# UNIVERSITÀ DEGLI STUDI DI CATANIA

**Dipartimento di Medicina Clinica e Sperimentale**

**DOTTORATO DI RICERCA IN BIOMEDICINA TRASLAZIONALE**

**XXXIV CICLO**

**Coordinatore: Prof. Lorenzo Malatino**


---


**Dott. Giuseppe Fabio Parisi**


# LUNG CLEARANCE INDEX:

## a new biomarker of ventilation inhomogeneity in pediatric respiratory diseases.

**PhD Thesis**


**Tutor:**
**Prof. Salvatore Leonardi**


---


**Anno Accademico 2020 – 2021**

## Abstract

Conventional spirometry has long been considered the primary test of respiratory function deficit in children and adults. However, performing forced breathing manoeuvres is challenging in an uncooperative child. Furthermore, recent evidence suggests that conventional spirometry is not sensitive for the early detection of lung damage affecting the small airways or the evaluation of homogeneity of air ventilation. For these reasons, techniques such as gas dilutions and multiple-breath washout (MBW) have been implemented over the last few years because they allow for the early assessment of damage to small airways. These methods permit the identification of ventilatory inhomogeneity in the lungs by analyzing the clearance of an inert gas used as a tracer. The equipment consists of a mass spectrometer combined with a flow meter. For all tests, the lung clearance index (LCI) is the parameter that is most often used to evaluate ventilatory inhomogeneity. Because it is sufficient to breathe with a normal tidal volume during the examination, this examination is particularly suitable for studying respiratory function, even in uncooperative children.

Considering the emerging role of the LCI in the evaluation of childhood respiratory diseases, the aims of my researches during the three years of PhD were: i) to evaluate this index in some pediatrics respiratory diseases including lung fibrosis in cancer survivors, cystic fibrosis, and finally in children healed from COVID-19; ii) to increase the scientific evidences on the role of LCI as a biomarker in pediatric respiratory diseases.

In this sense, the work of these three years of doctorate has contributed to increase the scientific evidence on the MBW test in childhood respiratory diseases. Based on this, we can certainly affirm that LCI can be considered to all intents and purposes a biomarker of ventilatory inhomogeneity in children with bronchopulmonary disease.



**Keywords**: lung clearance index, LCI, multiple-breath washout, MBW, children, lung, respiratory, disease, cancer, cystic fibrosis, COVID-19.

**List of publications**

This PhD thesis is based on the following original publications:

- **Parisi GF**, Cannata E, Manti S, Papale M, Meli M, Russo G, Di Cataldo A, Leonardi S. Lung clearance index: A new measure of late lung complications of cancer therapy in children. Pediatr Pulmonol. 2020 Sep 14. doi: 10.1002/ppul.25071. Epub ahead of print. PMID: 32926567
- Papale M, **Parisi G.F**, Spicuzza L, Licari A, Bongiovanni A, Mulè E, Rotolo N, Manti S, Leonardi S. Lung clearance index evaluation in detecting nocturnal hypoxemia in cystic fibrosis patients: Toward a new diagnostic tool. Respiratory Medicine 2020; Volume 164 Article number 105906
- Papale M, **Parisi G**, Spicuzza L, Rotolo N, Mulè E, Aloisio D, Manti S, Leonardi S. Nocturnal non invasive ventilation in normocapnic cystic fibrosis patients: a pilot study. Acta Biomed. 2021 May 12;92(2):e2021164. doi: 10.23750/abm.v92i2.11261. PMID: 33988138; PMCID: PMC8182613.
- Manti S, Galdo F, **Parisi GF**, Napolitano M, Decimo F, Leonardi S, Miraglia Del Giudice M. Long-term effects of bronchopulmonary dysplasia on lung function: a pilot study in preschool children's cohort. J Asthma. 2020 Jul 1:1-8. doi: 10.1080/02770903.2020.1779289. Epub ahead of print. PMID: 32508174.
- **Parisi GF**, Papale M, Tardino L, Nenna R, Midulla F, Leonardi S. Biomarkers in Pediatric Lung Diseases Including Cystic Fibrosis. Current Respiratory Medicine Reviews 2019; 15(3):163-173. DOI: 10.2174/1573398X15666190521112824
- **Parisi GF** and Leonardi S. Upper and Lower Airways Diseases in Childhood. Current Respiratory Medicine Reviews 2019; 15(3):161-162. DOI: 10.2174/1573398X1503191125160355
- Licari A, Castagnoli R, Tondina E, Testa G, **Parisi GF**, Marseglia A, Brambilla I, Marseglia GL. Novel Biologics for the Treatment of Pediatric Severe Asthma. Current Respiratory Medicine Reviews 2019; 15 (3):195-204. DOI: 10.2174/1573398X15666190521111816
- Manti S, Cuppari C, **Parisi GF**, Salpietro C. An Overview of HMGB1 and its Potential Role as a Biomarker for RSV Infection. Current Respiratory Medicine Reviews 2019; 15 (3):205-209. DOI: 10.2174/1573398X15666190603121448
- Giallongo A, **Parisi GF**, Licari A, Pulvirenti G, Cuppari C, Salpietro C, Marseglia GL, Leonardi S. Novel therapeutic targets for allergic airway disease in children. Drugs Context. 2019 Jul 9;8:212590. doi: 10.7573/dic.212590
- Pappalardo MG, **Parisi GF**, Tardino L, Savasta S, Brambilla I, Marseglia GL, Licari A, Leonardi S. Measurement of nitric oxide and assessment of airway diseases in children: an update. Minerva Pediatr. 2019 Jul 25. doi: 10.23736/S0026-4946.19.05513-0
- Licari A, Manti S, Castagnoli R, **Parisi GF**, Salpietro C, Leonardi S, Marseglia GL. Targeted Therapy for Severe Asthma in Children and Adolescents: Current and

Future Perspectives. Paediatr Drugs. 2019 Aug;21(4):215-237. doi: 10.1007/s40272-019-00345-7.

- **Parisi GF**, Portale A, Papale M, Tardino L, Rotolo N, Licari A, Leonardi S. Successful treatment with omalizumab of allergic bronchopulmonary aspergillosis in patients with cystic fibrosis: Case reports and literature review. J Allergy Clin Immunol Pract. 2019 Feb 15. pii: S2213-2198(19)30165-5. doi: 10.1016/j.jaip.2019.01.056.
- Spicuzza L, **Parisi GF**, Tardino L, Ciancio N, Nenna R, Midulla F, Leonardi S. Exhaled markers of antioxidant activity and oxidative stress in stable cystic fibrosis patients with moderate lung disease. *J Breath Res 2018.* Feb 20;12(2):026010. doi: 10.1088/1752-7163/aa9b39.

The publications have been adapted with the permission of the copyright owners.

**Acknowledgments**

The researches were carried out: i) at the Pediatric Pulmonology Unit of San Marco Hospital (Catania, Italy) directed by the professor Salvatore Leonardi; ii) at the School of Specialisation in Pediatrics (University of Catania, Catania, Italy) directed by the professor Martino Ruggieri; iii) at the Department of Clinical and Experimental Medicine (University of Catania, Catania, Italy) directed by professor Francesco Purrello.

The research activities were partially supported by the 2016/2018 Research Plan of University of Catania, Department of Clinical and Experimental Medicine (project #B Evaluation of long-term harmful effects of chemotherapy and radiotherapy on various organs in childhood cancer survivors).

# SUMMARY

# CHAPTER 1. The Lung Clearance Index

## 1.1 Introduction

Several respiratory disorders develop in the pulmonary system, especially during childhood. Lung diseases are responsible for about one-quarter of all pediatric clinic visits. The majority of these conditions are self-liming, particularly acute infectious diseases. An efficient immune system is capable of promoting efficient healing. By contrast, an increase in chronic diseases can lead to disorders that substantially impact later life [1,2]. Some of the most frequent chronic conditions affecting children are asthma and cystic fibrosis (CF). Their etiopathologies are different. However, small airway involvement appears to occur in both [3, 4].

In CF, peripheral airway damage begins in the first months of life because CF transmembrane conductance regulator (CFTR) gene defects are inherited [5]. CF pulmonary disease reduces the quality and length of a patient's life because respiratory decline can lead to pulmonary failure, including the inability to maintain normal oxygen and carbon dioxide levels [6,7]. Initially, pulmonary damage is partially reversible. However, the damage becomes irreversible when the patients develop bronchiectasis if they are under-treated. Lung function decline is more rapid if this decline is associated with exacerbations characterized by worsening symptomatology, loss of energy, weight loss, and changes in physical exam findings [8, 9]. When pulmonary failure occurs, patients are usually placed on a lung transplant list. These findings suggest that good treatment requires appropriate surveillance [10].

Conventional spirometry has long been considered the primary test of respiratory function deficit in children and adults. However, performing forced breathing manoeuvres is challenging in an uncooperative child. Furthermore, recent evidence suggests that conventional spirometry is not sensitive for the early detection of lung damage affecting the small airways or the evaluation of homogeneity of air ventilation [11–13]. For these reasons, techniques such as gas dilutions and multiple-breath washout (MBW) have been implemented over the last few years because they allow for the early assessment of damage to small airways. These methods permit the identification of ventilatory inhomogeneity in the lungs by analyzing the clearance of an inert gas used as a tracer. The equipment consists of a mass spectrometer combined with a flow meter. For

all tests, the lung clearance index (LCI) is the parameter that is most often used to evaluate ventilatory inhomogeneity. Because it is sufficient to breathe with a normal tidal volume during the examination, this examination is particularly suitable for studying respiratory function, even in uncooperative children [14,15].

## 1.2 Concept of biomarker

The basic definition of a biomarker is deceptively simple: "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention."3 This broad definition encompasses therapeutic interventions and can be derived from molecular, histologic, radiographic, or physiologic characteristics. Biomarkers are measures used to perform a clinical assessment such as blood pressure or cholesterol level and are used to monitor and predict health conditions in individuals or across populations so that appropriate therapeutic intervention can be planned.

Biomarkers can be used alone or in combination to assess a person's disease or health status.

A wide range of biomarkers are used today. Each biological system (for example the cardiovascular system, the metabolic system or the immune system) has its own specific biomarkers. Many of these biomarkers are relatively easy to measure and form part of systematic medical examinations.

For example, a general health check may include assessing blood pressure, heart rate, cholesterol, triglycerides, and fasting glucose levels. Body measurements such as weight, body mass index (BMI) and waist-to-hip ratio are routinely used for evaluating conditions such as obesity and metabolic disorders.

Currently, the gold standard for the measurement of airway inflammation is bronchoscopy with biopsy and/or broncho-alveolar lavage (BAL), but this method is invasive and certainly not to be used as a routine practice, especially in children. The collection and examination of sputum is not feasible in all subjects and the process is fairly time-consuming.

From the point of view of respiratory function indices, the possibility of having a biomarker that could early reveal a damage to the small airways could certainly improve the clinical management of these children. Nevertheless, a biomarker to be trusted should

have several features: (1) high positive and negative predictive values; (2) normalization after treatment; (3) reproducibility; (4) ease of collection; and (5) cost-effectiveness [16]. Despite the efforts to date, there are no biomarkers available with all of these characteristics.

## 1.3 Historical notes

At the end of the 1940s and the beginning of the 1950s, Fowler undertook a series of respiratory physiopathology studies that are still considered relevant today. Reading them is interesting because they are pioneering works that have laid the foundations for modern respiratory physiopathology [17–24].

The most important and well-known of these studies was published in 1952. This study was based on an analysis of the time course of equilibration of alveolar gas with a "foreign" inspired gas such as oxygen. When an individual inhales 100% oxygen, the nitrogen normally contained in the lungs (as present in a fixed percentage in the ambient air) is gradually exhaled. Thanks to the $N_2$ meter developed a few years earlier by Lilly and Hervey. It was possible to perform a breath-by-breath analysis of expired gas during and after the change from breathing air to uninterrupted breathing of $O_2$. Fowler compared the $N_2$ clearance of ten healthy young adults with 19 subjects with cardiopulmonary disease (predominantly patients with asthma, emphysema, and heart failure patients). He showed that the latter group's lungs were unevenly ventilated and showed that the extent of uneven ventilation could be measured quantitatively and was not influenced by the magnitude of the respiratory dead space [24]. Although conceptually interesting, this method was not much appreciated because it was considered too labor-intensive. The outlook changed when automatic volume and gas analysis techniques were developed, triggering renewed usage of the technique [25].

## 1.4 What is the LCI?

The LCI is used to evaluate the homogeneity of lung ventilation. It is obtained using the multiple breath washout (MBW) technique. This parameter indicates how many lung turnovers are needed to expel an inert gas from the lungs by breathing [26]. Serial LCI measurements are used for longitudinal lung function evaluation in obstructive

diseases, such as CF (especially in mild disease), asthma, and primary ciliary dyskinesia (PCD) [27–29].

Over the last fifteen years, the LCI has been expanded in the pulmonology field because of its simplicity and sensitivity. Simplicity is crucial, especially for pediatric patients, because children are less inclined than adults to perform tests such as spirometry, a benchmark functional test [11].

MBW is carried out at rest, and no collaboration is required. This detail is critical for children, particularly preschool-aged children. Those six years and older tend to be cooperative and can perform spirometry. In infants, pulmonary function tests are performed under sedation or during sleep [30]. By contrast, preschool-aged children are too old to be sedated and too young to be cooperative. This third age group has been mostly ignored from the functional testing point of view. In 2007, the American Thoracic Society (ATS) published a statement concerning pulmonary function testing in preschool children; various techniques were described, including the multiple-breath inert gas wash-out test [31]. Concerning the latter, the authors highlighted the necessity of standard criteria to establish procedures and medical staff education because only a few facilities have had experience with this technique [31].

## 1.5 Lung function notions: a premise for comprehension

### *1.5.1 Anatomy and physiology*

The respiratory tree can be divided into the intra- and extra-thoracic airways. The latter are the focus of otorhinolaryngology, while the former are within the present scope of interest. Large airways (main lobar and segmental bronchi), small airways, and alveolar regions constitute the various districts within the lungs. Gas exchange occurs in the alveoli, the main gas exchanging surface because bronchial and vascular compartment interactions occur in the alveoli; the alveoli are directly connected to the bronchial tree on one side and surrounded by capillaries on the other.

Weibel and Horsfield described the pulmonary tree division to simplify complex anatomy [32, 33]. The bronchial tree consists of ducts that progressively subdivide about 23 times to generate numerous continually narrowing structures that end in the acini, the respiratory units. Starting at the eighth generation and lower pulmonary branch divisions, airways are called “small airways,” characterized by diameters of < 2 mm. These airways

can be differentiated into conducting airways from the 8th to 16th branch divisions and into acinar airways from the 17th to the 23rd. This branching is essential because the resulting respiratory surface is magnified in this way; the total lung area can reach 100 $m^2$, and the peripheral airways contribute significantly to this phenomenon [34]. The respiratory zone begins at the 17th division and is the gas exchanging site, while the conducting zone consisting of both small and large ducts is responsible for gas transport and mixing, which occurs via convection. Convection can be thought of as a motor due to the production of a gas pressure differential within a duct. The gas molecule motor is based on an equal division between convection and diffusion [34]. Diffusion is driven by gradients of molecular concentrations from areas of higher to lower concentrations. Lung areas in which no gas exchange occurs, including conducting airways, are called dead space [35]; hence, conducting airways are defined according to that definition [35] as shown in **Figure 1**.

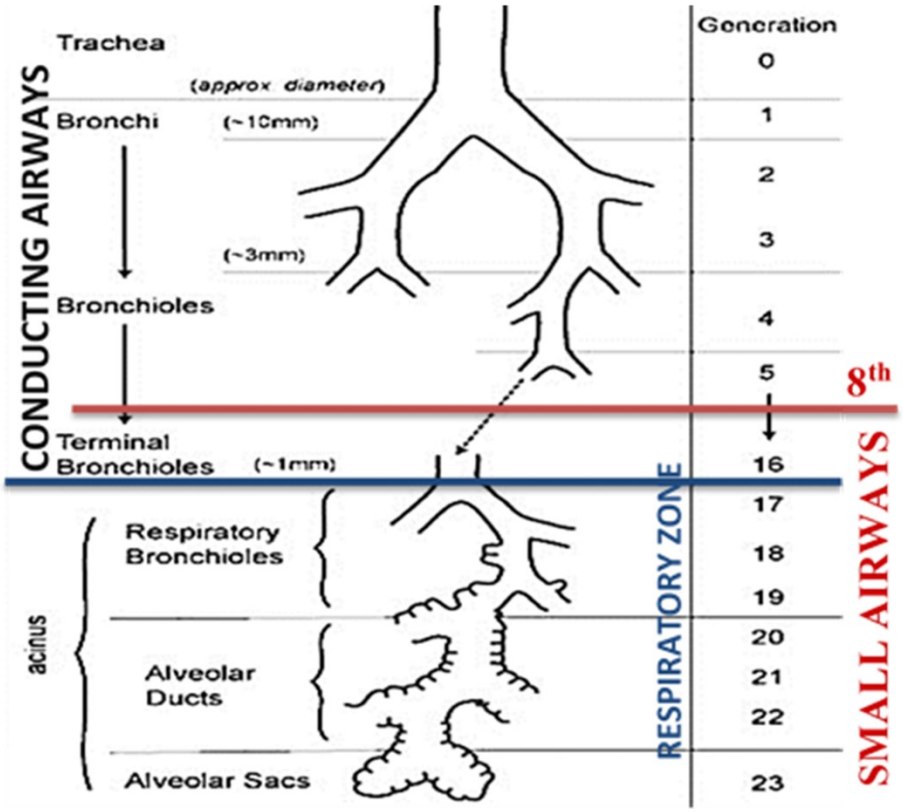


FIGURE 1. LUNG ARCHITECTURE: WEIBEL MODEL. "SMALL AIRWAYS" STARTING FROM RED LINE. WHILE THE BLUE ONE MARKS THE BEGINNING OF INTRA-ACINAR AIRWAYS, OR RESPIRATORY ZONE. [33]

The differences between large and small airways do not rely solely on their diameters. In small airways, velocity flow is slower than in large airways because the flow volume is the same for both the compartments; however, the small airway total cross-sectional area is greater than the large cross-sectional area [36]. Small airways have pathological characteristics distinct from large airways; small airways do not have cartilaginous support. The small airways are lined with a liquid with properties similar to surfactants and prevent airway collapse during expiration [36]. If mucus, inflammatory infiltrates, smooth muscle cell hypertrophy, or increased airway wall thickness increase, surface tension increases, causing the collapse of small airways. In this obstructive case, the downstream respiratory units are excluded from ventilation and consequently from gas exchange [36].

### *1.5.2 Ventilation and airways resistance*

Respiratory activity (or ventilation) consists of inspiration and expiration in which air moves from and to the lungs. Gas volumes are divided into several categories:

- Tidal volume (TV) is the quantity of gas volume moved during normal breathing;
- Inspiratory reserve volume (IRV) is the volume moved inside the lungs after normal inspiration;
- Expiratory reserve volume (ERV) is the excess volume exhaled after a normal expiration;
- Residual volume (RV) is the amount of gas that is not moved out of the lungs.

Lung capacities result from the sum of pulmonary volumes:

- Total lung capacity (TLC): TV+ IRV + ERV + RV
- Vital capacity (VT): ERV+ VT + IRV
- Inspiratory capacity (IC): TV+ IRV
- Functional residual capacity (FRC): RV+ ERV

FRC indicates the gas volume in the lungs at the end of exhalation at rest [37, 38]. FRC evaluation is critical in assessing several pathologies because some lung function indices depend on accurate FRC measurement [39]. FRC is derived from the following formula:

$$FRC = V(tracer)/(CInit-CEnd)$$

In which,

- V (Tracer) is the total volume of tracer gas eliminated at the end of the test;
- CInit is the starting fraction of tracer gas; and
- CEnd is the final fraction of tracer gas [40].

