microscopy (SEM). Seeded and unseeded devices were considered. Images are presented in Figure 4.

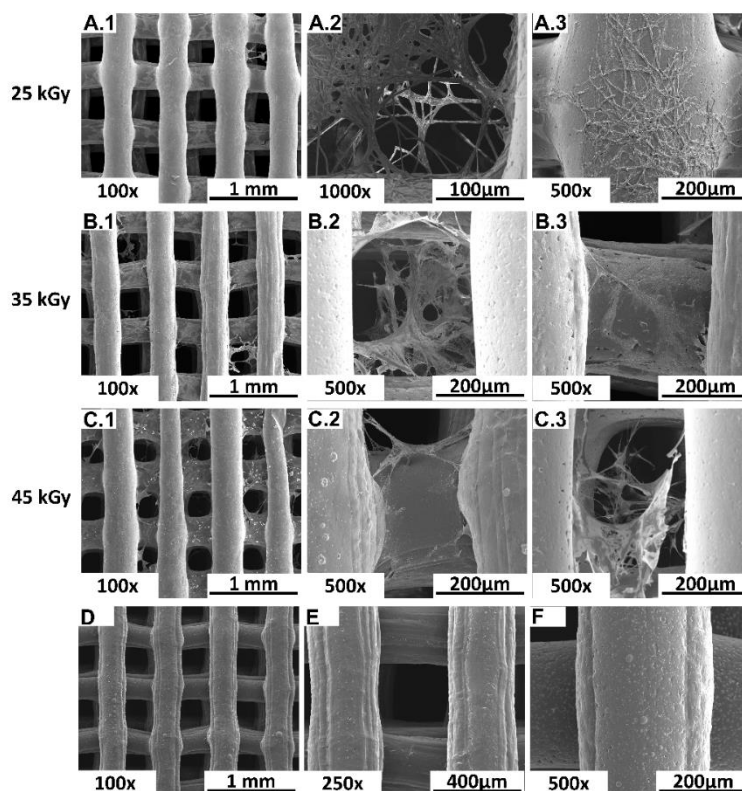


Figure 4. SEM images obtained from the 3D PCL devices seeded with hDPSCs. A, B and C representing seeded scaffolds sterilized by 25 kGy gamma radiation 35 kGy and 45 kGy, respectively. D, E and F represent the unseeded devices, sterilized by 25 kGy gamma radiation 35 kGy and 45 kGy, respectively. Left panel with 100x magnification and middle and right panel with 500x magnification.

SEM analysis allowed to visualize adhered cells to all the devices, presenting a fibroblast-like shape, normal morphology and adequate adhesion. Cells presented elongations of the cytoplasm, creating adhesion points between the device's fibers, thus creating a 3D cellular network. No differences could be qualitatively established between groups.

2.3. *In vivo* biocompatibility assessment

Following the *in vitro* assessment, devices were further assessed *in vivo* for their biocompatibility, according to ISO 10993-6:2016 guidelines for Biological evaluation of medical devices, Part 6: Tests for local effects after implantation. Scaffolds were implanted subcutaneously on the dorsum of Sasco Sprague-Dawley rats and analysed after 7 and 15 days post-implantation time. A semi-quantitative scoring analysis was performed, according to annex E of the referred guideline. Evaluation of the biological reaction to the devices included quantification of fibrosis, extent, inflammatory cells, necrosis, neovascularization, fat tissue infiltration, among others. Results are presented in Figure 6 and 7 and Table 2. Furthermore, samples were embedded in paraffin and analysed by SEM. The obtained images are presented in Figure 5.

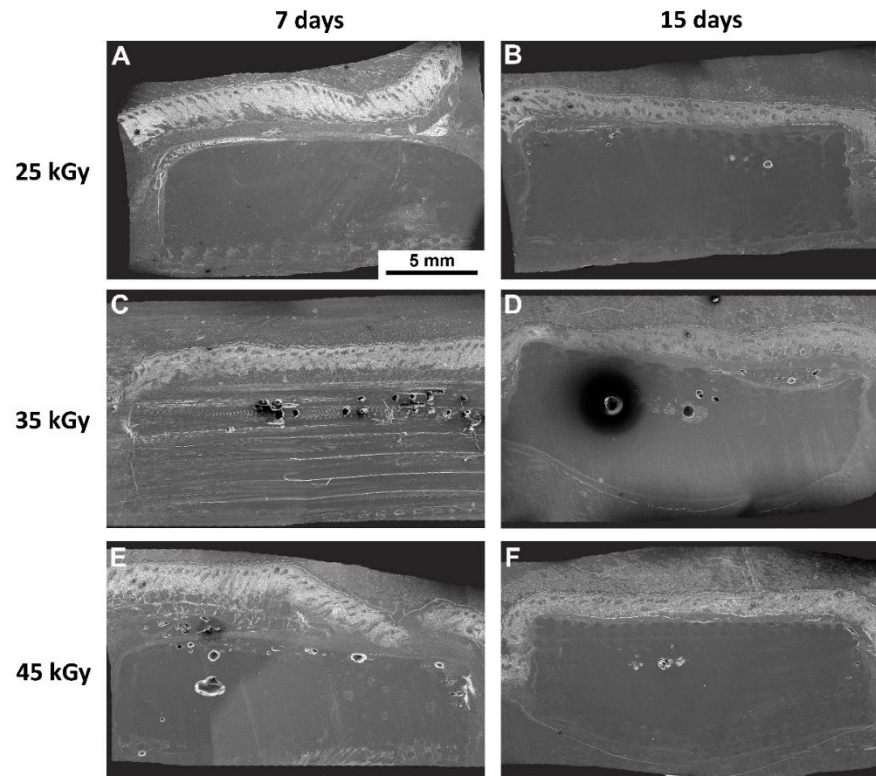


Figure 5. SEM images of the subcutaneously implanted 3D PCL devices in the rat animal model. Representing the left panel and the right panel, 7 days, and 15 days recovery time, respectively. A and B devices were sterilized by 25 kGy gamma radiation, C and D by 35 kGy and E and F by 45 kGy. Magnification of 20x and scale bar 5 mm.