FRC cannot be measured by spirometry because this technique provides results only for the volume moved by breathing rather than the amount (volume) of residual gas in the lungs. A procedure that permits data about the FRC to be obtained is the MBW. Based on the ratio between RV and TLC, the amount of "air trapping" also indicates obstructive conditions in the lung parenchyma [41]. Because RV is necessary for the ratio mentioned above, MBW is capable of providing these data.

Concerning spirometry, MBW provides data about lung function; however, there are two critical differences between MBW and spirometry: (1) the MBW technique is conducted at rest, while spirometry requires patient cooperation; and (2) different functional compartments are tested because spirometry provides data about the airway resistance. Considering that resistance depends 80%–90% on large airways, the resulting data reflect large airway function [42], whereas MBW provides data about the ventilation inhomogeneity and better reflects small airway function [34]. Hence, small airway obstruction affects ventilation distribution and, consequentially, gas exchange.

## 1.6 Focus on LCI calculation and the MBW technique

### *1.6.1 The multiple-breath wash-out technique*

The MBW technique was described for the first time in 1950 and principally used in adults to study lung physiology. Several years later, this technique was applied to study pulmonary physiopathology in adults and children, in whom substantial feasibility was established [34]. To use gas as a tracer, it must be inert, and only a minimal dispersion into the bloodstream is tolerable [40]. Resident lung $N_2$ was initially used to obtain the first measurements. Sulfur hexafluoride (SF6), another tracer gas, was adopted and has been used for a long time. Recently, the $N_2$-based technique came back into use because of its economic and environmental sustainability [43].

Tracer gas can be exogenous or endogenous; $N_2$ is an example of a tracer gas, an atmospheric gas that is normally inhaled. Examples of exogenous gases are helium (He), argon (Ar), and sulfur hexafluoride (SF6) [40]. This distinction is important because, for each gas, some changes in the technique are necessary.

In children, data are obtained through continuously monitoring exhaled tracer gas breath-by-breath during relaxed tidal breathing; in adults, data are obtained during a set TV [44]. A wash-in phase is necessary if an exogenous tracer is used; this phase consists of a gas mixture containing a defined quantity of gas inhaled by the patient. SF6 is used at 4%. The wash-in phase ends when SF6 reaches equilibrium in the lung. In the second part of the test, the wash-out phase, the patient breathes room air that does not contain any tracer gas, and in this way, the exhaled SF6 can be recorded by the machine. A photoacoustic sensor is used to measure SF6, while in the past, expensive mass spectrometry was used more frequently [45,46].

Only a wash-out phase characterizes the N2MBW technique. No wash-in phase is required because $N_2$ is one of the most representative atmospheric gases (78%–79%) [3]. The patient inhales oxygen at 100% during the test, so exhaled $N_2$ can be recorded using specific sensors [43].

### *1.6.2 LCI computing*

The LCI indicates the number of lung turnovers (TO) necessary to wash out the tracer gas, regardless of concentration, until it reaches $1/40^{th}$ of the initial concentration; for this reason, this parameter is called the LCI 2.5% [39, 40]. The measurement carried out at $1/20^{th}$ of tracer concentration is called the LCI 5%. Lung TO is the ratio between cumulative expiration volume (CEV) and FRC:

$$TO = CEV/FRC.$$

Thus, an accurate evaluation of FRC is used as an assumption for obtaining veritable lung function measures, not only for LCI [47]. A measurement ends when the LCI 2.5% value is recorded in triplicate for consecutive breaths. Usually, 5 min are necessary to complete a measurement (not considering the wash-in phase if SF6-MBW is used).

The test is performed while a patient is sitting with their mouth connected to a mouthpiece and the latter connected to the device. The patient is asked to breathe at TV while resting until the end of the test, which generally lasts between 3 and 15 min. In younger children, the measurement is taken using a face mask while they are in the supine position, preferably during sleep (spontaneous or induced). The examination is then repeated two or three times [39, 40].

A high LCI value suggests that pulmonary function is poor because of changes in small airway structure. Any alterations in peripheral lung architecture modify resident

gas mixture and create globally inhomogeneous pulmonary ventilation [39, 40]. The LCI 2.5% is the most viable cut-off value. This choice is because of the poor resolution of the first $N_2$ sensors, indicating that the cut-off was customized on the $N_2$-based technique. Yammine et al. reported that the cut-off could be modified for SF6MBW because of the varying natures and kinetics of tracer gases [48].

## 1.7 MBW and obstruction: is localization possible?

Inflammatory obstructive processes can involve the entire lung parenchyma with varying intensities. Modifications in the anatomical structure cause alterations in lung gas mixtures leading to inhomogeneous ventilation. The importance of MBW to the identification and localization of pathological processes provides insights into the type of information that spirometry can yield [39, 40]. Spirometry is a milestone among pulmonary function tests that provides insight into airflow resistance exerted by airways walls. Ten percent of the total resistance is attributable to small airways, even though they represent 95% of the total lung volume [42]. Because acinar and conducting airways contribute to heterogeneous ventilation, it appears evident that small airways cannot be extensively studied using only spirometry.

The MBW system provides regional ventilation inhomogeneity indices: (1) Scond and (2) Sacin, derived from phase III slope data reshaping. Concerning Scond and Sacin, knowledge of an expirogram is necessary to comprehend how to locate structural damage in the lung. An expirogram is a graph obtained initially using another inert gas wash-out technique, the Single Breath Wash-out (SBW) (**Figure 2**). Exhaled $N_2$ during expiration is a function of expired volume in a single breath. Lung ventilation occurs by convection and diffusion, as shown above.

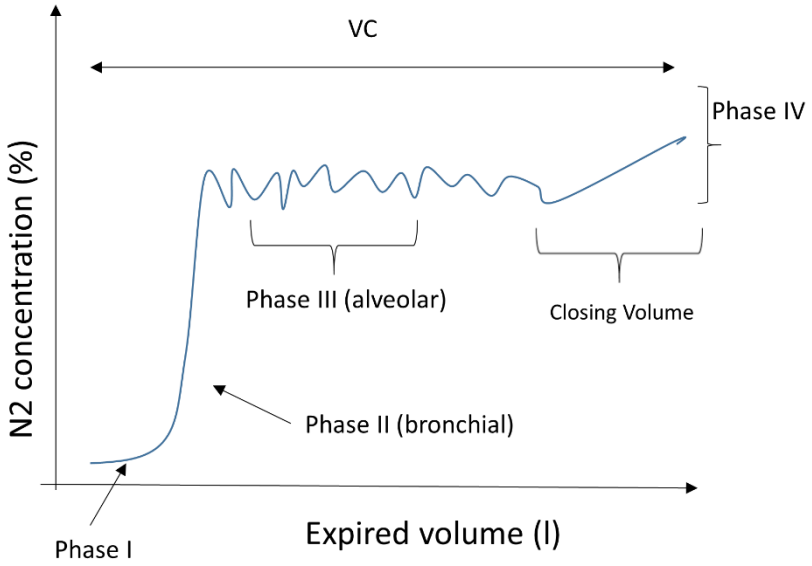


**FIGURE 2 NORMAL EXPIROGRAM. THE N2 CONCENTRATION MOVEMENT IN ONE BREATH. FOUR PHASES CAN BE DESCRIBED:**

- **FIRST PHASE (I) CORRESPONDS TO THE ABSOLUTE DEAD SPACE;**
- **SECOND PHASE (II), CALLED THE BRONCHIAL OR TRANSITIONAL PHASE, REPRESENTS THE ALVEOLAR GAS ARRIVING FROM THE LUNG UNIT;**
- **THIRD SPACE (III) IS ALSO CALLED ALVEOLAR SPACE;**
- **FOURTH PHASE (IV) STARTS WHEN AN INCREASE IN NITROGEN CONCENTRATION IS VISIBLE AND ENDS WHEN THE RESIDUAL VOLUME IS REACHED.**

**BASAL AIRWAY CLOSURE IS A POSSIBLE EXPLANATION OF THE MEANING OF PHASE IV. VOLUME BETWEEN THE START OF PHASE IV AND RESIDUAL VOLUME IS DEFINED AS "CLOSING VOLUME." IF CLOSING VOLUME OCCURS BEFORE THE RESIDUAL VOLUME REACHING, IT MEANS THAT PERIPHERAL AIRWAYS ARE OBSTRUCTED. IN PHASE III, THERE IS A SLOPE, A SENSITIVE INDEX OF PERIPHERICAL OBSTRUCTION. IN OBSTRUCTIVE DISEASES, THERE IS AN ELEVATION OF THIS INCLINATION.**

Phenomena that cause heterogeneous ventilation in the pulmonary structure are based on two mechanisms:

- Convection-dependent inhomogeneity (CDI) in the conducting airway zone (until terminal bronchioles); and
- Interactions between convection and diffusion (DCDI) in an intermediate zone at the level of the "diffusion-convection front," recognized as the alveolar "entrance."

Because the gas in narrowed airways remains in the airway longer than in normal ones, the consequence is slower emptying in damaged airways [27]. Therefore, the timing of gas concentration measurement is crucial. Thanks to the new analysis software, it is possible to obtain insights about gas movement into the lungs during MBW [49].

Raw data from the phase III slope is processed and normalized to obtain SnIII (normalized phase III slope), the ratio between the slope and the gas concentration during phase III. The computer then plots each resulting value recorded for each breath with the corresponding lung TO [49]. An increase in exhaled tracer gas concentrations during the specific range of time from 1.5 to 6 TO indicates that conducting airways are obstructed. Until 1.5 TO is reached, the increase in Sn could be due to a diffusion-dependent mechanism (inhomogeneity occurring between the acinar areas) and technically continues until the fifth breath is achieved, although their contribution appears to be negligible [50]. Hence, the evaluation of Scond can start from 1.5 lung TO because the emptying in damaged conducting airways does not occur rapidly. This emptying is not detected before the acinar airway contribution from the 1.5 TO because the emptying is not significant. Scond is a value that indicates how much the convection-dependent inhomogeneity (CDI) mechanism contributes to the obstruction, while the Sacin quantifies the diffusion mechanism. The difference in Sn per unit TO between TO 1.5 and 6 is defined as Scond [50]. Sacin is obtained by a calculation in which the CDI component is “translated” as the first breath (Scond x TO1 breath) [51]. More technical details are available [51, 52]. The LCI value reflects global inhomogeneity ventilation, while Scond and Sacin provide insights concerning damage localization.

## 1.8 Technical details for MBW equipment

Specific equipment is required to perform the MBW test. The entire wash-out system consists of separate units (**Figure 3**).

**FIGURE 3. MULTIPLE-BREATH N2 WASH-OUT DEVICE IN OUR DEPARTMENT: EXHALYZER-D (ECO MEDICS AG, SWITZERLAND)**

A gas analyzer is one of the most critical elements in this system, and detecting traces of the gas is its primary function. Initially, respiratory mass spectrometry (RMS), the gold standard, was only used, while several analyzers are currently available [39]. RMS allows concurrent quantification of different types of gas with high sensitivity and efficiency. The use of RMS has been limited because of elevated costs.

Other gas analyzers exploit the photoacoustic sensor (only for SF6) while the $N_2$ measurement is performed indirectly using concurrent $O_2$ and $CO_2$ quantification or using an ultrasonic flow meter (USFM) to evaluate changes in molar mass [39]. The gas analyzer can be situated along the main gas stream or on the side. Except in one case, most systems possess a side-stream analyzer. Response times and sampling rates of the analysis system (suggested > 100 Hz) are crucial for obtaining a quality test [39].

Another fundamental element is the flow meter that is used to quantify the gas volume moved during breathing. Data concerning the gas concentration and flow volume must be considered together because of their importance to data generation. This issue is solved using an Exhalyzer-D (ECO Medics AG, Switzerland) system equipped with a mainstream ultrasonic flow meter (USFM) sensor capable of detecting changes in molar mass (to evaluate the amount of nitrogen) and airflow measurements. $CO_2$ and $O_2$ sensors are also present to customize the analysis to the patient [39]. Europe, North America, and Australia have agreed on $N_2$ measurement standardization using the Exhalyzer-D [43].

The gas analyzer is linked to the analysis system, which consists of specific software that rearranges the data using algorithms and provides the results. During the test, the respiratory rate, flow, and volume of the airstream and the tracking of variations in the concentration of tracer gas are instantly reported and displayed using software [39]. In this way, the patient can be encouraged by an operator to accomplish better performance. Maintaining a regular respiratory rate is the only request. This is critical, especially in children, who can hyperventilate if they focus on their respiratory pattern. Entertaining children with audio-visual support is the best way to distract them from obtaining a regular respiratory rate. Other components of the MBW system include the gas delivery system and patient interface. This last component can adopt various shapes and dimensions [39]. Mouthpieces or facemasks are options for the interface (**Figure 4)**.

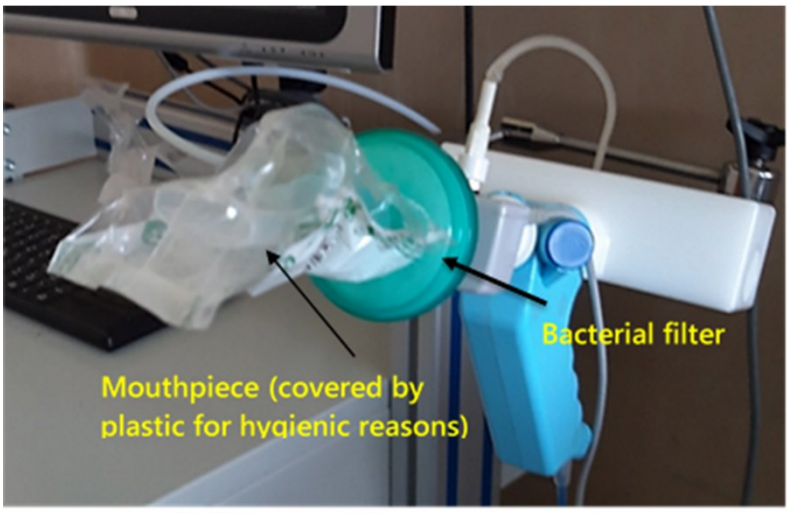


**FIGURE 4. MOUTHPIECE FOR THE TEST.**

The patient interface and the side-stream gas analyzer constitute external dead space. Some recommendations addressing dead space management (e.g., interface size to

avoid distorting data) are provided by the European Respiratory Society/American Thoracic Society (ERS/ATS). Depending on its position relative to the gas analyzer, equipment dead space is called dead space pre/post-gas-sampling [39]. The ERS/ATS provided recommendations regarding the way to obtain good quality measurements. Some aspects must be considered, including patient position during the test (infants in the supine position and the sniffing posture, while all other subjects remain seated), interface choice (facemask for infants and preschool children, mouthpiece plus nose clip in older subjects), technical criteria to adopt regarding the wash-in and wash-out phases, the moment to end the test, and gas leakage during the test [39].

## 1.9 Factors that can influence the results

The LCI value principally depends on lung function; however, it might be influenced by variables such as selected tracer gas, analysis software, and tool kits. The most studied variable is the tracer gas; several papers focused on the difference between the use of $N_2$ as opposed to SF6 [53, 54].

These studies highlighted that $N_2$MBW provides higher final values than those obtained using the SF6 technique in healthy and ill subjects. This difference is due to a phenomenon called "nitrogen back-diffusion," which exists because $N_2$ is excreted via the lungs [53, 55]. Differences in adipose tissue and muscle mass influence endogenous $N_2$, especially between adults and children [55, 56]. The cardiac output also impacts nitrogen exhalation; however, this is not an issue because the MBW test is performed at rest [53].

Some algorithms can be used to adjust $N_2$ concentrations; however, these adjustments are often inadequate. Guglani et al. noted that the instrument settings could increase the $N_2$-LCI value compared to the SF6-LCI [57]. Because the later algorithms have not yet been validated for children by the major scientific societies, no standardized adjustment is used for measurements using the $N_2$ technique [58]. By contrast, intrinsic $N_2$ is present in all lungs, even those with impaired ventilation; therefore, $N_2$ can be more noticeable and easier to detect in lung abnormality evaluations than an exogenous gas. This is because, during the wash-in phase, not all pulmonary units are reached by an exogenous gas [39]. Another variable is the choice of interface because (as previously shown) it is responsible for external dead space. This specification is necessary, especially

for adults, because Robinson et al. described how different interfaces could affect results in adult patients; however, there is no evidence concerning children [59]. Another variable is the difference between the various available software programs provided by the analyzers [60].

No standardized procedure or a universal normal/pathological LCI value has been established, and there are only normative data related to equipment, protocol, and analysis. Until technique standardization is reached, MBW will not be widely adopted because it cannot be officially validated in the guidelines. The challenge is to standardize the procedure to bypass this limitation as much as possible. For all these reasons, there are different reference data for each technique.

## 1.10 LCI values in healthy subjects

Many papers have been published concerning LCI values in healthy people. Data on children are examined in this section. Reference values differ between $N_2$-MBW and SF6-MBW. In 2004, Aurora et al. published LCI values regarding healthy school-aged children. Measurements were obtained from SF6-MBW and a respiratory mass spectrometer (AMIS 2000, Innovision A/S, Odense, Denmark). Thirty-three subjects were tested. LCI 2.5% values were expressed as mean ± standard deviation (6.45 ± 0.49). In another study by the same clinicians, in 30 preschool-aged children, the LCI 2.5% value was 6.89 ± 0.44 [14].

Fuchs et al. collected data from healthy school-aged children. To measure SF6, an ultrasonic flow sensor rather than a respiratory mass spectrometer was used. The mean ages were 12.1 and 12.2 years for the Hannover and Innsbruck Groups, respectively. **Table 1** displays the results of the first test with 44 subjects.

| | **LCI 2.5% - SFP6MBW** | ULN | LLN |
|---|---|---|---|
| Hannover | 6.13 ± 0.3 | 6.64 | 5.57 |
| Innsbruck | 6.27 ± 0.5 | 7.06 | 5.36 |

**TABLE 1. SFP6-MBW LCI VALUES IN HEALTHY CHILDREN IN FUCHS ET AL. COHORTS. ULN: UPPER LIMIT OF NORMAL; LLN: LOWER LIMIT OF NORMAL.**

To obtain an acceptable LCI value, measurements were performed in triplicate. Within-test repeatability was expressed as "coefficient of variation" (CV), and this value was expressed as the ratio between the standard deviation and the mean of the three measurements (CV = SD/Mean). This value should be < 10% [61].

To assay reproducibility, two or three tests were performed for each subject. Reproducibility in a subject refers to the matching between different longitudinal evaluations over time. Reproducibility and repeatability are assumptions to validate the LCI as a tool for a patient's longitudinal evaluation [61]. Lum et al. is a reference point in the LCI literature. The authors used the RMS with SF6 to evaluate 497 subjects (from 2 weeks to 19 years old) [62]. Indications about upper and lower limits of normal (ULN and LLN, respectively) and LCI z-score were provided with equations to normalize LCI and FRC height and age values. The authors specified that the equations were principally validated for equipment using SF6. The LCI value is influenced by height because the patients' spectra are vast, with robust differences between infants, children, and young adolescents, considering periods of growth and changing lung structures [62].