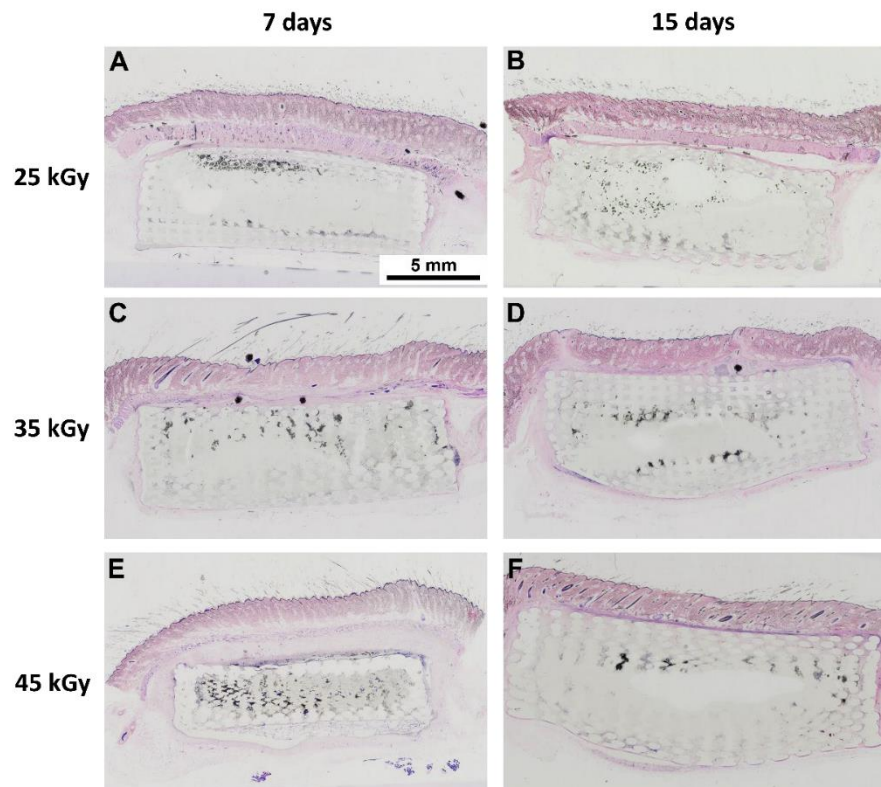


Figure 6. Histological images, stained with H&E, scanned using an Olympus Virtual Microscopy System VS110TM at 20x magnification, of the subcutaneously implanted 3D PCL scaffolds in the rat animal model. Representing the left panel and the right panel, 7 days, and 15 days recovery time, respectively. A and B scaffolds were sterilized by 25 kGy gamma radiation, C and D by 35 kGy and E and F by 45 kGy. Scale bar 5 mm.

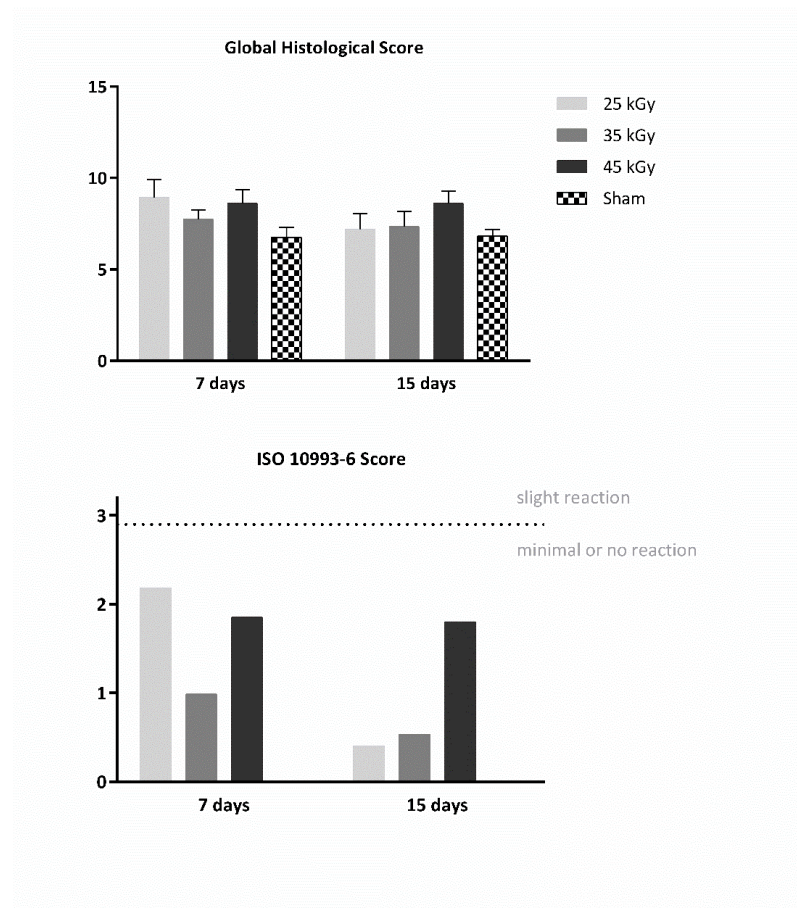


Figure 7. Global histological score (upper panel) and calculated score, according to guideline ISO 10993-6:2016 (lower panel), for subcutaneous implantation (biocompatibility assessment) of the PCL devices, after 7- and 15-days implantation time.

Table 2. Global histological scores presented as mean $\pm$ SE following ISO-10993-6 guidelines for the PCL devices groups and sham, at 7 and 15 days after implantation.

	Sham	25 kGy	35 kGy	45 kGy
7 days	6.750 $\pm$ 0.532	8.933 $\pm$ 0.959	7.733 $\pm$ 0.521	8.600 $\pm$ 0.748
ISO SCORE	-	2.183	0.983	1.850
15 days	6.800 $\pm$ 0.396	7.200 $\pm$ 0.846	7.333 $\pm$ 0.826	8.600 $\pm$ 0.675
ISO SCORE	-	0.400	0.533	1.800

The macroscopical evaluation of the samples revealed no signs of hemorrhage, infection or inflammation. All samples revealed, microscopically, minimal fibrosis extent, and non-detectable or rare necrosis or giant cells presence. Neovascularization was detected for all groups, as well as polymorphonucleated (PMN) cells, the latter decreasing in the latest timepoint. Following the pre-established criteria, all groups were considered biocompatible, presenting a score value contained in the “minimal or no reaction” category.

3. Discussion

FTIR analysis was performed before and after the use of different gamma irradiation conditions to evaluate any alterations in functional groups during sterilization. Comparing the spectrum of PCL scaffolds without sterilization with the spectra of sterilized PCL scaffolds, it can be seen that after gamma irradiation there were no evident modifications in the bands. These results corroborate with Tapia-Guerrero et al. and Paula et al. works that reported no significant changes in the PCL functional groups after sterilization using gamma irradiation [30]. SEM analysis was employed to determine the morphological features of the scaffolds. The microporosity observed on the surface of the filaments was caused by the material preparation method, more precisely due to the solvent addition (Biscaia). Thus, gamma irradiation had no influence on the filament morphology of PCL scaffolds.

A thorough cytocompatibility analysis was performed, including a qualitative SEM analysis of the *in vitro* seeded PCL-based scaffolds with hDPSCs. This assay allowed visualization of cellular adhesion, as well as a cellular layer formation in the 3D scaffolds, with a uniform distribution (Figure 4), thus confirming the analyzed scaffolds present potential cellular matrix conditions for 3D culture. Furthermore, SEM images of the subcutaneously implanted 3D PCL-based scaffolds in the rat animal model allowed visualization of the 3D structure integrity of the scaffolds (Figure 5), as well as tissue integration, which was validated by the histological scanning of the samples, with H&E staining (Figure 6).

As for the quantitative analysis, a Presto Blue™ viability assay confirmed the scaffolds cytocompatibility, thus sustaining the qualitative assessment by SEM analysis. All groups presented overall promising outcomes, with the 35 and 45 kGy presenting slightly better results, compared to the 25 kGy group. The control group was only considered to assess on the cellular population health, proliferation and normal behavior in culture, and direct comparisons between this group should be taken carefully, as in contrast with the tested groups, this control group is a 2D culture condition, as no matrix supporting biomaterial is considered. When considering tissue regeneration, 3D culture conditions are more reliable in mimicking the *in vivo* environmental conditions, when compared to 2D cultures. The latter are often associated with higher proliferation rates, but also with loss of diverse phenotype and metabolism alterations, thus compromising comparability with *in vivo* conditions [31, 32]. For this matter, 3D culture conditions are more likely to mimic the *in vivo* settings, as cell-extracellular, and cell-to-cell interactions are more precisely evaluated. Further, cells have variable access to nutrients and oxygen, as in *in vivo* conditions.

Considering the biocompatibility assay, the rat animal model was employed, and the scaffolds were implanted subcutaneously on the animal dorsum, considering 7- and 15-days recovery period. A semi-quantitative evaluation was performed, according to ISO 10993-6: 2016 guidelines for “Biological evaluation of medical devices, Part 6: Tests for local effects after implantation”. A scoring system was applied (according to Annex E) when evaluating the biological response, as the fibrosis extent, changes in tissue morphology, necrosis presence, vascularization, fatty infiltration, and the presence of inflammatory cells. Upon euthanasia, *ex vivo* tissue presented no abnormalities, with no visible hemorrhage or inflammation/infection. A global histological score was calculated for each group and a score was determined for each experimental sample, by subtracting

the sham group effect, associated with the intrinsic healing capacity (Table 2 and Figure 7). Microscopically, minimal fibrosis and neovascularization were detected in all samples at 7 days post implantation. Necrosis events and giant cells were rarely signalized, and mononuclear inflammatory cells (lymphocytes and macrophages) existed at a greater extent, when compared to polymorphonuclear cells, also for all groups and timepoints. A slight increase in neovascularization and decrease in fibrosis was detected at 15 days post implantation. According to the ISO 10993-6 scoring system, all samples were classified as “minimal to no reaction” at both timepoints, thus confirming its’ biocompatibility ability and suitability for *in vivo* implantation. In addition, several organs were further analyses, as to access to systemic effects through a necropsy exam and microscopic examination. Different organs were considered, as lungs, liver, heart, spleen, and pancreas. No alterations were detected.

4. Materials and Methods

4.1. Material Preparation and Devices Production & Characterization

Solid pellets of PCL (ϕ ~3 mm, MW 50 000, from Perstop Caprolactones (Cheshire, UK)) were dissolved in N,N Dimethylformamide (DMF, from Merck KGaA®, Germany) by solvent casting technique, using the amount of 1 g of PCL in 4 ml of DMF [25]. After the full dissolution of the PCL, the solution was deposited into petri dishes and left to dry in fume hood until solvent evaporation, for further use in a 3D printing system. The Biomate equipment was used to prepare the scaffolds [25, 33]. The 3D cylindrical scaffolds of 10 mm diameter and 2.5 height were produced using the following design parameters: 0°/90° laying pattern, 0.35 mm pore size and 0.3 mm filament diameter. Regarding processing parameters, the material was heated to 80 °C and deposited using a screw rotation velocity of 15 rpm at a working speed of 300 mm/min.