Anagnostopoulou et al. published outcomes from tests using the $N_2$-MBW technique obtained from school-aged Caucasian children (**Table 2**) [47].

| | **LCI 2.5% - N2MBW** | ULN | LLN |
|---|---|---|---|
| Caucasian children | 7.04 ± 0.45 | 7.91 | 6.16 |

**TABLE 2. N2-MBW LCI VALUES IN HEALTHY CHILDREN.**

The final sample consisted of 180 subjects despite initial recruitment of 285 subjects; 67 were excluded because of age or ethnicity, and 38 did not fulfil the inclusion criteria. The Exhalyzer-D (Eco Medics AG, Switzerland) was the ultrasonic flowmeter, and Spiroware version 3.2.1 was the software used to analyze data [47]. The authors found some differences from the study by Lum et al.. Anagnostopoulou et al. reported that the distribution of LCI values was narrow in their sample consisting of children ranging from 6 to 19 years in age; hence, the same ULN value could be used in this population [47].

Another paper that analyzed the results of the $N_2$-based technique was published in 2012 by another group that also used the Exhalyzer-D; however, the analytical software was different [63]. The study by Anagnostopoulou et al. is significant because no reference parameters were used for the equipment and software used in several medical

centers; therefore, normative data were needed [47]. Furthermore, details such as the correlation between LCI and height and LCI and weight were examined in that study. In both cases, a negative association was found [47]. As affirmed above, LCI reference values differ depending on the technique. Briefly, the LCI normal value is between 5 and 7.54, depending on the variables listed above. When the resulting LCI value is higher than the ULN, LCI is considered an index of ventilation inhomogeneity and an early pulmonary damage indicator.

In summary, MBW supplies information about the FRC, dead space, and global ventilation inhomogeneity and provides insight into the location of an obstruction. MBW allows the calculation of "trapped gas" (e.g., the exhaled tracer gas after the end of a normal wash-out test) because exhaled gas originates from the very slowly ventilated pulmonary portions.

## 1.11 LCI in obstructive lung diseases

Some pathologies involve small airways in their initial stages rather than the larger ones (e.g., CF, PCD, and asthma). Under these circumstances, the MBW technique is more sensitive than spirometry for obtaining lung function measurements, and LCI permits early detection of these respiratory diseases [64].

### *1.11.1 LCI in cystic fibrosis (CF)*

#### *1.11.1a Cystic fibrosis (CF)*

CF is an autosomal recessive disorder caused by mutations in the CFTR gene [10]. CF is the most common genetic disorder in the Caucasian population and is mostly found in Northern Europe. It is a systemic pathology for which different phenotypes have been described, from classic CF to atypical forms [65]. Lung disease, pancreatic insufficiency, and a pathological sweat test ([Cl] > 60 mmol/l) describe classic CF, although other organs can also be involved. Atypical CF forms are characterized by conserved pancreas functional, borderline, or normal sweat test values ([Cl] 40–60 mmol/l or < 40 mmol/l), preserved lung function, and congenital absence of vas deferens causing male infertility [9, 66].

A change in the CF-associated epidemiology is occurring; in the past, CF was recognized as a childhood disease, while currently, there is an increasing number of patients reaching adulthood with preserved lung function [67, 68].

*1.11.1b Lung involvement in CF: inflammation and infection leading to bronchiectasis*

Pulmonary infectious diseases have been widely studied, and various hypotheses have been suggested to explain them. Immune system defects can be excluded because these infections recur only in the pulmonary parenchyma [69]. One of the first theories was that mutated CFTR acted as a *Pseudomonas aeruginosa* receptor, favoring cellular internalization [47]. Normally, this process would lead to apoptosis; however, in some cases, CFTR is present on the bronchial epithelial surface, and patients are equally susceptible to *P. aeruginosa* infection. Therefore, this theory was not satisfactory [70]. Another hypothesis focused on the effectiveness of local antimicrobial protection. The airway-surface liquid (ASL) pH plays a role in antimicrobial peptide functionality; specifically, acidic ASL inhibits antimicrobial peptide activity. Considering that the ASL acidification mechanism is due to a defect in $HCO_3$/Cl exchange, another dysfunctional aspect associated with CFTR mutation, this hypothesis appears to be more realistic [71].

Reduced ASL volume leads to adherent mucus plaque formation and consequentially to impaired mucociliary clearance. Because another function of CFTR is epithelial sodium channel (ENaC) inhibition, a reduction in the ALS volume could be due to an enhancement in ENaC activity. CFTR is also dislocated in submucosal glands in which can influence mucus dilution [71]. CFTR defects are directly or indirectly responsible for exaggerated inflammatory reactions in the lung parenchyma.

In the mucus layer, a hypoxic environment due to mucus thickening has been demonstrated; hypoxia triggers inflammatory responses [48] and supports bacterial growth [71]. Accumulation of the sphingomyelin metabolite ceramide occurs in CF airways, and this accumulation leads to epithelial cell death, cytokine release (pro-inflammatory molecules), and DNA damage [72]. In these cases, mucociliary clearance is limited because bacteria can bind extracellular DNA.

When bacteria colonize airways, neutrophils are recruited to attack them. Despite this activation being directed at microorganism elimination, it can also lead to pulmonary parenchymal destruction because of an imbalance between the proteases released by immune cells, including human leukocyte elastase, and antiprotease host defense mechanisms [50] that are functionally incapacitated because ASL is acidic.

Neutrophil extracellular traps (NETs) constitute another mechanism used by neutrophils. This mechanism favors DNA release in extracellular environments via cell death mechanisms. The physiological role of NETs is a contextualized infection response, while in CF patients, it plays the opposite role [73]. It appears that CF patients are susceptible to pulmonary infections, accompanied by uncontrolled neutrophilic reactions, and on the other hand, a phlogistic environment encourages bacterial growth, initiating a vicious cycle. Abnormal neutrophilic responses characterize the nature of CF inflammation [74].

Airway obstruction, infections, and inflammation underlie CF lung pathology. Airway wall structural alterations are the result of this phenomenon. Small airways are involved earlier than other lung sites, as demonstrated by autopsies of infants with CF. Esterly et al. described the structural situation as "widespread severe dilatation of respiratory bronchioles and alveolar ducts present in 29 of the older infants." They noted that bronchiectasis was present in all of the 6-month-old infants [75]. Based on these findings, two other fundamental points emerged: (1) small airway involvement and (2) early-onset structural alterations.

A progressive increase in airway diameter defines bronchiectasis. Functional abnormalities are the consequences of airways remodeling, and usually, the changes in airways structure are irreversible. When bronchiectasis becomes symptomatic, the clinical manifestations include a chronic productive cough and recurrent respiratory infections [76]. In the early stages, bronchiectatic airways are localized to the right upper lobe; in the second stage, there is substantial involvement of the entire lung parenchyma, even if in an irregular pattern [77]. Advanced lung disease is characterized by severe and diffuse bronchiectasis.

The necessity to identify risk factors for initiation and progression of bronchiectasis is exemplified by the relationship between the severity of structural alterations and the risk of developing acute pulmonary exacerbations [78]. Despite better survival rates for CF patients, lung disease and pulmonary exacerbations remain the primary cause of morbidity and mortality [79]. The scientific community has proposed guidelines for treatment aimed at preventing and stabilizing bronchiectasis and lung disease. To achieve these objectives, tailored patient therapy and surveillance tools are necessary because CF is heterogeneous.

*1.11.1c Necessity of surveillance tools*

Despite advances in treatment, 60%–80% of CF children are affected by bronchiectasis before six years of life. Because asymptomatic lung infections and remodeling can occur, patient surveillance plays a crucial role in managing this disease. Because lung structural alterations have been observed during the earliest periods of life, efforts have been made to improve these children's outcomes.

The frequency of surveillance is tailored to a patient because a regular patient review improves their outcomes. Generally, visits once per month are recommended for patients with advanced disease or in young children, and in stable patients, visits every three months are recommended [10].

Keeping CF pathophysiology in mind, it is possible to identify critical features that need to be detected to perform accurate patient surveillance. Inflammation, obstruction, and infections occur initially in small airways and can cause respiratory symptoms. When lung disease is silent, clinical manifestations cannot explain how the disease evolves; therefore, diagnostic tools are necessary. In a regular review of patients, respiratory symptoms, if present, and pulmonary function are examined.

Spirometry is routinely used to evaluate pulmonary function, particularly the severity of obstruction. Spirometry yields information about the large airways that are not involved in the early disease; therefore, spirometry is inappropriate for these cases. Bronchoalveolar lavage (BAL) is the gold standard to evaluate inflammation and lower airway infections; however, it is an invasive method requiring sedation [80]. Alterations in lung airway structure are detectable by imaging, X-ray, or computed tomography (CT). CT is more accurate than X-ray, although the radiation burden is higher. High-resolution CT is the current gold standard for detecting bronchiectasis, defined as bronchial/arterial ratio > 1.55 [81,82].

To summarize, each technique described is not appropriate as an early disease surveillance tool. Spirometry is a non-invasive technique, repeatable, non-expensive, and normative data that allows values to be compared across healthcare centers exist. On the other hand, spirometry is not sensitive for early disease detection, and preschool-aged children do not achieve an adequate test because spirometry requires cooperation from the patient. A CT is a sensitive and specific test, but the clinicians' concern about the burden of ionizing radiation in children limits the widespread use of CT in this population [83]. BAL is invasive and not routinely used.

MBW is used to assess the quality of ventilation and is a valid candidate to cover the lack of surveillance in early disease. In recent years, MBW has been revived because it is non-invasive, does not require cooperation from the patient, and small airways are its target. LCI is an indicator of inhomogeneous ventilation that MBW measures. More widespread use of LCI among CF centers appears to be occurring [11–15].

#### 1.11.1d Focus on LCI in disease assessment: progression, exacerbation, and treatment response

LCI capabilities have been evaluated in terms of its capacity to detect and track early lung disease and indicate disease progression. Kraemer et al. determined whether LCI was an early index of disease progression. They designed a longitudinal study in which patients were evaluated using spirometry and plethysmography to estimate FRC and $N_2$MBW to evaluate LCI. Evaluations of airflow, static lung volume and intrapulmonary gas distribution were their objectives. Patients were monitored yearly for 18 years at ages ranging from 6 to 20. LCI was the earliest and strongest factor associated with disease progression [84].

Because LCI in serial measurements is frequently used in CF centers to study lung disease, it is urgent to understand its variability over time in CF patients with stable disease. LCI increases during disease evolution, as shown by Stanojevic et al. in pre-school-aged children; they analyzed the rate of LCI deterioration (expressed as slope) in CF patients compared to healthy controls [85]. The case-group deterioration rate (0.40 LCI units/year) was greater than healthy patients (−0.04 LCI units/year). In healthy patients, the LCI value was stable during the study (one-year duration). The grade of slope in the CF group depended on their health condition; a worsening in LCI values occurred during coughing episodes or pulmonary exacerbations. In healthy controls, coughing did not influence the LCI slope [85].

LCI intra-subject variation among different measurements was examined by Svedberg et al. They identified the upper limit of normal variability between measurements. An increase of 17% in LCI values compared to the previous measurement might indicate lung disease progression [86].

Fuchs et al. compared LCI, CT findings, and forced expiratory volume in 1 min (FEV1) in school-aged patients with mild disease. Measurements were carried out yearly. They used the same concept as previously described in which the LCI was used during the follow-up and proposed LCI as a surrogate factor to track disease evolution and treatment response [87]. Because longitudinal LCI measurements predict lung

remodeling as shown on CT scans, the authors speculated about reducing CT frequency and radiation burden in children with stable LCIs or normal values. It is unlikely that a normal LCI value would be associated with extensive structural damage; however, in a few patients, LCI cannot detect alterations as visualized on CT; therefore, these tools are not entirely interchangeable [87].

Ramsey et al. confirmed the relationship between high LCI value in school-aged children and lung disease based on CT findings. The authors demonstrated in infants that there was no relationship between LCI and structural lung disease based on their CT findings; in pre-school-aged children, LCI correlated with total disease extent, and in school-aged children, LCI was an index of bronchiectasis and reflected the total extent of the disease. LCI showed an excellent predictive positive value of 83%–86% in the latter two groups, while the predictive negative value range 50%–55% for bronchiectasis detection [88]. Although LCI is sensitive to bronchiectasis, CT remains mandatory to identify bronchiectasis in CF patients, after which LCI might be a surrogate to use during surveillance of an already assessed situation. Despite the finding that LCI in infants does not appear to be related to structural lung disease already identified by CT, defective CFTR function present since birth and functional abnormalities can also be highlighted in very young infants.

Hoo and colleagues studied pulmonary function in 3-month-old CF infants [89]. LCI was used to assess inhomogeneous ventilation; however, only 21% of these CF infants had abnormal LCI values. BAL was not obtained. Lung function was already reduced despite early diagnoses obtained by newborn screening and initial treatment for infections, prophylactic antibiotics against pulmonary infections, and nutritional implementation [89]. The authors suggested establishing a narrow surveillance plan for each child with impaired lung function to improve individualized treatment [89].

Belessis et al. explained the high LCI values in 21% of infants despite antibiotic treatment. Infants were studied using LCI and BAL. LCI appeared to be associated with lung inflammation (as shown by elevated interleukin-8 [IL-8] and neutrophilic enzymes and cells) [90]. The difference in LCI values between CF infants with and without infection was not statistically significant. However, LCI correlated with pathogen load. Infants affected by *P. aeruginosa* on BAL had higher LCI values (mean 7.92 ± 1.16) than children with no bacterial colonization (7.02 ± 0.56). Furthermore, *P. aeruginosa* infection was associated with higher inflammatory markers in BAL samples than in

children infected with other pathogens because this microorganism leads to an intense neutrophil response. If LCI values are less than the upper limit of normal, *P. aeruginosa* could be excluded (93% NPV) [90]. A substantial increase in LCI values was reported in older children and adults [91].

The same situation, dissociation between treatment for silent clinical infection and failure in LCI improvement, was shown in another study in subjects with a different age range [92]. A recent randomized pilot study of LCI-triggered intervention was published. Twenty-nine CF children ranging from 5 to 18 years old were randomized into two groups [92]. One unit of LCI increase was used as the trigger to perform BAL in the case group. If an infection were detected, antibiotic treatment would be initiated. Both groups received the standard of care. The only difference was that triggered-LCI BAL was performed in the intervention group. Results were not encouraging because the difference between the populations concerning the impact of an intervention on LCI rate deterioration was not statistically significant [92]. The authors interpreted these results given that other types of treatments (e.g., mucociliary clearance and hypertonic saline) improve the condition, and CFTR modulators cause a significant improvement in LCI, perhaps due to their indirect anti-inflammatory actions [93–95]. Hence, one of the explanations considered in a recent study of cumulative lung damage was inflammation and infection. Rosenow and colleagues published a paper on the role of inflammation and infection in extensive lung disease. CT and BAL were examined for 6 to 7 years. The findings supported the idea that inflammation, rather than infection, had a higher cumulative effect on the extent of structural lung disease [96]. Other authors agreed that chronic inflammation could be the primary determinant in lung pathogenesis. Inflammation could be "sterile," for example, due to hypoxia described by Montgomery et al. and not triggered only by infection [97].

An association between the baseline LCI value and clinical endpoints (pulmonary exacerbation and CF Questionnaire-Revised [CFQ-Rresp]) was established by Vermeulen et al. The authors reported that high LCI values, presented as quartiles, could be related to early pulmonary exacerbation (the authors considered only those who had required intravenous treatment), in patients with preserved and non-preserved FEV1. Because a relationship between LCI and the most common clinical endpoints (PE and CFQ-Rresp) was established, the authors proposed LCI as a surrogate outcome [98].

For specific instrumental measures to become outcome measures, they should be associated with clinical endpoints (a direct quantification of how a patient feels) [99]. Papale et al. showed high effectiveness in predicting nocturnal hypoxemia in stable patients with CF, particularly when compared with a traditional lung function parameter such as FEV1 [100]. Because progress has been made in clinical and pharmaceutical areas in recent years, a growing number of patients have preserved lung function, expressed as predicted FEV1. In recent years, the purposes of pharmacological treatments have changed from improvement in lung outcome to maintaining function. This change means that treatments are capable of preserving lung function. For all these reasons, new and more sensitive outcome measures are necessary. LCI appears to be the right tool to evaluate patients with early and mild disease; in fact, LCI is used in routine clinical settings to assess the response to the modulator/potentiator treatment and clinical trials to test new treatments.

Clinical responses to ivacaftor, an oral CFTR potentiator, were tested in children with preserved lung function (p-FEV1 > 90%) and evaluated using LCI as an endpoint, given that lung function was normal. The results indicated an improvement, represented as an LCI reduction in patients treated with ivacaftor [101]. In 2017, the first placebo-controlled phase 3 trial using LCI as the primary endpoint was performed with a 1-unit LCI decline as the objective. Efficacy and safety of lumacaftor-ivacaftor were tested in children with FEV > 70% [72]. The choice of LCI as the endpoint in clinical trials is more advantageous because a small sample size can be sufficient to achieve acceptable results as opposed to a sample studied using FEV1 as the endpoint [102].

In summary, LCI appears to be a valuable tool in early/mild disease surveillance, and international guidelines will probably recognize this value as a standard in conjunction with spirometry to track CF disease in clinical centers.

### *1.11.2 LCI in primary ciliary dyskinesia (PCD)*

Primary ciliary dyskinesia (PCD) is an inherited disorder with typically autosomal recessive genetics that usually involves chain dynein genes that encode various dynein chains. Cilia are composed of microtubules that interact with dynein to create movement [103]. As their name suggests, dynein acts as a motor molecule. It is understood that ultrastructural alterations in cilia caused by genetic alterations are responsible for deficits in movements. Ultrastructural defects consist mainly of the

absence or shortening of outer dynein arms (ODA) in 38.5% of cases or an ODA defect in conjunction with an inner dynein arm (10.5%) [103].

PCD is a heterogeneous disease because several genetic alterations can lead to dysfunction, and a relationship between the genotype and the phenotype has been described [80].

Since cilia are localized at different sites along the organism, ciliary dysfunction involves different organs. However, the most critical system from a clinical and prognostic point of view is the respiratory system from the upper (middle ear, sinus, nasopharynges) to the end of the small airways [104, 105].

Mucociliary clearance is a primary defense mechanism used by the airways against microorganisms and environmental particulates. In PCD, ciliary function is deficient, resulting in impaired mucociliary clearance. Clearance dysfunction has different grades of severity because the level of ciliary dysfunction depends on which/how the genes are involved (the genotype-phenotype relationship). An example of how a different genotype can confer a different phenotype is the presence of situs inversus in only 50% of patients. A condition characterized by chronic sinusitis, bronchiectasis, and situs inversus (one of the laterality defects) has been recognized (called Kartagener's syndrome) [81]. Respiratory features include neonatal respiratory distress, chronic cough, recurrent pneumonia, and bronchiectasis [79].

Because impaired mucociliary clearance occurs, airways are colonized by bacteria (e.g., *Staphylococcus aureus* and *Haemophilus influenzae*), and chronic bronchitis is the consequence. Chronic bronchitis favors the establishment of bronchiectasis. A PCD diagnosis is usually obtained after four years of age [79]. Because PCD is a genetic disease, researchers believe that large airways were less involved in the early stage than smaller ones since data obtained by spirometry were above the lower limit of normal [106]. In earlier stages, chronic cough might partially compensate for insufficient clearance of the upper airways; however, the small airways do not benefit from this mechanism because infections frequently occur in the lower airways [107].

Green et al. used MBW to assess small airway function to test their hypothesis [108]. Twenty-seven PCD patients were compared to normal data obtained from the literature. The authors demonstrated that LCI values in ill children were higher than in healthy subjects (9.44 versus 6.33). They then compared LCI values with data obtained from spirometry and found that ill children with normal FEV1 had abnormal LCI values

[108]. A focus on Scond was necessary because it was normal in only one PCD-patient while all of the others had abnormal values. Given these results, it appears that alterations occur in small airways and conducting and acinar airways [108].

Another group compared PDC patients to healthy controls and CF patients. Ill patients with CF and PDC had FEV1 values > 60%. These data agreed with a previous study [58]. LCI values proved to be more sensitive than spirometry and CT findings [109]. There are several analogies between LCI papers in PCD and CF, and because CPD is a rare disease that is less well studied than CF, clinicians follow the CF approach because impairments in mucociliary clearance characterize both diseases.

### *1.11.3 LCI in asthma*

Asthma is a chronic inflammatory disease characterized by respiratory symptoms and accompanied by reversible limitations in airflow. Genetic and environmental factors are involved in asthma pathogenesis [110]. Respiratory manifestations are wheezing, shortness of breath, cough, and chest tightness, and their presentation is intermittent over time. In 50% of asthmatic patients, respiratory symptoms began during childhood [111,112]. The Global Initiative for Asthma international guidelines define asthma as "a heterogeneous disease, usually characterized by chronic airway inflammation." It is considered heterogeneous because symptom severity varies among patients, and an intra-subject variability also exists whereby different asthma phenotypes have been described over the years [113].

Symptomatology exacerbations are due to intermittent airway closures because of their hyperresponsiveness, clarifying the sudden recurrence. Chronic inflammation can lead to airway remodeling with mucus hypersecretion and wall thickening (via smooth muscle cell hyperplasia/hypertrophy). When airway remodeling occurs, airflow limitation became irreversible [114]. Because asthma is the most frequent chronic pathology in children, understanding how pathology evolves is critical as it is an essential cause of emergency department visits; furthermore, 30%–40% of the patients remain asthmatic into adulthood. Airways in asthmatic children must be studied to establish tailored therapy. Appropriate management of the pathology to achieve reasonable control of symptomatology and prevent exacerbations and airflow limitation from becoming irreversible is the therapeutic goal [115].

For a long time, only large airways were studied from the functional perspective, although asthma involves both small and large ones confirmed by histological studies on transbronchial biopsies and autopsies studies on patients with asthma [116]. Severe asthma (SA) refers to those who are refractory to treatment, challenging to treat or have comorbidities [117]. Patients affected by SA have dyspnea that contributes to poor health-related quality of life. Data provided by spirometry, the gold standard to evaluate asthma severity, are weakly correlated with health status and dyspnea and are usually used as clinical outcomes [117]. Thus, researchers have begun to consider the role of small airways in the genesis, evolution, and clinical manifestations of asthma. Takeda et al.'s findings in adults were originally documented using impulse oscillometry (IOM) [116]. Another study demonstrated differences in asthma control between two patient groups that were treated with inhaled corticosteroids (ICS): (1) one group received extra-fine particle ICS (< 2-μm particle size) and (2) the other group received standard-particle ICSs. Patients treated with extra-fine particle ICSs had fewer asthma attacks and better control of symptoms [118]. Extra-fine particles probably reach peripheral airways better than standard-particle ICS [119]. These findings have been applied to pediatric populations. Children's small airways were studied using the MBW technique and are used for other pathology assessments because spirometry is insensitive to damaged peripheral airways [119].

Interestingly, there were differences in LCI use between asthmatic populations and CF/PCD patients; LCI was used to assess the status of the small airway in asthmatic children with SA and uncontrolled asthma. To define pathology uncontrolled by therapy, external factors such as poor environments, smoking, and gastroesophageal reflux that can undo treatment benefits must be excluded [120]. In these children, LCI can be used to monitor lung function evolution. In this way, LCI can be used to target the treatment choice. Extra-fine particle ICS and montelukast can be used to treat small airways. Belinelo et al. used LCI as one of several outcomes in evaluating SA children during inhaled therapy (biological or not) [117]. Their first purpose was to identify and examine therapy-refractive children and those not challenging to treat. At the first visit for therapy-refractory children, 79% had abnormal LCI while only 48% had altered FEV1% values, suggesting that LCI is more helpful in discriminating asthma severity. LCI values in SA children were higher than in healthy controls or other mild asthma children: (1) LCI in SA was 10.5 ± 2.3; (2) LCI in healthy children was 7.3 ± 1.0, and (3) LCI in children with

mild asthma was 7.6 ± 1.2. The Exhalyzer-D was used to obtain LCI measurements [117]. However, during the follow-up, LCI remained abnormal in most patients despite improvements in FEV1 and asthma control. Concerning abnormal LCI, two explanations were proposed by the authors: (1) high values were probably due to lack of fully-tailored therapy in these subjects; and (2) high values were correlated with a particular structural situation that does not allow the active ingredient to distribute to all lung compartments due to narrow airways. For the authors, the latter situation could mean that, in this scenario, inhaled therapy could not be recommended because small airways did not appear to benefit from inhalation treatment [117].

Steinbacher et al. highlighted the association between LCI and airway hyperresponsiveness. They also found an association with Scond [121]. Small airway involvement was not exclusively associated with SA. Takeda et al. demonstrated that dysfunction could also be found in mild asthma; despite this finding, LCI remained within the normal range in most moderate-asthma children [116]. Although LCI and Sacin were normal in preschool children, Vilmann et al. found abnormal Scond [122]. In summary, MBW in the evaluation of asthma has a role in diagnosing SA.

## 1.12 LCI and lung function assessment in other diseases

The incidence of cancer in children has been increasing in recent years because of improvements in diagnosis and treatment. Treatments can be pharmacological, radiotherapeutic, or surgical, and in some cases, hematopoietic cell transplantation is performed. Adverse reactions to therapies include organ dysfunction, subsequent malignancies, and psychosocial complications. The onset of these reactions can be short- or long-term and lead to sequelae and worsening quality of life until an early death. For this reason, the Children's Oncology Group Late Effects Committee suggested long-term follow-up guidelines for pediatric cancer survivors [123].

Lung dysfunction is one sequela of chemotherapy. Lung disease cumulative incidences were described in the Childhood Cancer Survivor Study (CCSS) in a 45-year-old population who complained of pulmonary symptoms, for example, chronic dry cough. Pulmonary fibrosis was associated with the cough. The CCSS found lung dysfunction in 29.6% [124]. Bleomycin, nitrosoureas, and radiotherapy are widely prescribed for pulmonary dysfunction. Children treated with these molecules are classified as at-risk

patients whose pulmonary damage could be restrictive, obstructive, or diffusion-defective [125]. Cyclophosphamide, an alkylating agent, appears to be responsible for developing pulmonary fibrosis in patients treated with this drug [99]; however, patients are not considered at risk for fibrosis [126].

The most common type of malignant neoplasm during childhood is acute lymphoblastic leukemia (ALL). Due to the development of new therapeutic protocols, patient surveillance has improved. Cyclophosphamide is used during the consolidation phase [127]. The association between lung disease and cyclophosphamide treatment has been studied by our group using the $N_2$MBW technique for the first time to study this association [128]. The original off-treatment cancer survivor sample was restricted to those children who had a history of ALL. Because the damage caused by cyclophosphamide appears to be localized to peripheral airways, LCI was considered for use in this study. Small airway injury was described by Segura et al. as the "presence of atypical cells in the alveolar and bronchiolar epithelium, hyperplasia of type II pneumocytes, and interstitial and intra-alveolar edema and fibrosis" [126].

LCI values expressed as z-scores and spirometric parameters (FEV1 and FVC) were examined following the final drug administration. The correlation between increasing LCI values and years since the final dose was statistically significant ($r = 0.35$; $p \leq 0.05$) while those who underwent spirometric examinations showed no correlation for FEV1 ($r = 0.16$; $p$ = n.s) and FVC ($r = 0.20$; $p = 0.23$) [128]. Damage in small airways usually develops over time because pulmonary fibrosis has been described as a late-onset sequela. This result could be interpreted as a probable consequence of cyclophosphamide treatment. Thus, in this study, LCI was more sensitive than spirometry; hence, the MBW technique should be considered in patient surveillance during periods of no treatment [128].

# CHAPTER 2. RESEARCH AIMS

Considering the emerging role of the lung clearance index (LCI) in the evaluation of childhood respiratory diseases, the aims of my researches during the three years of PhD were: i) to evaluate this index in some pediatrics respiratory diseases including lung fibrosis in cancer survivors, cystic fibrosis, and finally in children healed from COVID-19; ii) to increase the scientific evidences on the role of LCI as a biomarker in pediatric respiratory diseases.

# CHAPTER 3. RESEARCH # 1: Lung clearance index: a new measure of late lung complications of cancer therapy in children

## 3.1 Introduction

In recent years, the improvement of diagnostic techniques and therapeutic strategies has led to an increase in the number of pediatric cancer survivors (CSs). The latest data show that more than three-quarters of children diagnosed with malignancy survive 5 years after diagnosis, and 1 in 600 young adults are estimated to be pediatric CSs in Western countries. In the face of improved survival, a downside is the complications that childhood CSs might experience as a result of the same life-saving treatments. In fact, the treatment-related complications represent one of the main causes of morbidity, they have a strong impact on the quality of life, and they predispose CSs to higher mortality in adulthood [129].

Pulmonary complications in children with malignant neoplasms can be distinguished as acute (if they occur during treatment) or as late. Different causes related to both the neoplasm itself and the treatment are recognized. Regarding acute complications, infections are the most common cause of lung damage. The use of cytotoxic and immunosuppressive drugs alters the body's innate and adaptive physiological defense mechanisms, and it frequently causes neutropenia, a type of cell-mediated and humoral immunity deficiency [130].

Long-term complications are the result of lung surgery, mediastinal radiation therapy, the immune phenomena following the transplantation of hematopoietic cells (HCT), and chemotherapy drugs (where provided) [131]. For these reasons, the Children's Oncology Group (COG) developed the COG Long-Term Follow-Up (COG-LTFU) Guidelines to identify risk categories for patients who have undergone cancer treatment and thus establish the stages of follow-up. Regarding respiratory complications, follow-up is recommended for patients who have been treated with bleomycin, busulfan, nitrosoureas, chest irradiation, or allogenic HCT with chronic graft versus host disease, if associated with chest X-ray abnormalities (scarring of pulmonary parenchyma or pleura) or impairment in lung function (Forced Expiratory Volume in 1 second, FEV1, < 80% of

predicted, Forced Vital Capacity, FVC, < 80% of predicted, Total Lung Capacity < 80% of predicted, or Diffusing Capacity of the Lungs for Carbon Monoxide, DLCO, < 80% of predicted).

As for the other categories of patients, no specific recommendations are given [132]. However, more recently, cyclophosphamide has also been reported as toxic to the lungs. This drug is widely used in the treatment of Acute Lymphoblastic Leukemia (ALL), the most common type of pediatric tumor [133]. Conventional spirometry is considered the main examination method for respiratory function to evaluate the degree of any obstructive or restrictive deficit. However, there is growing evidence that conventional spirometry is insensitive when detecting early damage to the small airways and assessing the distribution of ventilation. This response has also been described in many other pathologies, such as cystic fibrosis. In this context, there is growing interest in gas dilution techniques, especially multiple breath-washout (MBW), for the evaluation of peripheral airway function and to evaluate the possible inhomogeneity of ventilation [134,135].

The MBW technique was used for the first time more than half a century ago but was set aside for many decades until recently. It has returned to the fore in the field of pediatric pulmonology as an essential exam to obtain the Lung Clearance Index (LCI). High LCI values are an expression of ventilatory inhomogeneity reflecting damage to the small airways. Furthermore, since tidal breathing is sufficient for the examination, it can be performed on uncooperative children [136,137]. Several studies have evaluated respiratory complications in childhood CSs with conventional spirometry, but to our knowledge, there are no studies on LCI in this cohort of patients. The aim of our study was to evaluate this index in a cohort of patients with a history of childhood cancer who do not belong to only the categories defined as at risk. We also compared them to a group of healthy controls of the same age. The findings could indicate whether this approach offers any information beyond that obtained by conventional spirometry.

## 3.2 Materials and Methods

### *3.2.1 Study design and participants*

We designed a case-control study in which child CSs were compared with healthy controls (HCs). This study is part of a departmental project of the University of Catania, Department of Clinical and Experimental Medicine, with the aim of studying the

long-term complications of chemotherapy and radiotherapy in patients who have recovered from cancers diagnosed in the pediatric age range. CSs (0–18 years old) were recruited from the Pediatric Hemato-Oncology Unit at the Polyclinic University Hospital of Catania, Italy. All children had a history of pediatric cancer, undergoing chemotherapy, or radiotherapy treatment.

The eligibility criteria included an interval time of at least 1 year from the end of the cancer treatment. The exclusion criteria included prematurity, congenital heart disease, smoking, other chronic lung diseases not apparently related to or consequent to pediatric cancer; however, we decided to keep those patients with any pulmonary involvement following cancer therapy. All these patients usually undergo one spirometry session per year at our Pediatric Bronchopneumology Unit of the hospital. To carry out this study, we also performed the MBW test in addition to conventional spirometry. Frequency-matched HCs for sex and age with no history of cancer were recruited from the general population. The study has been approved by the local committee for clinical investigations, and informed consent was obtained from all parents of participants.

### *3.2.2 Clinical and pulmonary function evaluation*

A physician collected the medical history and performed a detailed physical examination of the study participants. The medical history included the type of cancer, date of diagnosis, type of treatment, date of suspension of treatment, and any respiratory symptoms. Subsequently, MBW and then the conventional spirometry were performed (always in that order).

MBW testing was performed during relaxed and stable tidal breathing using the Exhalyzer D (EcoMedics AG, Duernten, Switzerland) and an inert intrinsic gas (nitrogen). All subjects underwent the test until the test gas reached $1/40^{th}$ of the initial gas concentration to obtain the LCI value. Testing was performed in triplicate, and the mean LCI is reported from a minimum of two (but aiming for three) technically acceptable tests [138-140]. LCI was analyzed as both raw scales and z-scores calculated based on published reference equations [141]. Spirometry was performed in the laboratory according to ERS/ATS guidelines [142, 143]. The best spirometric measure of at least three attempts was recorded for the analysis. FEV1 and FVC were expressed as a percentage of predicted values and z-scores using the GLI equations [144].

### 3.2.3 Statistical analysis

Statistical analyses were performed with the software Graph Pad Prism version 8.3.0. CSs and HCs were matched by sex, age, and height. Subjects' characteristics are presented as the median (interquartile range) for continuous variables or a frequency for categorical variables. Comparisons between groups were calculated using the student t-test or Mann-Whitney U test for continuous variables, while Fisher's exact test was used for categorical variables. The degree of association was determined by applying a linear regression model and calculating the Pearson correlation coefficient (r). P-values $< 0.05$ were considered to be significant.

## 3.3 Results

The baseline clinical characteristics of the study subjects are summarized in **Table 3**.

| Variables[a] | Cancer Survivors (CSs) N = 57 | Healthy Controls (HC) N = 50 | P value[b] |
|---|---|---|---|
| **Age (years)** | 13.1 (11.1, 15.8) | 12.5 (11.4, 14.4) | 0.45 |
| **Weight (kg)** | 48.0 (40.0, 62.0) | 51.5 (42.0, 62.0) | 0.90 |
| **Height (cm)** | 156.0 (144.0, 165.0) | 157.5 (148.0, 170.0) | 0.20 |
| **Male, N (%)** | 35 (61%) | 31 (62%) | 0.95 |
| **Asthmatic children, N (%)** | 3 (5%) | 2 (4%) | 0.99 |

**TABLE 3. DEMOGRAPHIC FEATURES OF CANCER SURVIVORS (CSS) AND HEALTHY CONTROLS (HCS).**

[A] VARIABLES ARE PRESENTED AS MEDIAN (INTERQUARTILE RANGE);

[B] COMPARISONS BETWEEN GROUPS WERE CALCULATED USING STUDENT T-TEST (FOR AGE AND WEIGHT) OR MANN-WHITNEY U TEST (FOR HEIGHT) FOR CONTINUOUS VARIABLES; FISHER'S EXACT TEST WAS USED FOR CATEGORICAL VARIABLES.

We enrolled 57 off-treatment CSs and 50 HCs matched for sex, age, and height. Three CSs and two HCs were also asthmatic. Among the patients with a history of cancer, those with a history of ALL were the most common (n = 38, 67%), followed by four (7%)

patients with a history of Acute Myeloid Leukemia (AML). 15 (26%) patients had solid tumors (**Figure 5**).

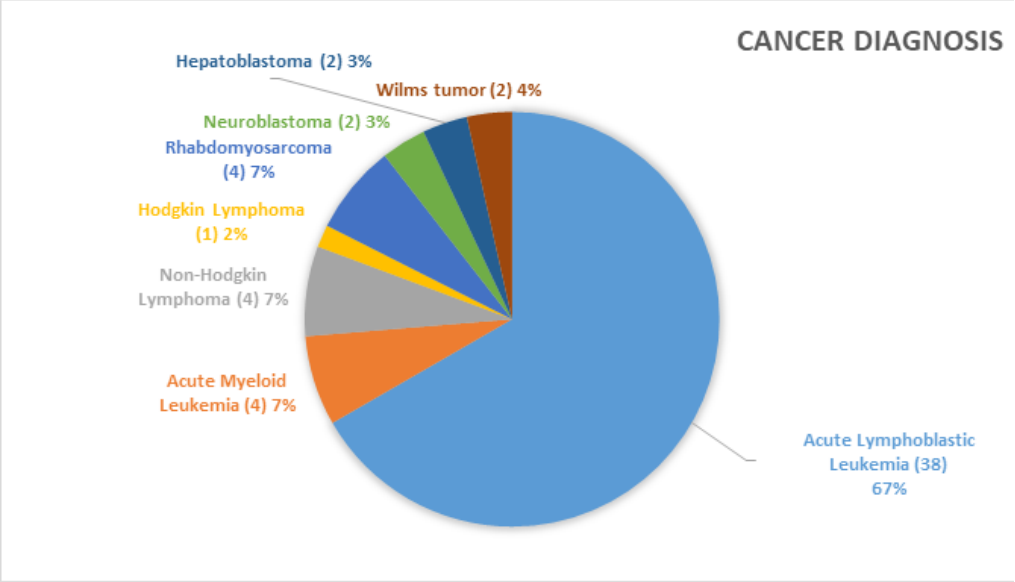


FIGURE 5. CANCER DIAGNOSIS DISTRIBUTION.

The median age at diagnosis was 3.2 years, and the median number of years since the last treatment was 6.2 years. Cyclophosphamide was the most frequently used chemotherapeutic agent (**Table 4**).

| Variables | Cancer Survivors (CSs) |
|---|---|
| **Age at diagnosis, median year (interquartile range)** | 3.2 (2.2, 5.2) |
| **Years from last chemotherapy median year (interquartile range)** | 6.2 (4.6, 8.8) |
| **Hematopoietic cell transplantation, N (%)** | 3 (5.3%) |
| **Bleomycin, N (%)** | 2 (3.5%) |
| **Busulfan, N (%)** | 2 (3.5%) |
| **Nitrosoureas, N (%)** | 0 |
| **Cyclophosphamide, N (%)** | 46 (80.7%) |
| **Lung Irradiation, N (%)** | 1 (1.8%) |

TABLE 4. FOLLOW-UP DATA OF CANCERS SURVIVORS (CSS) AND NUMBER OF THEM WHO HAVE UNDERGONE TREATMENTS WITH RISK OF LUNG COMPLICATIONS.