The 3D polycaprolactone (PCL) scaffolds were sterilized by gamma radiation (25 kGy, 35 kGy and 45 kGy), in a Red Perspex Dosimeters. Fourier Transform Infrared Spectroscopy (FTIR) were performed in the sterilized PCL scaffolds using Alpha FT-IR spectrometer (Bruker, Kontich, Belgium) and Opus Software. Samples were analyzed at room temperature, in a spectral range of 400-4000 cm⁻¹, with a resolution of 4 cm⁻¹ in a total of 64 scans.

PCL scaffolds morphology was also observed using a scanning electron microscope (SEM) (VEGA 3, TESCAN, Kohoutovice, Czech Republic) that was operated at a voltage of 15 Kv. Before observation, the scaffolds were coated with gold-palladium.

4.2. Sample Sterilization

The 3D PCL scaffolds were sterilized by gamma radiation (25 kGy, 35 kGy and 45 kGy), in a Red Perspex Dosimeters.

After sterilization, the surface morphology of all 3D constructs was analyzed by using a scanning electron microscope (SEM) (VEGA 3, TESCAN) that operated at a voltage of 15 kV, after coating the powders with gold-palladium.

4.3. *In vitro* assays

4.3.1. Cell culture and maintenance

Human Dental Pulp stem/stromal cells (hDPSCs) obtained from AllCells, LLC (Cat. DP0037F, Lot N° DPSC090411-01) were maintained in MEM α , GlutaMAX[™] Supplement, no nucleosides (Gibco, 32561029), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, A3160802), 100 IU/ml penicillin, 0,1 mg/ml streptomycin (Gibco, 15140122), 2,05 μ m/ml amphotericin B (Gibco, 15290026) and 10 mM HEPES buffer solution (Gibco, 15630122). All cells are maintained at 37°C, 80% humidified atmosphere and 5% CO₂ environment. Campos et al previously described the characterization of these cellular population [29].

4.3.2. Cytocompatibility evaluation

The cytocompatibility between the cellular system and the scaffolds was assessed by a Presto Blue™ assay, to determine the impact of the sterilization intensity on the adhesion and cellular viability. The Presto Blue™ assay, a viability assessment, analyses the permeability of cells to a resazurin-based solution. This solution allows to quantify cellular viability, by modifying the media color after metabolization of the reagent by viable cells.

This assay was conducted as described in previous works [25-27]. Briefly, scaffolds are pre-hydrated in complete FBS medium for 24 hours and then seeded through dynamic seeding, where the cellular suspension is incubated with the scaffolds in a roller bank, each with a 2.5×10^5 cellular suspension, for 8 hours, at 37°C, 80% humidified atmosphere and 5% CO₂ environment. Later, seeded scaffolds are transferred to a non-adherent 24 well plate and concealed with complete medium. Culture media is removed, and fresh media is added to every cultured well, at each time-point (24, 72, 120 and 168 hours). 10% (v/v) 10x Presto Blue™ cell viability reagent (Invitrogen, A13262) is added to each well and plates are incubated for 1 hour. Following, 100 µl of media is transferred to a 96 well plate absorbance is read at 570 and 595 nm. Dulbecco's phosphate-buffered saline solution (DPBS, Gibco, 14190169) is used to wash and remove the reagent from the wells, prior to adding fresh media. Absorbances were read at 570 nm and 595 nm with a Multiskan™ FC Microplate Photometer (Thermo Scientific™, 51119000), following manufacturing instructions. For this assay, blank wells were considered, containing the respective scaffold, but no cells. Absorbance data were collected for each well by subtracting values obtained at 595 nm from values obtained at 570 nm. Data was further corrected, by subtracting the average of the blank wells (average of 570 nm – 595 nm), from the absorbance values (570 nm – 595 nm) of each experimental well (seeded scaffolds).

A control of the cellular population was considered, where cells were seeded in 10% FBS supplemented media, directly in a tissue-treated 24 well plate, with a density of 7000 cells per cm², as to control cell normal growth and proliferation.

4.3.3. Scanning Electronic Microscopy (SEM)

Further, seeded scaffolds were fixated for SEM analysis, as described in previous works [25, 27]. Scaffolds were rinsed 3 times with a 0,1M HEPES (Merck®, PHG0001) buffer solution and left overnight in a fixative solution containing 2% glutaraldehyde (Merck®, G5882). A dehydration crescent alcohol series (50%, 70%, 95% and 99%) was conducted previously to the incorporation of hexamethyldisilazane (HMDS, Alfa Aesar, A15139). Samples were left overnight, as to evaporate remaining residues of the reagents.

Following, samples were coated with Au/Pd by sputtering (SPI Module Sputter Coater) for SEM analysis with a high resolution (Schottky) Environmental Scanning Electron Microscope with x-ray microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M in high vacuum mode.

4.4. *In vivo* biocompatibility assessment

Animal testing assays were conducted in conformity with the Directive 2010/63/EU of the European Parliament and the Portuguese DL 113/2013 with previous approval by the ICBAS-UP Animal Welfare Organism of the Ethics Committee (ORBEA) and by the Veterinary Authorities of Portugal (DGAV). Humane endpoints in agreement with the OECD Guidelines (2000) were followed. The *in vivo* biocompatibility assessment was performed in adult male Sasco Sprague-Dawley rats (Charles River, Barcelona, Spain) weighing 250-300g, as described in previous works [26-28]. An adequate environment for the animals was considered, with controlled temperature, humidity, and 12-12 hours light/dark cycles. Feeding included standard chow and water *ad libitum*. For the surgical procedure, anesthesia was administered intraperitoneally: Xylazine/Ketamine (Rompun®/Imalgène 1000®; 1,25mg/ 9mg per 100 g b.w.), following an aseptic skin

preparation of the dorsum. 15–20 mm long incision were performed, and scaffolds were implanted subcutaneously, following incision suture. Animals were recovered, evaluated, and returned to their housing groups. Shams were considered, where the surgical access was performed, but no medical device implanted. At 7- and 15-days recovery time, animals were subjected to anesthesia, as described above, and sacrificed, by lethal intracardiac injection (Eutasil® 200 mg/ml, 200 mg/kg b.w.). Skin and subcutaneous tissue was collected and fixated in 4% formaldehyde (Merck®, 100496).