Compared with HCs, CSs' mean LCI values were 0.46 units higher (95% confidence interval (CI): 0.06–0.85), and their z-scores were 0.003774 units higher (95% CI: 0.000160–0.007388). However, these differences were not statistically significant. For conventional spirometry, we observed that CSs maintained good levels of respiratory function indices in comparison with HCs (**Table 5**).

| Variables[a] | Cancer survivors (CSs) N = 57 | Healthy Controls (HC) N = 50 | Δ (95% CI) CS - HC | P value[b] |
|---|---|---|---|---|
| **LCI** | 6.78 (1.35) | 6.32 (0.44) | 0.46 (0.06, 0.85) | 0.09 |
| **LCI z-score** | 0.001674 (0,011659) | -0.002099 (0,005850) | 0.003774 (0.000160, 0.007388) | 0.12 |
| **FEV1 (% of predicted)** | 99.9 (11.3) | 103.0 (5.9) | -3.1 (-6.6, 0.5) | 0.09 |
| **FEV1 z-score** | -0.14 (0.94) | 0.09 (0.59) | -0.23 (-0.55, 0,06) | 0.06 |
| **FVC (% of predicted)** | 98.2 (10.3) | 101.1 (3.3) | -2.9 (-5.90, 0.12) | 0.06 |
| **FVC z-score** | -0.16 (0.89) | 0.04 (0.40) | -0.20 (-0.47, 0.07) | 0.05 |

TABLE 5. LUNG FUNCTION TESTS IN CANCER SURVIVORS (CSS) AND HEALTHY CONTROLS (HCS).

We found 8 patients with abnormal lung function values (FEV1 <90% predicted) at baseline. The details of these patients are summarized in **table 6**.

| Patients' diagnosis | Cyclophosphamide | HCT | LCI (absolute value / z-score) | FEV1 % of predicted / z-score) | FVC % of predicted / z-score) |
|---|---|---|---|---|---|
| **ALL** | Yes | No | 6,96 / 0,006 | 7 / -1,96 | 0 / -1,81 |
| **ALL** | Yes | No | 6,93 / 0,006 | 9 / -1,78 | 1 / -1,58 |
| **ALL** | Yes | No | 10,07 / 0,030 | 7 / -1,05 | 3 / -1,51 |
| **ALL** | Yes | No | 6,99 / 0,007 | 3 / -2,29 | 3 / -1,45 |
| **Neuroblastoma** | No | No | 6,85 / 0,003 | 8 / -1,79 | 5 / -1,40 |
| **Rhabdomyosarcoma** | No | No | 13,18 / 0,044 | 9 / -0,98 | 8 / -1,01 |
| **Wilms' tumor** | No | No | 7,00 / 0.006 | 1 / -1,59 | 9 / -0,86 |
| **AML** | Yes | Yes | 7,43 / 0.009 | 9 / -1,95 | 1 / -2,33 |

TABLE 6. PATIENTS WITH ABNORMAL LUNG FUNCTION (FEV1 <90% OF PREDICTED) AT BASELINE.

Next, we assessed whether there was a correlation between the respiratory function indices and the years since the last chemotherapy session. To make the sample homogeneous, we only considered patients with ALL as having undergone similar treatment. In these patients, we observed that the LCI z-scores were closely related to the

years that had passed since the end of chemotherapy treatment (r = 0.35, P < 0.05, **Figure 6**). This correlation was not shown for conventional respiratory function indices, such as FEV1 (r = 0.16, P = not significant (ns)) and FVC (r = 0.20, P = 0.23, **Figure 7**).

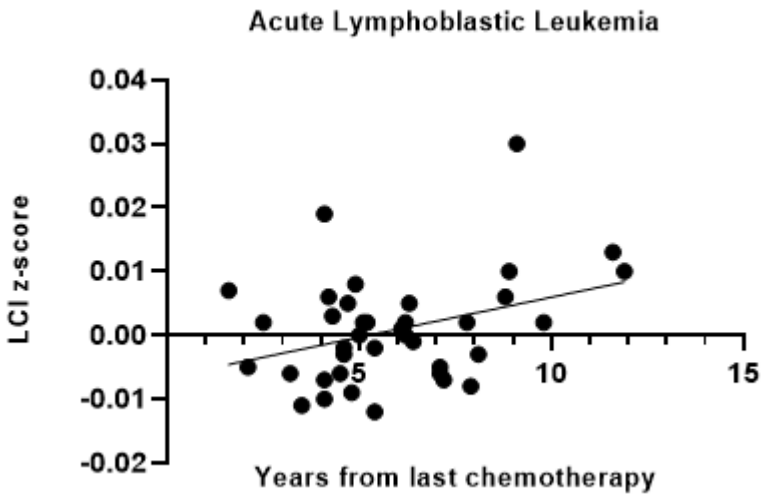


FIGURE 6. CORRELATION BETWEEN LCI Z-SCORE AND YEARS SINCE THE END OF THE CANCER TREATMENT (R = 0.35, P < 0.05).

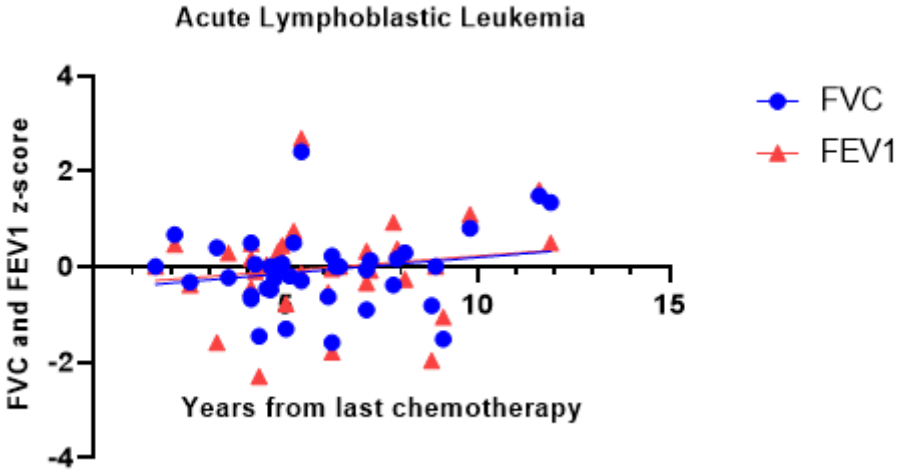


FIGURE 7. CORRELATION BETWEEN FVC AND FEV1 Z-SCORES AND YEARS FROM THE END OF THE CANCER TREATMENT (FVC: R = 0.20, P = 0.23; FEV1: R = 0.16; P = 0.34).

## 3.4 Discussion

The results of our study show that off-treatment CSs maintain good respiratory function values during childhood according to conventional spirometry and the LCI, which is a sensitive index of damage to the small airways. The respiratory function values obtained in both methods (MBW and spirometry) were comparable to those of healthy subjects. With regard to drug-induced respiratory complications, the most frequent clinical conditions described in CSs in the literature are hypersensitivity pneumonia, pulmonary edema, pulmonary hypertension, pleural effusions, pulmonary veno-occlusive disease (VOD), restrictive diseases, and obstructive pulmonary diseases [145, 146].

Among these complications, the most frequent clinical presentation is Drug-induced Interstitial Lung Disease (DILD) [147]. Although this pathological condition occurs in a minority of subjects treated with anticancer drugs, it can evolve towards severe respiratory insufficiency and acute respiratory distress syndrome. From a histopathological [148] and radiographic [149,150] profile, DILD can present in various ways, such as diffuse alveolar damage, chronic interstitial pneumonia, eosinophilic pneumonia, hypersensitivity pneumonia, and granulomatous pulmonary disease. The two main etiopathogenetic factors responsible for lung damage from drugs are direct lung toxicity mechanisms (production of reactive oxygen species, reduction of inactivation of metabolites in the lungs, and the same drugs) and immune-mediated mechanisms [147, 150].

Several chemotherapeutic agents have toxic effects on the lungs. These include bleomycin, a drug used in therapy for Hodgkin lymphomas and germ cell tumors. This drug accumulates in the lungs due to the reduced presence of the enzyme that performs detoxification in these organs. Pneumonia induced by this drug is a serious and often fatal complication. Pulmonary fibrosis is actually rare in children, whose bleomycin dosages are lower than in adults. Obstructive airway diseases and pulmonary hyperinflation are more frequently observed but are symptomatic in only a minority of patients [151,152].

Another drug that is believed to be toxic to the lungs is cyclophosphamide, one of the most common alkylating agents in the treatment of pediatric tumors. It is also frequently used in preparatory regimens for hematopoietic cell transplantation. Cyclophosphamide is responsible for interstitial pneumonia (early onset), which can evolve into pulmonary fibrosis (late complication) [133]. Also, for busulfan and

nitrosoureas (carmustine and lomustine), there is a possibility of conditions similar to those previously described [128].

Most of the follow-up studies of such patients have assessed the onset of respiratory complications in only categories of at-risk patients defined by the COG-LTFU guidelines (i.e., patients who have undergone treatment with thoracic radiation therapy, thoracic surgery, HCT, busulfan, bleomycin, and nitrosoureas) [132]. In such patients, studies have shown a percentage of lung complications varying between 45% and 85% [153-157]. Mulder et al. identified restrictive lung disease in patients treated with radiation only, bleomycin and radiation, and radiation with surgery and compared them to those treated with bleomycin only [153]. Landier et al. studied 370 childhood CSs and applied the COG-LTFU guidelines to identify patients at risk for pulmonary complications, of which 84% experienced lung complications over the years [154].

A report from the St. Jude Lifetime Cohort Study on 1713 adult survivors of childhood cancer showed that 65.2% have abnormalities in pulmonary function among survivors exposed to pulmonary-toxic cancer treatments. The highest prevalence occurred among those treated with lung radiation (74.4%), followed by those treated with bleomycin (73.3%) and thoracotomy (53.2%) [155]. In a study by Armenian et al., the percentage of patients in the risk category for lung complications experienced restrictive dysfunctions in 45% of cases [156]. The Childhood Cancer Survivor Study (CCSS) published in 2016 reported a cumulative incidence of pulmonary symptoms (chronic cough, oxygen need, lung fibrosis, and recurrent pneumonia) of 29.6% among a population of 14,316 CSs at 45 years of age (vs. 26.5% in siblings) [157].

In our patient series, few belonged to the risk categories since few patients had undergone treatments known to be associated with pulmonary complications. There is an exception with cyclophosphamide, however, which instead represents one of the main treatments of ALL and was associated with neoplasm occurring more frequently in our case history. In this regard, studies that have assessed respiratory complications in patients with a history of ALL are mainly dated and thus involve patients undergoing chemotherapy regimens with different drugs than those used today.

In 1998, Nysom et al. studied 94 survivors of ALL and showed that several of their participants had a subclinical, restrictive ventilatory insufficiency or restrictive flow-volume curve patterns [158]. Previously, in a study by Miller et al. on 15 patients with a history of ALL, 48% had lung function abnormalities [159]. Jenney et al. demonstrated

that at a median of 6 years after diagnosis among 70 survivors of childhood ALL, more than 50% had lower lung volumes and impaired maximal exercise capacity [160].

The strength of our study is that it evaluated not only patients at risk but all patients with a history of cancer for the first time. Furthermore, a more sophisticated method, MBW, was used and allowed for the study of the LCI. These data suggest that the chemotherapeutic agents used in the treatment of tumors previously analyzed have less toxicity than expected, at least in childhood. Furthermore, this study allowed for validation of the MBW method in this category of patients since it can also be carried out on preschool children who have difficulties performing the forced expiratory maneuvers, unlike traditional spirometry.

Also, the LCI study showed that this index increases and worsens as the years pass after the end of the treatment. This correlation is not evident with conventional spirometry and probably expresses a greater sensitivity than MBW in identifying lung damage, albeit minimal, for small airways 5-6 years after the end of treatment. In this age range, the degeneration to pulmonary fibrosis becomes more evident.

Finally, this study adds scientific evidence on the applicability of this method not only in patients undergoing cancer therapies but more generally in populations at risk of lung damage. In this sense, there are studies on the use of the MBW for infants with recurrent wheezing [161], symptom-controlled asthma [162], post-infectious bronchiolitis obliterans [163], spinal muscular atrophy [164], late preterm birth [165].

## 3.5 Conclusions

Our study described the trend of LCI in a variegated cohort of off-treatment cancer survivors and compared it with the results obtained from HCs. The results showed that patients maintain good values of respiratory function and good homogeneity of ventilation during childhood. However, as LCI increases and worsens as the years pass after the end of the treatment could identify the tendency towards pulmonary fibrosis, which is typical of adult CSs, at an earlier time than spirometry.

Our study also assessed not only classically defined at-risk patients but all cancer patients, including those with previous ALL treated with cyclophosphamide, a drug for which toxic effects of the lungs have been were described but not included in the list of COG-LTFU guidelines. Finally, the study allowed for the validation of MBW

for the calculation of the LCI in these patients since it can also be performed on preschool patients who are unable to perform forced expiratory maneuvers, unlike conventional spirometry.

# CHAPTER 4. RESEARCH #2: LCI IN OUR COHORT OF CYSTIC FIBROSIS PATIENTS

## 4.1 Aim of the study

As mentioned before, the Multiple Breath Washout (MBW) test seems to be a helpful tool in CF surveillance as it allows to calculate the LCI. In view of this, the aim of this study was to evaluate LCI sensitivity in detecting lung function and lung structure abnormalities, like *bronchiectasis,* in our cohort of Sicilian school-aged children suffering from CF comparing to conventional spirometry.

## 4.2 Matherials and Methods

### *4.2.1 Study design and participants*

This was a *monocentric*, cross-sectional, retrospective, and observational study in which lung function in healthy controls (HC) and in Cystic Fibrosis patients (CFp) was compared. This last group, made up of 16 CF patients (6-18 years old), has been followed in our center from their diagnosis. Neither low FEV1 (% of predicted) value nor genotype-phenotype are restrictions in CFp choice. Healthy controls group (50 children) was originally recruited to carry out another clinical study, in which exclusion criteria were congenital heart disease, chronic lung diseases, and smoking. Limit of age was 18 years old ($18^{th}$ years inclusive) in both groups. Data were collected since 2018,in both HC and CFp, in Paediatric Respiratory Unit at the San Marco Hospital of Catania, Italy. All data have been treated according to privacy normative.

### 4.2.2 Lung function evaluation

All measurements have been carried out during stable disease periods. MBW test and spirometry were performed during the same day. All LCI value were carried out during tidal breathing using the Exhalyzer D (EcoMedics AG, Duernten, Switzerland). Nitrogen was the tracer gas used to evaluate lung washout. Flow and CO2 sensors have been calibrated before testing patient. Mouthpiece plus nose clip were used because of age patients. Current recommendations, provided by ERS/ATS societies, have been followed to accomplish and select measurements [138]. For each patient, measurements were carried in *duplicate*, or triplicate and the coefficient of variation between them had to be under thresholds which are provided by the same consensus of above. Spiroware 3.2.1 version has been used to record measurements, as suggested by manufacturer. Final LCI value, expressed as raw data, is reported as mean of all acceptable measures. Equation provided by Lum et al. has been used to quantify LCI z-score [141].

Spirometry was performed in duplicate or triplicate following the current guidelines and the optimal performance has been considered for the study [142-144]. Both FEV1 and FVCare expressed as percentage of predicted and as z-score. GLI equation was used to calculate z-score of FEV1 and FVC. In addition, at least one Computer Tomography (CT) report per patient has been considered in the study since TC is the method of choice to detect bronchiectasis.

### 4.2.3 Statistical Analysis

Anthropometric features were summarized as median, for continuous data, and as percentage for categorical ones, such as sex. Continuous data (age, weight, height), since their normal distribution in both groups, have been compared using T-student test while to compare categorical one Fisher's exact test has been used. Lung function values, both spirometric and LCI, have been presented as median or means, depending on their distribution and compared using Mann-Whitney U test or T-student test. For each variable, Shapiro-Wilk test has been chosen to evaluate normal distribution values. Upper Limit of Normal (ULN) for LCI is 7.64 and it was obtained taking into account LCI values collected from HC as predicted. Equation provided by Lum et al. has been used [141]. Association between z-FEV1 and LCI values has been calculated using Spearman correlation coefficient (rho), because of the non-normal

distributions of FEV1 z-score values in entire CF population. In two subgroups, since normal distribution of FEV1-zscore, Pearson's coefficient (r) has been used to evaluate relation between the variables. Scatter diagram has been used to represent correlation. LCI and FEV1 sensitivity and specificity in bronchiectasis detecting have been evaluated using ROC (Receiver operating characteristic) curve. MedCalc and Excel were used to reshape data. P-values $< 0.05$ were considered statistically significant.

## 4.3 Results

### *4.3.1 Participants*

The original cohort was composed by 16 children while actually, in the final one, the amount of patients is 14 due to the lack of measurements quality criteria. Percentage of *patients drop-out* is 12,5%. After all data collecting, both spirometric and MBW ones, quality checking and selection have been done (**Figure 8**).

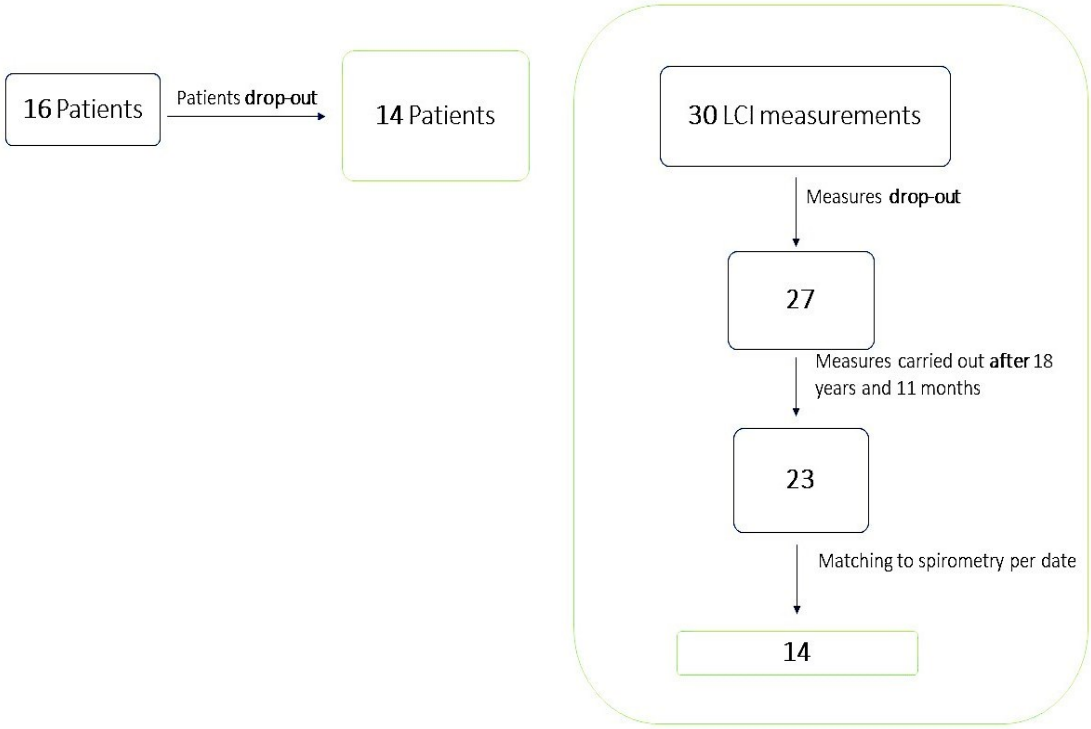


**FIGURE 8. PATIENT AND MEASURES SELECTION SCHEMATIC VIEW**

For 14 patients, 30 LCI measurements have been controlled to test their authenticity and3 of these were excluded because of the high Coefficient of Variation. Measures drop- out rate is 10%. The 27 remaining measures were selected to obtain single measure for child, since only seven of them had multiple LCI measurements while the rest of children were evaluated once. Some measures were excluded because of the age of patient at the test date, specifically four measures in two patients. Spirometry is usually carried out more often than MBW, hence LCI measures have been firstly chosen and then matched with spirometric measure. Matching was done *per* date since the tests have been often done the same day. The most recent measurements have been chosen. Anthropometric data, like weight and height, were evaluated in the same day of lungfunction testing, both per CFp and Healthy control. They are summarized in **table 7**.

| Anthropometric Features | | | |
|---|---|---|---|
| Variables | CFp (14) | HC (50) | p-Value |
| Age | 15 (12,5 - 17,25) | 12,5 (11,4 - 14,4) | *0,03* |
| Weight | 45,55 (37,65 - 59) | 51,5 (42 - 62) | 0,67 |
| Height | 157,5 (141,375 - 165,75) | 157,5(148 -170) | 0,4737 |
| Male | 9 (64,28% ) | 31 ( 62%) | 1 |

TABLE 7. ANTHROPOMETRIC FEATURES IN CFP AND HC

Checking CT reports, we have found that only 14,28% of patients had not bronchiectasis and they had normal values of LCI and FEV1, while 85,71% of patients had airways architecture alterations and only one of them had normal LCI and FEV1 values.

### *4.3.2 Spirometric values*

Considering the entire CFp group, children with FEV1>90% are 50% while the remaining patients have FEV1<90%. Among the last group, 42,85% of patients had FEV1>80% whereas 57,14% have FEV1 value less than 60%. FEV1 values between 60% and 79% are not present in our population. Median FEV1 value in HC is 103 (IQR 100 to 106) while the median value in CFp is 89,55 (57,70 to 98,50). Comparing this value in both groups, CFp and HC, the difference between them is 14 (IQR 7,4 to 22) and it is statistically significant. In 2012, ERS Task Force set out -1.64 as the Lower Limit of normal (LLN) for FEV1 z-score values [144]. Patients who had a FEV1 z-score above the LLN (i.e. pathological value) are 42,85%. Comparing FEV1 z-score values between two groups, the difference was statistically significant too. Difference between CFp and HC about FVC z-score value is statisticallysignificant, except in FVC which it is also expressed as percentage of predict value. Comparisons are summarized in **Table 8**.

| Variables | CFp (14) | HC (50) | Δ (C.I. 95%) | pValue |
|---|---|---|---|---|
| FEV1 | 89,55 (57,70 ; 98,50) | 103 (100 ; 106) | 14 (7,4 to 22) | <0,0001 |
| FEV1 z-score | -1,35 (-4,4250 ; -0,9760) | 0,14 (0,010 ; 0,44) | 1,5925 (1,1340 to 2,3220) | <0.0001 |
| FVC | 98,40 (87,30; 104,80) | 101 (100 ; 103) | 2,85(-1,80 to 8,30) | 0,2214 |
| FVC z-score | -0,3140 (-2,26; 0,18) | 0,10 (0,010 ; 0,30) | 0,481 (0,11 to 1,17) | 0,0305 |

TABLE 8. DIFFERENCES BETWEEN SPIROMENTRIC VALUES IN CYSTIC FIBROSIS PATIENTS (CFP) AND HEALTHY CONTROL (HC). FEV1 AND VALUES ARE EXPRESSED AS MEDIAN (IQR) BECAUSE OF THE NOT NORMAL VALUES DISTRIBUTION AMONG VARIABLE AND THEIR RESIDUALS.

### 4.3.3 LCI Values

Median LCI values in CFp is 11,42 (IQR 9,15 to 15,53) and it is considerably higher than HC (6,48 with IQR 5,95 to 6,63). LCI z-score values has been calculated in boththe group and difference between values is statistically significant. Results of comparisons are summarized in **Table 9.**

| Variables | CFp (14) | HC (50) | Δ (C.I. 95%) | pValue |
|---|---|---|---|---|
| LCI 2.5% | 11,42 (9,15 ; 15,53) | 6,48 (5,95 ; 6,63) | -5,195 (-7,2400 to -3,9500) | <0,0001 |
| LCI z-score | 0,7327 (0,5924 ; 0,8516) | 0,0002767 (-0,006225 ; 0,002355) | -0,7362 (-0,7898 to -0,6411) | < 0,0001 |

TABLE 9. DIFFERENCE IN LCI VALUES BETWEEN CFP AND HC GROUPS. VALUES ARE EXPRESSED AS MEDIAN (IQR) BECAUSE OF THE NOT NORMAL VALUES DISTRIBUTION AMONG VARIABLE

### 4.3.4 Comparison of the results

Making an analysis at glance, hence taking in to account the entire CFp population, LCI value was abnormal, i.e. higher than ULN, in 78,57% while pFEV1 z-score is lower than -1.64 in 54,54% of children. 62,5% of CFp with normal pFEV1 z-score values have *abnormal* LCI values. Then, specifically, we have done a stratification of all patients considering their pFEV1 values. So, we have analysed CFp values in different subgroups between each other,and with HC. Subgroup are three:

- CFp with FEV1>90: LCI abnormal value is present in 57,14% of them

while, to be exact, the rest of children have normal values;

- CFp with FEV1>80: only one patient has normal LCI value, who has FEV189%;
- CFp with FEV1<60: in this population LCI values are *always* higher than ULN.

Comparison between HC and subgroups have been done.

Firstly, in patients with FEV1<60%, differences came out by comparisons between variables are statistically significant, for every examined value, spirometric and LCI derived too. Table of results is not shown.

For patients who belong to FEV1>80% and >90 subgroups, data are shown in **Table 10** and **Table 11**. Another comparison has been done between FEV1>90 and FEV<90subgroups (**Table 12**)

| *Variables* | *CFp with FEV>80% (10)* | *HC (50)* | *Δ (C.I. 95%)* | *pValue* |
|---|---|---|---|---|
| FEV1 | 95 (8,78) | 103 (5,9) | 8 (3,53 to 12,54) | 0,0007 |
| FEV1 z-score | -1,04 (-1,39 to -0,59) | 0,09 (0,59) | 0,14(0,010 to 0,44) | <0.0001 |
| FVC | 101,20 (97,60 to 110,17) | 101 (100 to 103) | 3, 71 ( 0.11 to 7.30) | 0,6183 |
| FVC z-score | -0,12 (-0,47 to 0,21) | 0,10 (0,010 to 0,30) | 0,1670 (-0,1950 to 0,4810) | 0,5117 |
| *Variables* | *CFp with FEV>80% (10)* | *HC (50)* | *Δ (C.I. 95%)* | *pValue* |
| LCI 2.5% | 10,63 (7,49 to 11,70) | 6,48 (5,95 to 6,63) | -4,2600 (-5,18 ; -2,19) | <0,0001 |
| LCI z-score | 0,7699 (0,7229 to 0,9843) | 0,0002767 (-0,006225 to 0,002355) | -0,7737 (-0,8647 to -0,7366) | < 0,0001 |

**TABLE 10. COMPARISON BETWEEN CFP FEV1>80% AND HC. MANN-WHITNEY U TEST WAS USED TO MAKE COMPARISONS.**

| Variables | CFp with FEV>90% (7) | HC (50) | Δ (C.I. 95%) | pValue |
|---|---|---|---|---|
| FEV1 | 98,83 (7,51) | 103 (5,9) | -4,17 (-9,10 to 0,76) | 0,0957# |
| FEV1 z-score | -0,9760 (-1,05 ; -0,57) | 0,14 (0,010 to 0,44) | -0.89 (-1.35 to -0.42) | 0,0005 |
| FVC | 104,80 (98,77 to 113,86) | 101 (100 to 103) | -4,8 (-12,1 to 1,7) | 0,1176 |
| FVC z-score | 0,1810 (-0,3795 to 0,4900) | 0,1 (0,01000 to 0,3000) | -0,081 (-0,48 to 0,33) | 0,6785 |
| Variables | CFp with FEV>90% (7) | HC (50) | Δ (C.I. 95%) | pValue |
| LCI 2.5% | 10,18 (7,01 to 11,12) | 6,48 (5,95 to 6,63) | -3,69(-4,79 to -0,90) | 0,0001 |
| LCI z-score | 0,7903 (0,7443 to 1,0302) | 0,0002767 (-0,006225 to 0,002355) | -0,7900 (-1,0411 to -0,7472) | <0,0001 |

TABLE 11. COMPARISON BETWEEN CFP FEV1>90% AND HC. MANN-WHITNEY U TEST; # T-TEST

| Variables | CFp with FEV>90% (7) | CFp with FEV<90% (7) | Δ (C.I. 95%) | pValue |
|---|---|---|---|---|
| FEV1 | 98,83 (7,5) | 64,2857 (21,3092) | -53,1511 to -15,9403 | 0,0016 |
| FEV1 z-score | -0,8006 (0,4533) | -3,5557 (1,7715) | -2,7551(-4,2610 to -1,2493) | 0,0018 |
| FVC | 107,72 ( 11,59) | 87,3571 (11,6163) | -20,3671 (-33,8812 to -6,8531) | 0,0065 |
| FVC z-score | 0,2109 (0,7325) | -1,6756 (1,2420) | -1,8864 (-3,0739 to -0,6990) | 0,0047 |
| Variables | CFp with FEV>90% (7) | CFp with FEV<90% (7) | Δ (C.I. 95%) | pValue |
| LCI 2.5% | 9,4371 (2,3771) | 15,5386 (4,3384) | 6,1014 (2,0275 to 10,1753) | 0,0068 |
| LCI z-score | 0,8646 (0,1606) | 0,6196 (0,1311) | -0,2450 (-0,4157 to -0,07423) | 0,0088 |

TABLE 12. COMPARISON BETWEEN CFP FEV1>90% AND ALL THE OTHER PATIENTS. T-TEST WAS USED FOR ALL COMPARISONS (SINCE THAT FOR EACH VARIABLE VALUES ARE NORMALLY DISTRIBUTED

Spearman's coefficient of rank correlation (rho) has been calculated to evaluate relation between FEV1 z-score and LCI in our CFp. In the entire CFp, the two variables appear related r = -0,793 (95% C. I. for rho -0,932 to -0,454; p= 0,0007). (**Figure 9**)

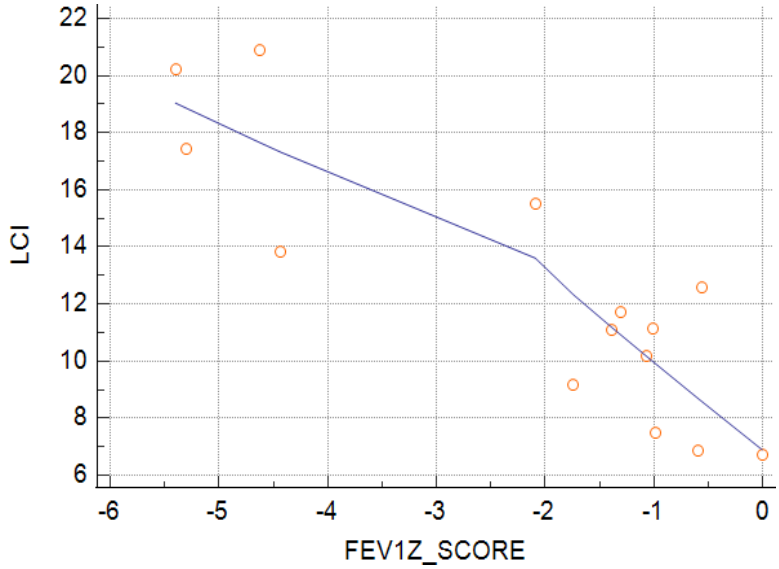


**FIGURE 9. CORRELATION BETWEEN FEV 1 Z-SCORE AND LCI IN ENTIRE CFP GROUP. R = -0,793; P= 0,0007**

In the same population, correlation between FEV1 z-score and LCI z-score is alsostatistically significant r= 0,807 (95% C.I. for rho 0,483 to 0,936; p= 0,0005). (**Figure 10**)

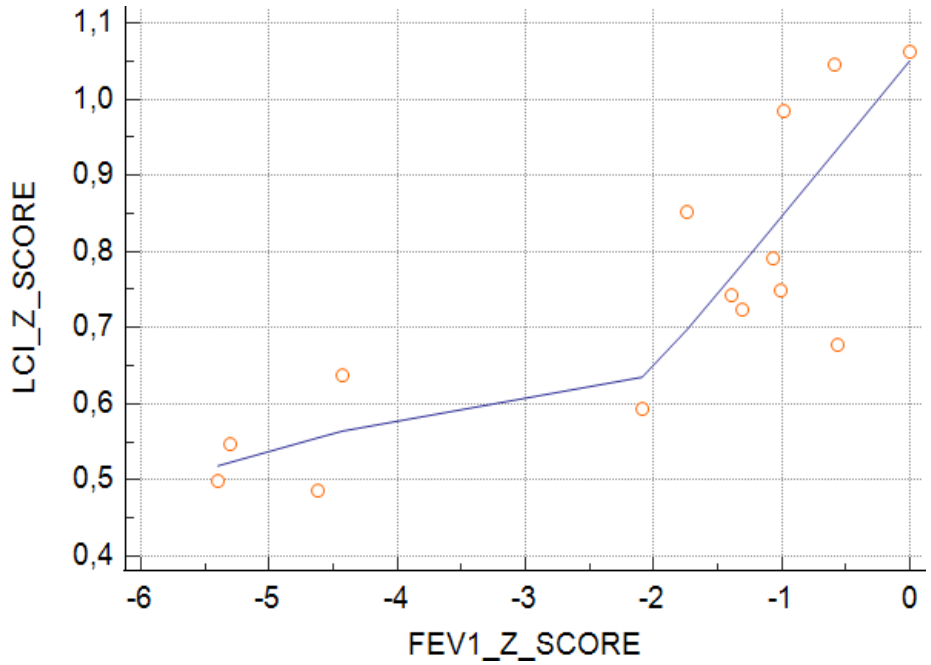


FIGURE 10. **CORRELATION BETWEEN FEV 1 Z-SCORE AND LCI Z-SCORE IN ENTIRE CFP GROUP.**

In subjects who belong to FEV1>80 subgroup, correlation between FEV1 z-score and LCI has been done, r= -0,6373 (95% C. I. for r -0,9041 to -0,01277; p= 0,0475).

Correlation has been verified also between expressed as z-score variables, r=0,6471 (95% C.I. for r 0,02952 to 0,9071, p= 0,0431). (**Figure 11**).

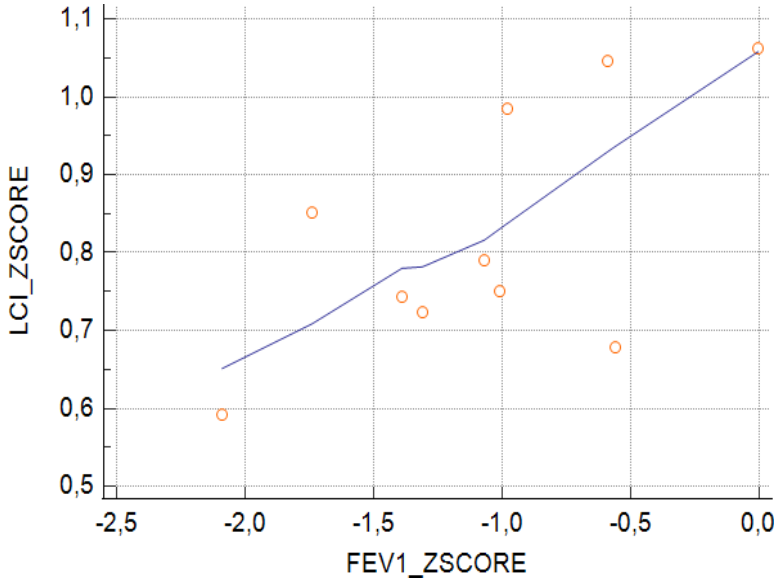


**FIGURE 11.** CORRELATION BETWEEN FEV1 Z-SCORE AND LCI Z-SCORE IN PATIENTS WHO BELONG TO FEV1>80 SUBGROUP. R= 0,6471; P= 0,0431

Relation between FEV1-zscore and LCI in the FEV<60 subgroup is not statistically significant r= -0,4366 (95% C.I. for r -0,9846 to 0,9037; p= *0,5634*). Also doing correlation using variables expressed as z-score no correlation is highlighted, r=0,4900 (95% C.I. for r -0,8904 to 0,9865; p=0,5100). (**Figure 12**)

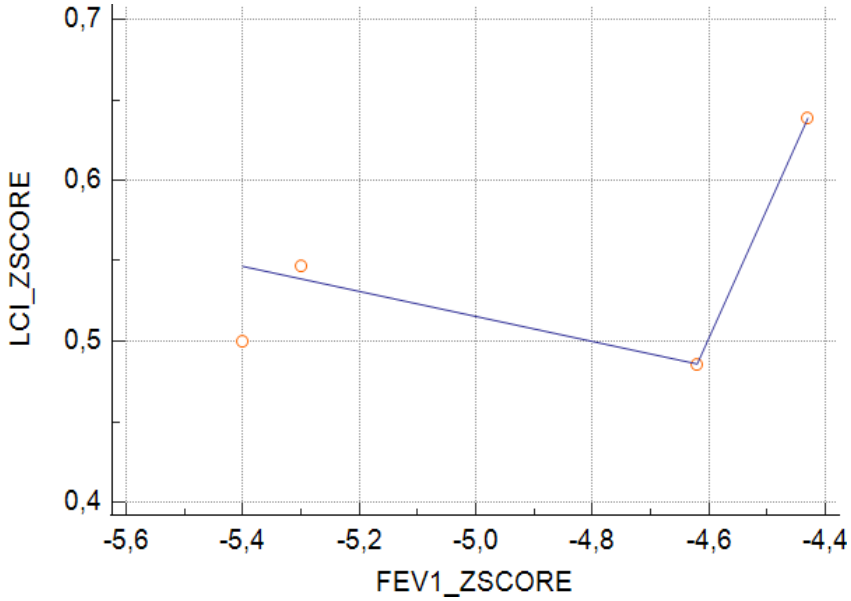


**FIGURE 12. CORRELATION BETWEEN FEV1 Z-SCORE AND LCI Z-SCORE IN PATIENTS WHO BELONG TO FEV1<60 SUBGROUP. R= 0,4900; P= 0,5100**

Receiver operating Characteristic (ROC) curve for LCI and FEV1 for the detection of bronchiectasis has been made to establish sensitivity and specificity of both tests. Area under the curve (AUC) is statistically significant in LCI (0.958, **Figure 13**) and FEV1(0.792 **Figure 14**) too. In particular, LCI sensitivity and specificity, in our cohort, are very significant 91,67% and 100% respectively. FEV1 has a lower sensibility (58,33%) than LCI, while specificity is the same (100%).