Following, samples were processed for histopathological analysis, and stained with hematoxylin-Eosin (H&E). Stained sections were analyzed with a Nikon microscope (Nikon Eclipse E600) equipped with $\times 2$, $\times 4$, $\times 10$ and $\times 40$ objectives and coupled with a photo camera (Nikon Digital Sight DS-5M) equipped with a lens (Nikon PLAN UW 2X/0.06). Evaluation followed ISO-10993-6:2016 guidelines, annex E, and included inflammatory infiltration, fibrosis, angiogenesis and/or necrosis surrounding the implant. A scoring system was established, following a semi-quantitative classification of the implants as “minimal or no reaction” (score 0,0 up to 2,9), “slight reaction” (score 3,0 up to 8,9), “moderate reaction” (score 9,0 up to 15,0) or “severe reaction” (score > 15).

4.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6,00 for Mac OS x, GraphPad Software, La Jolla California USA. Triplicates were considered and results are presented as Mean $\pm$ Standard Error of the Mean (SE). Analysis was conducted by Two-Way ANOVA analysis with Tukey multicomparison test. Differences were considered statistically significant at $P \leq 0,05$. Results significances are presented through the symbol (*), according to the p-value, with one, two, three or four symbols, corresponding to $0.01 < p \leq 0.05$; $0.001 < p \leq 0.01$; $0.0001 < p \leq 0.001$; and $p \leq 0.0001$, respectively.

5. Conclusions

Tissue engineering of customized 3D printed medical devices relies on the premise of producing safe and implantable biomaterials, where the sterilization process plays an important role. Gamma radiation is one of the most employed sterilization processes, although the most suitable radiation level must be previously established for each study, depending on various conditions, such as the biomaterials' properties and medical condition in scope. The authors intend to investigate the potential of developing customized 3D printed PCL-based scaffolds for tracheal occlusion in CAO scenarios. For this purpose, this preliminary study was conducted, as to assess on the most suitable gamma radiation condition for PCL-based scaffolds sterilization. Higher levels from 35 to 45 kGy have presented better cytocompatibility outcomes, although 25 kGy presented equally good outcomes regarding biocompatibility after subcutaneous implantation in a rat animal model. Results suggest radiation levels of 35 kGy or 45 kGy to be safer and more suitable for the sterilization of these devices. Thus, the authors intend to continue this study, by applying these production and sterilization conditions to customized 3D-printed PCL scaffolds for trachea occlusion.

Abbreviations

AM – Additive manufacturing
 CAO – Central airway obstruction
 DMF – Dimethylformamide
 DPBS – Dulbecco's phosphate-buffered saline solution
 FBS – Fetal bovine serum
 hDPSCs – Human dental pulp stem/stromal cells
 HMDS – hexamethyldisilazane
 H&E – Haematoxylin-eosin
 MSCs – Mesenchymal stem cells
 PCL – Polycaprolactone
 SE – Standard deviation of the mean

SEM – Scanning electron microscopy

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Directive 2010/63/EU of the European Parliament and the Portuguese DL 113/2013. All the procedures were approved by the ICBAS-UP Animal Welfare Organism of the Ethics Committee (ORBEA) (Project 288/2018) and by the Veterinary Authorities of Portugal (DGAV). Humane end points were followed in agreement to the OECD Guidelines (2000).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author on request.

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Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of this article.

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Chapter IV

General Discussion

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Interventional Pulmonology

Access to the airways in the living patient was tried by Hippocrates (460–370 BC), who advised the introduction of a pipe into the larynx in a suffocating patient. At the end of the 19th century, and combining three important inventions, the local anaesthesia, the use of electricity as a light source and the development of instruments for inspection of the upper respiratory tract, Gustav Killian showed to the world the first bronchoscopy. After that, generations of physicians were taught about technique of bronchoscopy, thereby spreading his beneficial invention all over the world. Throughout his life, he continued to improve instruments and applications. In the following years, Chevalier Jackson in the US (1) and Ino Kubo in Japan gave important steps forward (2). They continued Killian's works, improving the instruments and technologies for diagnosis and treatment of tracheobronchial and pulmonary diseases. It was only after Shigeto Ikeda had introduced the flexible fiberscope in 1966 that bronchoscopy became routinely performed in specialized centres. The advent of interventional techniques such as Nd:YAG laser application, cryotherapy and stenting, has led to the spread of the term "Interventional Pulmonology". Interventional pulmonology is a field of pulmonology which describes the use of techniques for the diagnosis and treatment of thoracic disorders.

In the context of this document, the term Interventional Pulmonology describes a field within pulmonary medicine focused on the use of advanced bronchoscopic and pleuroscopic techniques for the treatment of a spectrum of thoracic disorders.

CAO is a challenge far from being overcome

As extensively debated in the article "*Central airway obstruction: is it time to move forward?*", CAO represents a challenge which demands a multidisciplinary approach.

Its complexity related to its multiple aetiologies and different expressions makes its therapeutic approach a challenge. It is recognized that management should have a multidisciplinary team discussion to cover all possible treatments and to select the best therapeutic options, as previously stated. As discussed, in our study, definitive management may include from simple observation in the pauci-

symptomatic cases to medical or surgical options. Tracheal bronchial sleeve resection is considered to be the first option and the definitive treatment in the treatment of complex airway stenosis (3). Nevertheless, bronchoscopic management in selected patients is reported to have high success rate (4, 5). Complex stenosis may also require stent placement, sometimes working as a bridge to definitive treatment, or only in a palliative way (6).