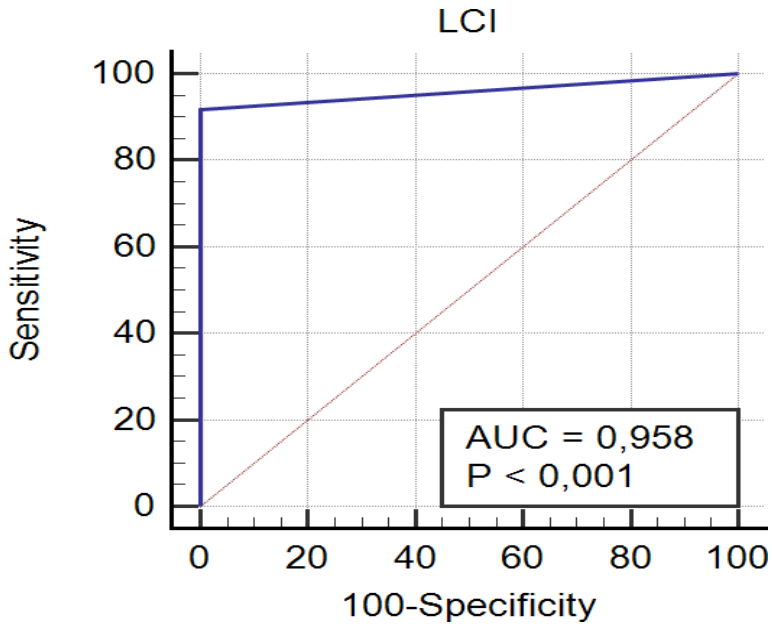


FIGURE 13. LCI SENSITIVITY AND SPECIFICITY FOR BRONCHIECTASIS DETECTION

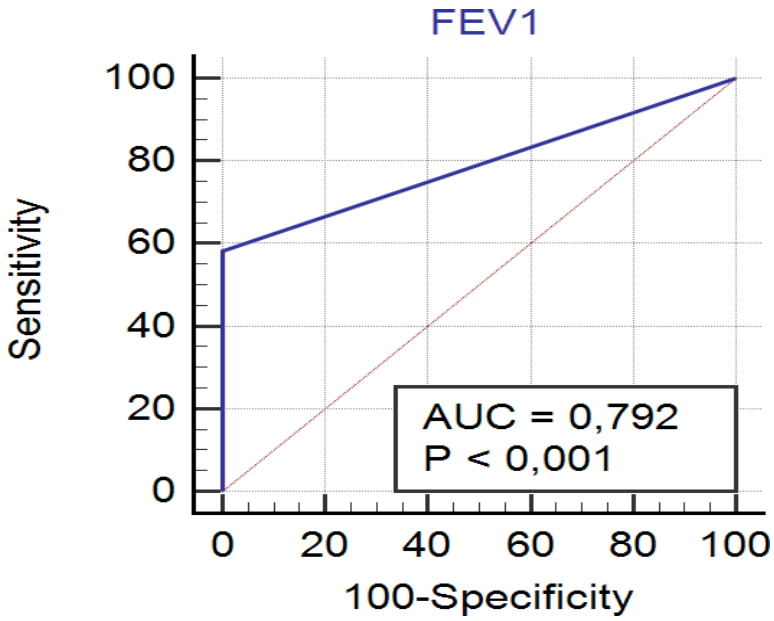


FIGURE 14. FEV1 SENSITIVITY AND SPECIFICITY FOR BRONCHIECTASIS DETECTION

## 4.4 Discussion

Lung Clearance Index seems to be more sensitive than FEV1 and FEV1 z-score values in detecting lung involvement in patients with Cystic Fibrosis, since only 50% and 42,85% of children have respectively pathological FEV1 and FEV1 z-score values, while 78,57% of them have *abnormal* LCI.

Patients who have FEV1 z-score above the LLN (i.e. pathological value) are 42,85% but, as seen before, the percentage of patients with pathological values rises to 50% if we consider FEV1=90% as LLN. This difference is due to the presence of a limit value between "normality" and disease in a patient who has FEV1 89% and -1,31 as FEV1 z- score.

In **Table 13** we can see how LCI and LCI z-score are the most statistically significant variables compared to all spirometric ones; even if FEV1 z-score value seems to be statistically significant, despite *none* of the patients in that *subgroup* have pathological FEV1 z-score value.

| *Variables* | *CFp with FEV>90% (7)* | *HC (50)* | *Δ (C.I. 95%)* | *pValue* |
|---|---|---|---|---|
| **FEV1** | 98,83 (7,51) | 103 (5,9) | -4,17 (-9,10 to 0,76) | **0,0957** |
| **FEV1 z-score** | -0,9760 (-1,05 ; -0,57) | 0,14 (0,010 to 0,44) | -0.89 (-1.35 to -0.42) | **0,0005** |
| **LCI 2.5%** | 10,18 (7,01 to 11,12) | 6,48 (5,95 to 6,63) | -3,69(-4,79 to -0,90) | **0,0001** |
| **LCI z-score** | 0,7903 (0,7443 to 1,0302) | 0,0002767 (-0,006225 to 0,002355) | -0,7900 (-1,0411 to -0,7472) | **< 0,0001** |

**TABLE 13. COMPARISON BETWEEN CF PATIENTS WHO HAVE WELL PRESERVED LUNG FUNCTION AND HC**

This difference could be due to the variability in FEV1 values within normal range (since lowest value is 80% while the highest is 120%) and how z-score varies in proportion, since it indicates how much a value is far from the predicted one. The most part of CFp has values near to 90% rather than to 100%. The most interesting results concern subjects with *early disease*: among patients characterized by FEV1 and FEV1 z-score within normal range values (i.e. patients commonly recognize who have preserved lung function) there is a wide portion of them, 57,14% and 62,5% respectively, who have an abnormal LCI values. Hence, in this circumstance, LCI is the only index of disease. The same point of above cannot be affirmed for the other spirometric parameters (FVC and FVC z-score), as seen in tables before.

During disease progression, in according to CF disease pathophysiology, FEV1 values decline occurs meanwhile LCI values increasing, i.e. worsening. LCI is indirectly proportional to the FEV1, as demonstrated also in our study, r= -0,793, using Spearman's correlation, while r= 0,807 is the result of correlation using z-score of the same variables. This result has been highlighted by *Singer et al.,* using the same equipment too. In this study, they have correlated FEV1 z-score and LCI z-score, reversing x-axis scaling and for this reason their coefficient is negative r= -0.49 (p=<0.001) [166].

During the full-blown disease, correlation between these variables does not seem to be evident and this occurrence could be explained making a focus on LCI detection mechanism. LCI is an index of inhomogeneity ventilation that describes how lung zonescan be well or inadequately ventilated. So, it represents small airways global functional status, specifically those ones which *contribute to ventilation*. Hence, if some areas are excluded from ventilation, because of mucus plugging presence, they

will be excluded from detecting during LCI test. Thus, when pathological processes involve some branches, it can lead to a complete obstruction, so LCI value could be better than expected. This phenomenon should often happen in advance disease, so, in other word, there is a high LCI variability in these patients.

In our study, even if its limitations, no correlation has been resulted between FEV1 z- score and LCI in patients belong to FEV1<60% subgroups. (**Figure 15**).

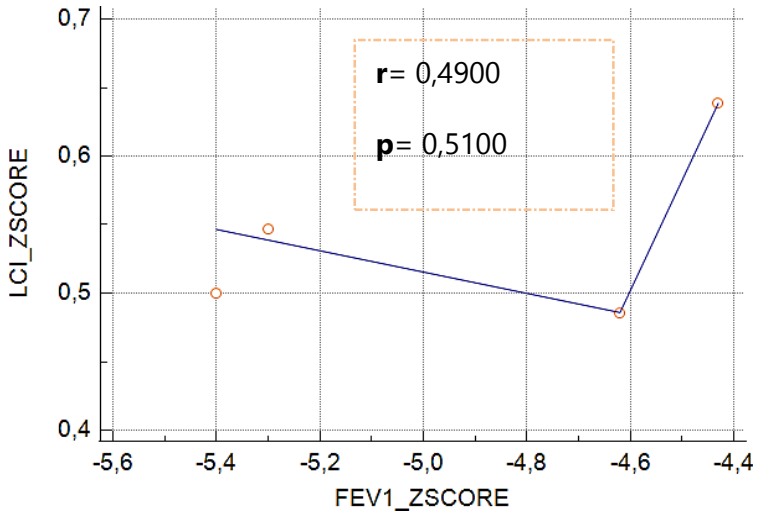


**FIGURE 15. FOCUS ON OVERT DISEASE. SCATTER DIAGRAM SHOWS NO CORRELATION BETWEEN LCI AND FEV 1 (BOTH EXPRESSED AS Z-SCORE VALUES) IN OVERT DISEASE**

Alteration in LCI value seems to be an index of lung pulmonary remodeling, i.e. overt bronchiectasis or similar modifications. Several papers have investigated the relationship between LCI abnormal value and the presence of the aforementioned alterations. Depending on the range of age, different types of alterations seem to be related to pathological Lung Clearance Index, as described by Ramsey et al. In school-aged children, high LCI value is an indirect index of bronchiectasis [88]. We have tried to measure how much LCI and FEV1 could be related to radiological reports, making a focus on bronchiectasis detecting. In our study, LCI appears to be more sensitive (91,66%) than FEV1 (58,3%) in detecting children who have bronchiectasis, while no differences were highlighted about specificity. Despite LCI AUC is higherthan that of FEV1, this difference (Δ: -0,166) is not statistically significant (p= 0,0514). LCI shows a good predictive positive value (p.p.v.) 100% while the predictive negative

value (p.n.v.) is 66,6%. Comparing our results with those published by *Ramsey et al*., their predictive values are softly lower (p.p.v. 83-86% vs p.n.v 50-55%) than ours, but strongly significant too [88]. We hypothesize that these differences are due to our small sample of patients.

Starting from the observation of our results, namely that LCI is more sensitive than FEV1 and it has a higher predictive negative value, we can align to another study, made by Fuchs et al., in which they assume that *normal LCI value* could be capable to indicate the absence of bronchiectasis in patients [11, 61, 87].

The most relevant limitation is that data collection has been forcefully limited becauseof the COVID-19 pandemic. This event has driven to analyze data which were in our possession. For this reason, a small sample of patients has been examined. Otherlimitations are in part related to its retrospective design: the control group was designed for another study, in fact age of patients is different. Anyway, we have chosen to adopt data form available control group, despite the aforementioned difference, because the inter-subject LCI variability in healthy children, for this range of age, is narrow. Patients and measures drop-out testifies a stringent quality control. The diversity of examined patients have allowed to us to emphasize the role of LCI in early disease rather than in the advanced. These two points are the strengths of this study.

Hence, in view of LCI sensitivity in patients with lung preserved function, firstly, and LCI values losing significance during disease progressing, secondly, we support its *emerging role* in early surveillance of disease.

Despite the small sample of patients, all the obtained results are in accordance with the recent scientific papers and this could signify that LCI potentiality are suitable to be utilized in routine clinical practice.

# CHAPTER 5. RESEARCH#3: LCI EVALUATION IN DETECTING NOCTURNAL HYPOXEMIA IN CYSTIC FIBROSIS PATIENTS

## 5.1 Introduction

In CF the deterioration of lung function, due to structural parenchymal abnormalities and to airway obstruction leads, through the time, to increased work of breathing, alveolar hypoventilation and impaired gas exchange [167, 168]. As in other chronic respiratory diseases, early signs of respiratory functional impairment occur during the night and, in the last years, sleep has been increasingly recognized as a vulnerable state in patients with CF. During sleep, these patients often experience a significant decline in oxygen saturation ($SpO_2$), higher respiratory rate and a variable number of upper airway obstructive events as compared to healthy subjects [167-169]. Nocturnal hypoxemia has been mainly attributed to hypoventilation and ventilation/perfusion mismatch, particularly during REM sleep [170]. As pulmonary impairment progresses nocturnal severe hypercapnia can also occur [171]. It has been shown that nocturnal hypoxemia may cause disrupted sleep with poor daytime function and, if not reversed, may leads to pulmonary hypertension and right ventricular failure [170]. Therefore, the early recognition of nocturnal hypoxemia and sleep disordered breathing, particularly in the first phases of the disease, has been the topic of many studies [169-171]. Most of the studies have looked at the traditional lung function measure expressed by the forced expiratory volume in 1 second ($FEV_1$). However, this is a poor predictor of nocturnal hypoxemia, unless respiratory function is not severely impaired [170-172]. Specifically, only a $FEV_1$ value below 65% predicts the risk of hypoxemia during sleep [170,171]. A predictive role for diurnal values of $SpO_2$ has also been investigated, however in patients with awake SaO2 >93% the nocturnal trend of $SpO_2$ is heterogeneous [170-173]. Therefore, in patients with normal diurnal gas exchange it might be difficult to suspect nocturnal desaturation.

The LCI provides a reliable measure of the overall degree of ventilation inhomogeneity [39]. Recently this test has been widely used in patients with CF as it offers information on lung pathology complementary to that obtained from conventional lung function tests. The LCI value reflects the distal airway status thus providing a valid tool to detect lung damage more precociously and more sensitively than spirometry [40].

Most of the previous studies on potential predictors of sleep hypoxemia in CF were based on traditional functional parameters such as $FEV_1$ or daytime $SpO_2$, producing often uncertain results, particularly in patients with mild disease [170-172]. Therefore, the aim of this study was to establish whether the LCI may be reliable in predicting nocturnal hypoxemia in patients with stable, mild to moderate CF, with normal diurnal gas exchange.

## 5.2 Materials and Methods

### *5.2.1 Participants*

We enrolled in the study a total of 31 caucasian patients (15 males, mean age 17.4±5.2 years) affected by CF in stable phase followed in our Cystic Fibrosis Unit, University of Catania, Italy

Diagnosis of CF was based on a sweat chloride level above 60 mmol/L and a genetic test showing two pathogenic mutations in the CF transmembrane conductance regulator gene [10].

We defined as "stable" patients showing: 1) no disease exacerbation for at least one month before enrolment; 2) no decrease in $FEV_1$ after at least one month since the last clinical evaluation. We excluded patients with respiratory failure requiring chronic oxygen administration.

For all patients clinical records were available since they were followed in our Center from long time. We assessed demographic data, comorbidities, and the occurrence of pulmonary exacerbations. In addition, we reviewed all microbiological data, such as chronic colonization with Pseudomonas, St. Aureus or Burkholderia cepacia.

Written informed consent was obtained from the patients or, in children and adolescents, from their parents. Ethical Committee from the Institutional Review Board approved the study.

### *5.2.2 Study protocol*

In the morning of the study day we performed physiological measurements. Lung function was evaluated by standard spirometry (Cosmedsrl), according to the American Thoracic Society criteria [143]. Values of forced expiratory volume in 1 second ($FEV_1$) were expressed as percentage of the predicted normal values adjusted for age, sex,

height, and weight. An arterial blood gas test was also performed and resting oxyhemoglobin saturation ($SpO_2$) was recorded for five minutes using a finger pulsoxymeter (Nonin Medical, Inc). To evaluate lung ventilation distribution inhomogeneity, the Multiple Breath Washout (MBW) test was performed using the Exhalyzer D (EcoMedics AG, Duernten, Switzerland) and an inert intrinsic gas (nitrogen). All subjects performed the test during relaxed and stable tidal breathing until the test gas reached $1/40^{th}$ of the initial gas concentration. Testing was performed aiming to obtain at least three acceptable trials according to ERS standards [138]. The results of the test were analyzed through the Spiroware software (EcoMedics AG, Duernten, Switzerland) and recorded by the physician. The main parameters reported from the MBW tests were the functional residual capacity, the moment ratios and the LCI, which was the outcome of our study. LCI values >7 were considered abnormal [40].

During the night in the same study day we performed nocturnal cardiorespiratory polygraphy (PG) using a SomnoScreen® Plus TM Domino Software, v.2.3.1. In brief, thoracic and abdominal excursions were detected by inductance plethysmography bands. Airflow was detected by nasal prongs attached to a flow sensor, and $SpO_2$ was determined using finger pulse oximetry. Respiratory events, obstructive or central apneas and hypopneas, were scored according to the American Academy of Sleep Medicine (AASM) standards [13, 14]. The apnea/hypopnea index (AHI) was defined as the number of apneas and hypopneas per hour of recording. Oximetry parameters included mean nocturnal $SpO_2$, the time of the night spent with $SpO_2$ below 90% (T90 $SpO_2$) and the minimum $SpO_2$. A drop in >3% SpO2 was considered significant. Nocturnal hypoxemia was defined as $T90SPO_2$>5%. Transcutaneous $CO_2$ was also recorded through the night.

### *5.2.3 Data analysis*

Data were statistically analyzed using the SPSS software (version 15.0) and presented as mean ± standard deviation (SD). Shapiro-Wilk normality test was used to assess data distribution patterns. QQ-plots and attendant regression were adopted [174]. Data were also log distributed. A real-space data were adopted to make our conclusions. The non-parametric Mann-Whitney test was used in addition to the Pearson's correlation coefficient. A p value < 0.05 was considered statistically significant. Fit was assessed in each model using the associated $R^2$ and P values. To assess the prediction performance of LCI or $FEV_1$ for nocturnal hypoxemia, a receiver operating characteristic (ROC)

analysis was performed. The area under the curve (AUC) was calculated [175]. In addition, the Youden's J index was calculated to provide an objective assessment of optimality of the threshold calculated [176].

## 5.3 Results

All 31 patients completed the study and were considered in the final analysis. Demographic, clinical and laboratory data are summarized in **table 14**.

| Patients (31) | |
|---|---|
| $PaO_2$ (mmHg) | 86.21±10.9 |
| $PCO_2$ (mmhg) | 35.61±3.81 |
| LCI | *13.1*±5.1 |
| FEV1% | *66.3*±19.6 |
| **Polygraphic variables** | |
| Mean awake $SpO_2$ | *97%*± 0.01 |
| Mean nocturnal $SpO_2$ | *93%*±0.02 |
| Time with $SpO_2$ <90% | *10%*±0.2 |
| Mean $TcCO_2$ (mmHg) | *32.6*±3.21 |
| ODI | *7.2*±0.92 |
| AHI | *1.1*±1.7 |
| Mean awake RR | *20.6*±1.8 |
| Mean nocturnal RR | *18.5*±7.0 |

**TABLE 14. CLINICAL AND POLYGRAPHIC VARIABLES OF THE ENROLLED PATIENTS**

M: male; F: female; BMI: body mass index; P.a.: Pseudomonas aeruginosa; S.a.: Staphylococcus aureus; B.c.:*Burkholderia cepacia;* N.b.f: normal bacterial flora; LCI: lung clearance index; FEV1: forced expiratory volume in 1 s; $TcCO_2$: transcutaneous partial pressures of carbon dioxide; ODI: oxygen desaturation index; AHI: Apnea Hypopnea Index; RR: respiratory rate. Data presented as mean ± standard deviation

We found that in the entire population the AHI was within a normal range (1.1±1.7), thus excluding the occurrence of upper airways obstruction. However, nocturnal desaturation occurred (mean $T90SpO_2$ 10%±0.2, mean nocturnal $SpO_2$ 93%±0.02), which can be considered of mild to moderate degree, given the young age of

the population. All patients were normocapnic during the night (mean Tc $CO_2$ 32.6±3.21 mmHg).