We presented three representative cases of different causes and severity of CAO, in which the decision was the placement of a silicone airway stent. In the first case, a typical post orotracheal intubation stenosis, the therapeutic option was to perform mechanical dilatation with a balloon through a rigid bronchoscope. Although an immediate effect of relief was achieved, it only lasted a few days. In this case, because of the co-morbidities of the patient, the option was to place a stent, which was effective during the first month. After that, complications, such as migration and stent infection, unfortunately lead to patient death. In the second case, we describe a clinical case of CAO caused by extrinsic compression caused by a thoracic aorta aneurism. A silicon Dumon stent was placed as a bridge to endovascular correction of the aneurism, but the aneurism correction was not effective, and the compression persisted, as well the need of the stent. In this case, the outcome was the patient death caused again, by a stent complication, in this case, a fistulisation of considerable diameter. In the third clinical case, a patient with a large tracheal stenosis, caused by a voluminous adenopatic conglomerate of the mediastinum irresponsive to chemotherapy and radiotherapy, demonstrated a relief of dyspnoea with an effective palliative airway stenting. The death of patient resulted by the progression of the cancer.

In our opinion, the response to que question “is time to move forward?” is between the lines considering the three cases presented. Although the procedures were all “by the book” and using all the options according to the “state-of-the-art”, the outcome was not the desired one. Even if we consider that the third case was a small cell lung cancer whose prognosis is very poor (7), that was the only one that we can consider of success because our objective was only a palliative relief of the dyspnea, which was achieved for a short period of time. In the other cases the fatal outcome was caused by known complications of airway stenting (8).

The step forward, in our opinion, could be based on tissue engineering and regenerative medicine. When stenting is not possible, there are other options that range from autografting and allografting to the use of prosthetic materials (as

showed in Table 4 of our article “*Central airway obstruction: is it time to move forward?*”).

Our purpose is to create a tissue-engineered tracheal absorbable synthetic 3D bioprosthesis coated with MSCs to promote *in vivo* tracheal regeneration in the cases of CAO. The scaffolds to be used to promote regeneration of tracheal tissue must have some specific characteristics, namely: design that mimics the anatomical shape of the trachea or defects to be filled; mechanical strength and flexibility identical to native tracheal tissue to prevent collapse; porosity that allows good vascularization and cell proliferation. In addition, the scaffold must be biocompatible, biodegradable, non-toxic and non-immunogenic, to ensure a good cytocompatibility and to not trigger organic reactions and rejections (9).

The article “***Gamma irradiation processing on 3D PCL devices - a preliminary biocompatibility assessment***” represents our preliminary work and it is developed later in this chapter.

Although this is an exciting new world waiting to be discovered, this reality is far away from being achieved. A recent evolutionary step was made when manufacturers offered modifications of their products according to patient’s needs, with rapid prototyping 3D printing techniques, allowing stents to be tailored to the individual’s airway in days or even hours (10). If on one hand 3D printing technology could become accessible for physicians, on the other hand there are no certified manufacturers nor a direct access between parts. Maybe a creation of a software easy-to-use between hospitals and manufacturers could provide that answer but there are still ethical and legal issues to overcome.

The COVID-19 pandemic

On 2nd March 2020 a medical doctor which came from Italy from vacances tested positive from COVID-19 in Centro Hospitalar e Universitário do Porto (CHUP). It was the first case of the pandemic which emerged in Wuhan at the beginning of December 2019 and in that day the pandemic officially started in Portugal.

Coronavirus disease 2019 (COVID-19) is an emerging infectious disease caused by SARS-CoV-2 pathogen. Its capacity for human-to-human transmission through respiratory droplets, coupled with a high-level of population mobility, has resulted in a rapid dissemination worldwide. Healthcare workers have been particularly

exposed to the risk of infection and represent a significant proportion of COVID-19 cases in the worst affected regions of Europe(11).

Like other open airway procedures or aerosol-generating procedures, bronchoscopy poses a significant risk of spreading contaminated droplets, and medical workers must adapt the procedures to protect patients and staff (12)

The impact in IP procedures were impressive, with some units stopping to perform exams during the first weeks. Although the panic generated of the pandemic, immediately the patients with more serious pathology like oncologic patients starts to feel the negative effect of not having the procedures available, either diagnostic and therapeutic(13).

The urgency of rapid responses led the scientific societies of pulmonology around the world to publish recommendations for the IP procedures during pandemic. In Portugal, the Portuguese Society of Pulmonology empowered me as Coordinator of the Bronchoscopic Working Committee to chair a consensus group. Seven national IP specialists were selected based on their clinical expertise and different settings (private/state, pulmonologists/critical care specialists, general hospital/oncological hospital). In this context it was published the document ***“Recommendations for interventional pulmonology during COVID-19 outbreak: a consensus statement from the Portuguese Pulmonology Society”***.

This document, focused on needs of the community of IP, purpose adaptations in the department in administrative and organizational issues, environment control and individual protection. Administrative procedures as phone call to every patient, RT-PCR tests 24-48h before and reorganization of space are other important measures to avoid prolonged exposition to the patients and diminishing the risk. The environmental organization considered rooms with negative pressure and HEPA filters, with air renewal twelve times/hour or alternatively natural ventilation of 160l/s. 30-minute wait between exams for aerial decontamination was advised. The individual protection was purposed to be biological safety level 3, with disposable and waterproof material, two pairs of gloves, googles or facial mask, cap, and masks FFP2/FFP3. A prioritization model according to the urgency of the exam was suggested.

Those recommendations were of the utmost utility, mostly considering that the information regarding the disease and its transmission, was far away from the knowledge we have nowadays. Rapidly they were implemented on a vast majority

of IP units as observed in the study **“Pre- and post-COVID practice of interventional pulmonology in adults in Portugal”**. Although we do not have official reports about the number of healthcare professionals of the IP units, we have the notion that they were very few, which may demonstrate the relevance of our study.

Concerning the way the pandemic potentially could cause or increase the incidence of central airway obstruction, we hypothesise two different situations: in the first, because many centres opted to limit in-person consultations and avoid endoscopic and surgical procedures, a decrease in both the diagnoses of all types of cancers, including lung cancer occurred. Actually, a decrease of number of new cancer diagnoses that has been described during the pandemic (14,15); that led to achieve cancer diagnosis in more advanced stages and a considerably delay between the diagnosis and the beginning of the treatment (16); secondly, because seriously ill COVID-19 patients need long periods of intubation with mechanical ventilation, the CAO caused by post-extubation stenosis also tend to increased(17).

The pandemic and CAO: drawing of a country

With the awareness of the potential impact of the COVID-19 pandemic in the pathology of central airway and taking advantage of the fact that we do not have a characterization of the practice of interventional bronchology in Portugal, we conducted the study **“Pre- and post-COVID practice of interventional pulmonology in adults in Portugal”**.

A structured questionnaire with 46 questions was developed by a group of experts in IP and 41 units of Portugal mainland were invited to respond. Of whom, we had response of 37, meaning a total of 90.2% The questionnaire was based on IP characterization, staff characterization, equipment, types of procedures and diagnostic and therapeutic techniques, including airway stenting and pathology of central airway. Then we compare the number of procedures and level of protective healthcare the year before the pandemic and during the year which pandemic starts.

It was evident that the number and type of procedures performed decreased during the pandemic period. It was less 30% of flexible bronchoscopies and 27% of rigid bronchoscopies. Most units equipped themselves with disposable bronchoscopes

and there was a shift in the type and level of protective equipment used to increase the safety for staff and patients. That may represent the impact of our previous work ***“Recommendations for interventional pulmonology during COVID-19 outbreak: a consensus statement from the Portuguese Pulmonology Society”*** in the pneumological community. It is reported in a previous survey made in UK in 2002 that only 9% of physicians used four protective items: face mask, gloves, eye protection and gown(18). In our survey and during 2020, 100% of the centres use the referred protection.

In this study we were unable to draw conclusions on the impact of the pandemic on central airway pathology because of the decrease in the number of examinations overlapped to such an extent that even in ad-hoc analyses we did not obtain statistically significant data. A relevant fact that is worth mentioning is that in the only hospital that in 2020 performed more than one thousand procedures, the number of rigid bronchoscopies increased compared to 2019 (from 210 to 250). Bearing in mind that rigid bronchoscopy is frequently used in cases of pathology of the central airway, mechanical dilatation of stenoses or the insertion of stents, one may assume that the patients who required procedures in that unit were already at a more advanced stage of the disease, although there is no information as to whether the disease was oncological or non-oncological.

Tissue Engineering and Regenerative Medicine – The new horizon

The traditional and available approaches and treatments for CAO we have been discussing have proved to have limitations and some important challenges to patients and physicians, like COVID-19. The results are not as successful as desired and the procedures itself like airway stenting have their own problems like infection, migration, or fistulisation. Clinically we find ourselves at an impasse in which it is necessary to take a step forward.

The spectrum of the disease is changing and moving from acute and infective to chronic and degenerative. Nowadays the medium survival even for advanced oncologic patients could be of several years. So, as clinicians, we are expected to change our therapeutic approach from rescuing to supporting.

For that reason, in the last few years Regenerative Medicine represents the forefront of health sciences and holds promises for the treatment of many challenging

situations. Along with additive manufacturing techniques, 3D printing enables customize fabrication of 3D supporting scaffolds that will work as templates for cell interaction, providing support while recreate the damaged and functionally impaired tissue.

In our quest to create a biocompatible, biodegradable, non-toxic, non-immunogenic and mechanically adapted scaffold, we made a preliminary study presented in Chapter III, “***Gamma irradiation processing on 3D PCL devices - a preliminary biocompatibility assessment***”.

In this preliminary work the authors intended to study the cytocompatibility and biocompatibility of 3D printed polycaprolactone (PCL) based devices (19) after different gamma radiation conditions. The choice for the biomaterial relied on the premise that, in future assays, the device to be studied as to promote regeneration of tracheal tissue must have a design that mimics the anatomical shape of trachea, must have mechanical strength and flexibility like a native trachea and porosity that allows good vascularization and cell proliferation. In addition, must be biocompatible, biodegradable and non-immunogenic (9). Furthermore, PCL allows the production of 3D devices with interconnected porous network and high reproducibility (20). Following the biomaterial production and characterization studies, in vitro and in vivo assays were conducted to assess on the gamma radiation effect on the device's properties, the first applying mesenchymal stem cells (MSCs) from the dental pulp tissue of human origin and the latter considering subcutaneous implantation on a rat animal model.

Among the various synthetic non absorbable polymers available, we decided to use polycaprolactone in our study. That is a polymer approved by Food and Drug Administration (FDA) and offers great advantages such as biocompatibility and good mechanical properties (21). Because of PCL ability to lower elastic modulus and to soften other polymers, PCL can be used as a polymeric plasticizer. Besides its characteristics, our group has vast experience with this biomaterial (22, 23).

Solvent casting method was used for the preparation of PCL and cylindrical scaffolds of ~2mm thick were produced using an additive manufacturing system. The sterilization was performed with gamma radiation since it is crucial the achieving of sterilized medical devices without compromising the biomaterials' structure and mechanical properties. Gamma radiation is highly penetrative and kills bacteria breaking down bacterial DNA (24). A minimum of 25KGy is routinely

applied to be effective, but higher levels of radiation which are safer in terms of decontamination, may lead to decrease mechanical properties of PCL (25). We decided to test three levels of radiation: 25K Gy, 35K Gy and 45K Gy. Choose of higher radiations relies on the fact that we are considering a possibility of a future medical device that could be used in humans. In this case we preferred to use a stronger radiation to maximize and ensure the sterilization capacity, without compromising the mechanical and chemical properties of the biomaterial, but also its cito- and biocompatibility. Devices were further characterized *in vitro*, assessing on their cytocompatibility properties, and *in vivo*, by subcutaneous implantation on a rat animal model, following ISO 10993-6:2016 guidelines, assessing on the devices' biocompatibility.

A thorough biocompatibility assessment for PCL devices was addressed, according to ISO 10993-6:2016 guidelines for biological evaluation of medical devices, Part 6: Tests for local effects after implantation". Devices were subcutaneously implanted at the dorsum of the animals, as described in annex B: "Test methods for implantation in subcutaneous tissue" of the referred guideline. Implanted samples were analysed at 7- and 15-days post-implantation time, and a semi-quantitative scoring system was applied, according to annex E: "Examples of evaluation of local biological effect after implantation". PCL devices were considered for each animal and were used as negative control for each timepoint. The scoring system implied a global histological scoring of each sample, with a detailed histopathologic evaluation of the bio-logical tissue response, including fibrosis extent, tissue morphology changes, inflammatory cell types, necrosis presence and extent, neovascularization, fatty infiltration, and other relevant parameters, according to the guideline. The evaluation of the biocompatibility is crucial to ensure that the medical device does not elicit any local or systemic reaction.

The cytocompatibility assessment for the devices was performed by the PrestoBlue™ viability assay and with use of hDPSCs. Although the reagent used is not referred in the ISO 10993-5:2009 guidelines, the PrestoBlue™ was employed for this assay, as it allows to perform live-cell assays, in contrast with, for example, the MTT assay, that only allows for endpoint assays. Thus, by using this method, the exact same cell population can be evaluated over time in addition to using fewer devices. As such, this assay is less time consuming and implies an inferior financial investment. Nevertheless, the PrestoBlue™ has comparable performance with other cell viability reagents (26). Despite not being listed on the guidelines, this assay

was adapted from ISO 10993-5:2009 “Biological evaluation of medical devices” – Part 5 – “Test for in vitro cytotoxicity”.

A thorough cytocompatibility analysis as performed, including a qualitative scanning electron microscope (SEM) analysis of the *in vitro* seeded scaffolds with hDPSCs, as to assess on the scaffolds-cellular population interactions *in vitro*. This assay allowed visualization of cellular adhesion, as well as a cellular layer formation in the 3D scaffolds, with a uniform distribution, thus confirming the analysed scaffolds to present potential cellular matrix conditions for 3D culture. EDS analysis detected nitrogen when cellular layers were assessed, mainly carbon and oxygen, and sodium and phosphate at a minor extent, when analysing the biomaterial itself. Furthermore, SEM images of the subcutaneously implanted 3D PCL scaffolds in the rat animal model allowed visualization of the 3D structure integrity of the scaffolds, as well as tissue integration, which was validated by the histological scanning of the samples, with H&E staining. That means that as we expected the PCL disk was biocompatible and cytocompatibility. Concerning the sterilisation doses, the preferred is 45K Gy because is the most efficacy maintaining the structural and biological properties.

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Chapter V

Conclusions and Future Perspectives

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The basilar idea of our work was clearly the CAO problem and what we can do to provide longer lasting and less morbid treatment for our patients. With that problem in our minds, we thought: what else can I do? Effectively we saw our patients, sometimes young ones, that for some reason had the need of a prolonged intubation or a tracheotomy and suddenly find themselves with a post-intubation or post-tracheotomy tracheal stenosis. Patients otherwise completely healthy but with a stenosis that must undergo to our units on a regular basis to perform mechanical dilatations because if not they suffer major limitations in their daily life. In others, we end up putting a stent, but complications such as mucous plugging, infection, migration, or persistent cough - due to the presence a foreign body in the trachea - limit their effectiveness. The examples provided on our first study *“Central airway obstruction: is the time to move forward?”* reflect the poor outcomes. So, there is a major clinical problem, and it is time to move forward. In every IP unit one of the most challenging challenges is to deal with CAO.

A disease that affected the humanity and our IP units was the COVID-19 Pandemic because the safe of healthcare professionals and patients were in risk. We contributed to a document of recommendations which had a positive impact in the pulmonology community, providing rules that had been followed by most of the Portuguese centres with a clear impact in the safety of healthcare workers and patients. This was directly related to CAO theme, which was the augment of patients post COVID-19 with CAO after prolonged intubation and the delay of diagnosis and treatment of oncological patients. The study we performed *“Pre- and post-COVID practice of interventional pulmonology in adults in Portugal”* proved that the number of procedures clearly diminished during the pandemic with a negative impact for the patients, inclusively with an increased number of rigid bronchoscopies in the hospital that performs more exams in Portugal.

The last work of this thesis represents the beginning of our answer to the question. In that study we introduced the theme of Regenerative Medicine (RM) and Tissue Engineering (TE).

We tested a synthetic polymer, polycaprolactone (PCL) for the fabrication of tracheal prothesis. We concluded that although the minimum gamma radiation required is 25 KGy, we can use securely 45 KGy which promotes a better sterilization without affecting the quality of the scaffold and inclusively, not

affecting the growth of the dental pulp mesenchymal stem cells (DPSCs) that we seeded in PCL discs.

We expect this preliminary work to be continued and to obtain a prototype of a stent coated with stem cells that would have a crucial role in the regeneration of the central airway.

Normally medical advances need the convergence of multiple innovations from different areas and a multidisciplinary approach. In this case, and like transplantation medicine some years ago, we need to congregate the knowledge of molecular biology, stem cell biology and bioengineering. Significant potential of cell-based therapies has been shown in several areas such as haematology or neurology. In the case of central airway, we clearly need the support of novel biofabrication strategies, as 3D bioprinting. The next issue is how to make it available to every patient. Perhaps the creation of a friendly use software for medical institutions and in net with manufactures, could solve the problem of 3D printing.

Certainly, Regenerative Medicine is not yet the *El dorado*, but concerning our specific problem, might shift the paradigm from symptomatic to curative treatment in CAO.