### *5.3.1 Nocturnal hypoxemia and Lung Clearance Index*

Most of the patients had abnormal LCI values (>7). Since some previous studies reported that a $FEV_1$ value below 65% predicts nocturnal desaturation, we divided our patients in two groups according to the severity of airway obstruction, with a $FEV_1$ cut-off value of 65% of the predicted. Data for each group are summarized in **table 15**.

| Patients* | $FEV_1$<65% | $FEV_1$>65% | p value |
|---|---|---|---|
| N. | 14 | 17 | |
| Gender (M:F) | 7:7 | 8:9 | n.s. |
| Age (years) | 18.9±4.7 | 16.1±5.4 | n.s. |
| BMI (<18/>18) | 11/3 | 10/7 | n.s. |
| Pulmonary colonization | | | |
| P.a | 9 | 2 | <0.01 |
| S.a. | 4 | 10 | <0.05 |
| B.c. | 1 | 0 | n.s. |
| N.b.f | 0 | 5 | <0.05 |
| LCI | 17.4±3.1 | 9.6±3.5 | <0.01 |
| FEV1% | 48.2±12.2 | 80.1±10.8 | <0.01 |
| Mean awake $SpO_2$ | 96%±0.009 | 98%±0.007 | n.s. |
| MeanNocturnal $SpO_2$ | 91%±0.01 | 94%±0.01 | <0.01 |
| Time with $SpO_2$ <90% | 8%±0.09 | 2.38%±0.28 | <0.01 |
| Mean $TcCO_2$ (mmHg) | 32.6±3.33 | 32.6±3.09 | n.s. |
| ODI | 7.2±0.7 | 7.2±1.0 | n.s. |
| AHI | 0.5±0.3 | 1.7±1.2 | n.s. |
| Mean awake RR | 21.3±2.2 | 20±1.3 | n.s. |
| Mean nocturnal RR | 27.4±3.8 | 21.9±2.1 | <0.01 |

**TABLE 15. COMPARISON OF DEMOGRAPHIC, CLINICAL AND POLYGRAPHIC VARIABLES OF THE PATIENTS**

M: male; F: female; BMI: body mass index; P.a.: Pseudomonas aeruginosa; S.a.: Staphylococcus aureus; B.c.:Burkholderiacepacia; N.b.f: normal bacterial flora; LCI: lung clearance index; FEV1: forced expiratory volume in 1 s; TcCO2: transcutaneous partial pressures of carbon dioxide; ODI: oxygen desaturation index; AHI: Apnea Hypopnea Index; RR: respiratory rate. Data are presented as mean ± standard deviation

Sex, age, and BMI distribution was similar in the two groups. Significantly higher LCI values were found in patients with more severe airway obstruction (17.4 and 9.6 in patients with $FEV_1$<65% and >65%, respectively, P<0.01) and in the whole population LCI inversely correlated with $FEV_1$ (r=-0.809 p<0.01) (**figure 16**).

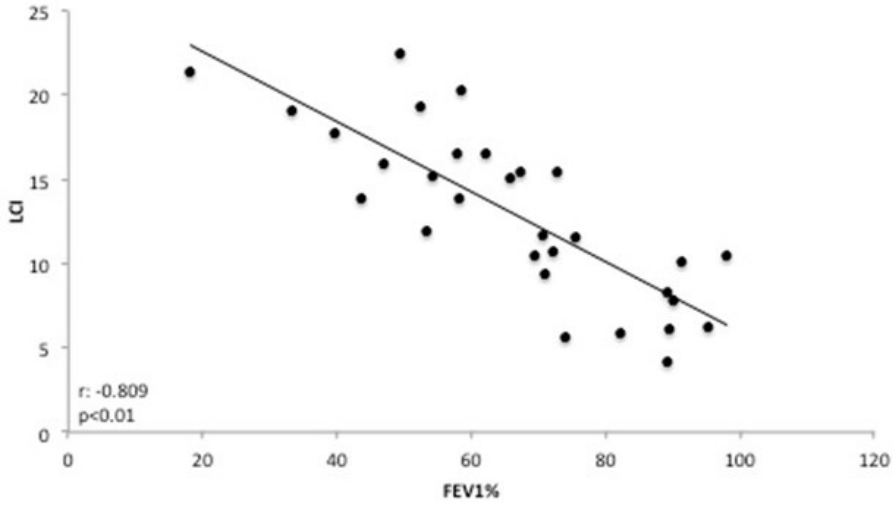


**FIGURE 16. CORRELATION BETWEEN LUNG CLEARANCE INDEX (LCI) AND FORCED EXPIRATORY VOLUME IN THE 1ST SECOND AS % OF THE PREDICTED VALUE (FEV1%).**

However, it is noteworthy that in four patients with normal $FEV_1$% values the LCI values were abnormal. In the entire population we found a significant negative correlation between LCI values and the mean nocturnal $SpO_2$ (r=-0.880 p<0.01) (**figure 17**).

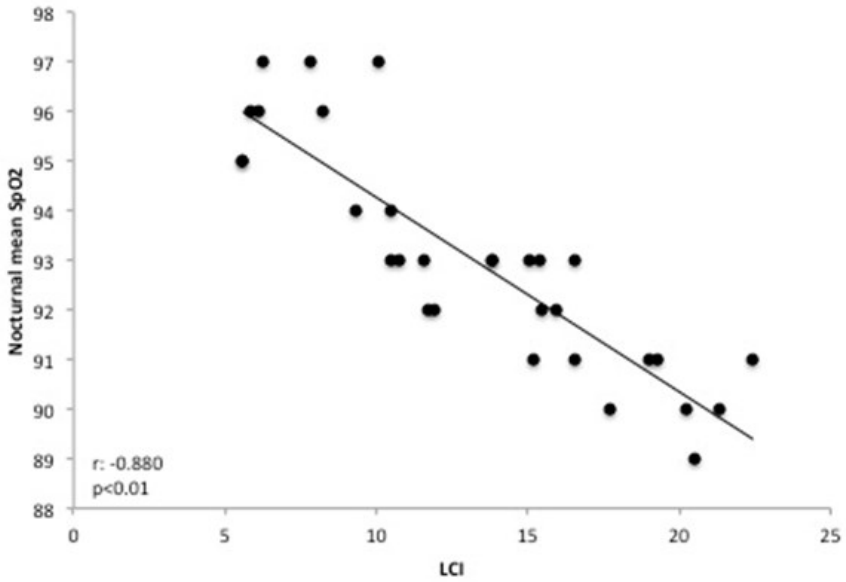


**FIGURE 17. CORRELATION BETWEEN LUNG CLEARANCE INDEX (LCI) AND NOCTURNAL MEAN SPO2.**

Of greatest relevance is the observation that, when we performed a ROC analysis to assess whether LCI predicted nocturnal hypoxemia ($T90SPO_2$>5%), we found that the AUC was 0.96, which indicates an excellent, high predictive accuracy. In addition, the Youden index for the ROC was 0.79.

### *5.3.2 Nocturnal hypoxemia and airway obstruction*

As expected, patients with more severe airway obstruction ($FEV_1$ <65%) had more severe nocturnal hypoxemia with significantly lower nocturnal mean $SpO_2$ and higher $T90SpO_2$.

In the entire population, we found a negative correlation between $FEV_1$% and nocturnal mean $SpO_2$ ($r=-0.805$ $p<0.01$). However, when we performed a ROC curve analysis we found that the best predictive cut-off value of $FEV_1$ for nocturnal hypoxemia was 63.9% and the AUC was 0.71 which indicates only a moderate degree of accuracy.

## 5.4 Discussion

In this study we have shown for the first time that the LCI, derived from a multiple-breath washout technique, is a good predictor of nocturnal hypoxemia in patients with CF and normal diurnal gas exchange. This functional tool, recently implemented in CF, allows early recognition of nocturnal hypoxia in young adults with mild to moderate disease, more accurately than a traditional functional parameter such as $FEV_1$.

The causative events leading to a progressive lung dysfunction in CF are complex. At some point, during the disease progression, severe lung dysfunction produces hypoxemia, which is latent in the first stages and evident successively, often associated with hypercapnia. Chronic hypoxia in turn negatively affects biological and lung mechanical features, thus generating a vicious circle [177]. Hypoxemia causes disease progression worsening the pulmonary circulation, affecting the bacterial profile/activity and antibiotic resistance, enhancing a pro-inflammatory status and skeletal muscles dysfunction. [178].

As compared to other chronic respiratory conditions, in CF hypoxemia can occur precociously triggered by physiological stress including sleep [170]. Therefore, both adults and children with different degree of lung dysfunction and normal daytime $SpO_2$ may experience nocturnal episodes of desaturation [179-181]. To date, differently from

other obstructive lung diseases, an univocal definition of physiologically significant nocturnal hypoxemia in CF has not been reported. Some authors define nocturnal hypoxia as the detection of $SpO_2$< 90% recorded for more than 5%, 10% or 30 % of the total recording time, while some others take into account the lower value of nocturnal $SpO_2$ or a given cut-off of mean $SpO_2$ [179, 181]. From a physiological point of view it is unclear what level of hypoxemia becomes relevant, however it is clear that a repeated hypoxic insult, even if mild, in such patients may negatively affect the pulmonary circulation, sleep quality, as well as lung inflammation and Pseudomonas growth [168]. In addition to a critical variability in the cut-off values to define significant nocturnal hypoxemia it is also unclear at which stage of the disease latent hypoxemia must be suspected.

The effect of the severity of lung dysfunction on sleep has been investigated by several studies [182-184]. Although nocturnal desaturation is more frequent in patients with advanced lung disease, it has been reported also in patients with mild to moderate disease [180, 181]. Similarly, our clinically stable patients, with mild to moderate disease severity, exhibited nocturnal desaturation episodes. The degree of airway obstruction, expressed by the $FEV_1$% value, has been widely used to predict sleep desaturation [171,172]. Generally, it has been reported a sensitivity and specificity around 80% for a $FEV_1$ value below 65% [170-172] or even below 53% in children [173]. However, our group of patients with $FEV_1$>65% also exhibited nocturnal desaturation, although of mild degree. In agreement with previous studies, we found a predictive threshold value for $FEV_1$ of 63.9% but only a moderate degree of prediction accuracy, as shown by the AUC value. Therefore, the $FEV_1$ value is not the best marker to suggest sleep hypoxemia in patients with mild lung dysfunction. Other studies have tried to correlate awake $SpO_2$ or $PaO_2$ and nocturnal saturation, though, in one study diurnal $SpO_2$ predicted sleep hypoxemia only in 26% of the patients [170]. Given this uncertainty, we decided to investigate the predictive value of an alternative marker of lung dysfunction such as the LCI, an index of uneven ventilation distribution.

The multiple-breath washout technique has been used since the 60s to evaluate the distribution of ventilation in the lungs [174]. Nevertheless, only in more recent years this technique has been used for the functional evaluation of patients with CF being non-invasive, repeatable, harmless and feasible in all age groups [40]. The LCI, reflecting the extent of structural lung disease, is more sensitive than $FEV_1$ in indicating, in an early

stage, the severity of the disease, particularly in children which often exhibit normal spirometric values [40]. In one study the LCI correlated better than $FEV_1$ with the lung structural changes detected by the high-resolution computer tomography [40]. Furthermore, more recently, a close correlation between overall structural chest magnetic resonance imaging (MRI) scores and LCI was reported [185-187]

Herein, LCI showed a better accuracy than $FEV_1$ in predicting nocturnal hypoxemia. It is noteworthy that if only $FEV_1$ is taken into account patients with a value >65% should not be at risk of nocturnal desaturation. In our study some of patients with normal $FEV_1$ (>80%) exhibited nocturnal desaturation and this was associated with abnormal LCI.

The mechanism linking changes in LCI and nocturnal desaturation of course remains to be cleared.

Ventilation inhomogeneity occurs in the lung at branch points in large and small airways, close or within gas exchange zones, due to the presence of bronchiectasis and airway thickening [170]. Ventilation inhomogeneity has been shown through LCI changes in CF as early as the first year of life and it has been correlated with an increased respiratory rate [188]. It has been reported that ventilation-perfusion mismatch in the lung is one of the mechanism underling nocturnal desaturation [170] and this maybe a putative link between altered LCI and nocturnal hypoxemia. Data from LCI and respiratory rate in CF also indicate that this index may reflect the greater breathing load in patients with CF, and this may be another putative link. Of course this is only speculation and further studies are needed to clarify this point.

Our study has some limitations. First, the sample size is small and the group of patients enrolled might not be entirely representative of the wide spectrum of the disease. Indeed patients with CF exhibit a great variability in clinical presentation and respiratory impairment, even in the presence of identical genetic mutation. In addition, CF is a rare disease, therefore a smaller size of patients is generally included in the studies It is also noteworthy that the MBW test to derive LCI, is an emerging technique to monitor the disease progression and standardized protocols in CF are still unavailable even though several CFTR modulator studies have been done with MBW as endpoint [95]. The lack of a testing method able to intercept patients at risk of nocturnal hypoxemia is probably due to the heterogeneous nature of the disease and justifies the present study. Finally, our

observations, limited to a certain time in the clinical history of these patients, cannot predict future outcomes. Further studies are necessary to address this point.

Nevertheless, our data add a further element to define the LCI as a reliable tool for a comprehensive clinical assessment of CF, particularly in that stage in which spirometric parameters do not reliably reflect the degree of lung dysfunction.

# CHAPTER 6. RESEARCH#4: LCI AS OUTCOME MEASURE FOR NON INVASIVE VENTILATION IN NORMOCAPNIC CYSTIC FIBROSIS PATIENTS

## 6.1 Introduction

In patients with CF non-invasive ventilation (NIV) improves lung mechanics and gas exchange, and decreases the work of breathing [193, 194]). Domiciliary NIV is mainly used in hypercapnic patients with severe disease, because it counteracts the progression of lung functional impairment and it is often used as a useful "bridge" to lung transplantation [195]. However, to date, there are no standardized criteria to indicate the effect of a precocious starting of NIV in patients with functional ventilation inhomogeneity without hypercapnia. On this regard, the LCI, derived from a MBW test, reflecting the distal airway status, has been recently proposed as a new tool to assess the ventilation inhomogeneity in the lungs of patients with CF [44]. In fact, abnormal LCI values occur early in the life of these patients and reflect, more sensitively than spirometric values, the structural changes occurring in the lungs of patients with CF [12, 40]. The aim of this study was to assess whether a precocious starting of nocturnal NIV can be useful to improve clinical condition even in normocapnic awake CF patients and it can represent a procedure to counteract the progression of lung diseases as well as quality of life score.

## 6.2 Materials and Methods

### *6.2.1 Participants*

We studied a total of 6 nocturnal hypoxemic and daily normocapnic awake patients (2 males, 4 females, age 15-34 years) followed in our Cystic Fibrosis Centre, Respiratory Unit, Department of Clinical and Experimental Medicine, University of Catania. Diagnosis of CF was based on a sweat chloride level above 60 mmol/L and a genetic test showing two pathogenic mutations in the CF transmembrane conductance regulator gene [196]. All patients were stable at initiation of the treatment. We defined as "stable" patients showing: 1) no disease exacerbation for at least one month before enrollment; 2) no decrease in FEV1 after at least one month since the last clinical evaluation. Exclusion criteria included: diurnal hypoxemia and/or hypercapnia,

significant nocturnal events of obstructive apneas and severe nocturnal hypoxemia (time of the night spent with a SaO2 below 90% >30% for adults and >10% for adolescents), history of pneumothorax/blebs and/or experiencing pulmonary exacerbations defined in accordance to the Fuchs criteria [197]. For all patients clinical records were available since they were followed in our Center from long time. We assessed demographic data, comorbidities, and the occurrence of pulmonary exacerbations. Written informed consent was obtained from the patients or, in children and adolescents, from their parents. Ethical Committee from the Institutional Review Board approved the study.

### *6.2.2 Study protocol*

At the baseline (day 1) arterial gas analysis, spirometry, MBW to derive LCI, and nocturnal cardio-respiratory polygraphy (PG) were performed in accordance to ERS guidelines (Exhalyzer D (EcoMedics AG, Duernten, Switzerland) [39], and American Academy of Sleep Medicine (AASM) standards [198]. The Pittsburgh Sleep Quality Index (PSQI) was also recorded [199]. A NIV session for acclimatization pressure support ventilation (PSV)-ST was also performed by using a bi-level domiciliary ventilator (Astral 150) with nasal mask. The following parameters were adopted: inspiratory positive airway pressure (IPAP) 10 cmH2O and expiratory positive airway pressure (EPAP) 6 cmH2O. Following the night of adaptation to NIV, set parameters for the use of home NIV were defined for each patient. The adherence to the treatment was defined as at least 4 hours per night. Both the clinical status and the adherence to the treatment were assessed every 3 months. At the end of the study (year 1), all enrolled patients underwent to spirometry, LCI, and nocturnal cardiorespiratory PG. The PSQI was also administered.

### *6.2.3 Spirometry*

Pulmonary function was measured in the laboratory using spirometry (Lungtest 1000, MES Ltd. 30- 390 Krak.w, 56 Zawia street). FEV1% was measured as gold standard parameter for airflow obstruction assessment. The best spirometric measureof at least three attempts was recorded for analysis. Values were expressed as percent of predicted values, based on child age, gender, weight and height [200].

### *6.2.4 Lung Clearance Index*

MBW was performing using 100% oxygen to washout resident nitrogen from the lung with the Exhalyzer D system (EcoMedics, Duernten, Switzerland) and associated

Spiroware software (version 3.1.6). Patients performed the test seated using a noseclip and low dead space snorkel-like mouthpiece (EcoMedics). At each visit patients aimed to achieve three acceptable trials according to ERS/ATS consensus criteria and underwent qualitative quality control assessment [201].

### 6.2.5 The Pittsburgh Sleep Quality Index (PSQI)

The PSQI was used for both an initial assessment and ongoing comparative measurements in enrolled patients across the health care continuum [199].

### 6.2.6 Data analysis

Data were statistically analyzed using the SPSS software (version 15.0) and presented as mean±standard deviation (SD). Shapiro-Wilk normality test was used to assess data distribution patterns. QQ-plots and attendant regression were adopted [202]. The non-parametric Mann-Whitney test was used in addition to the Pearson's correlation coefficient. A p value $< 0.05$ was considered statistically significant. Fit was assessed in each model using the associated R2 and P values.

## 6.3 Results

All 6 patients (male:female 2:4, mean age ± SD: 23.3±5.8) completed the study with full satisfaction. All patients had a good adherence to the treatment and no adverse effect was reported.

### 6.3.1 Effects of NIV on clinical and laboratory findings

The mean pressures used for NIV were IPAP 10 cmH20 and EPAP 5 cmH20 in spontaneous/controlled mode. NIV significantly reduced the nocturnal respiratory rate (28.4±4.2 vs. 23.5±1.9, $p<0.01$) and improved mean nocturnal SaO2% (91±1.0 vs. 94±1.0, $p<0.001$). The mean number of exacerbations was 4.7 in the pre-treatment year and 2.2 during the treatment year ($p<0.001$). PaO2 and PaCO2 also remained stable after one year.

### 6.3.2 NIV and lung function tests

After one year of nocturnal NIV polysomnografic parameters showed a significant improvement detected in mean nocturnal SpO2 and SpO2 <90% values. The FEV1% values were stable after one year, whereas the LCI values decreased by 12% but not significantly (from 17.4 to 15.5; p: n.s).

### *6.3.3 NIV and PSQI*

After one year of nocturnal NIV, a significant improvement in PSQI was also recorded (17.1±2.1 vs.13.5±2.1; $p<0.05$).

## 6.4 Discussion

In our pilot study nocturnal NIV treatment appeared a preventive and useful therapeutic strategy to reduce the number exacerbation/year and polysomnografic parameters, as well as quality of life in normoxiemic and normocapnic awake patients with CF. Infectious exacerbations play an important role in the progressive loss of functional lung tissue and in the disease's progression, and predictive biomarkers for clinical decay are not still available [203]. Although pursuing a reduction in the number of exacerbations is pivotal, no study conducted on patients with CF in treatment with NIV has clearly addressed this outcome. In light of the evidence that the use of NIV is not without risk, especially in patients infected with Pseudomonas aeruginosa and/or experiencing severe airflow obstruction, to date, the use of NIV in patients with CF is limited to acute or chronic lung failure as well as a bridge to transplantation at the end stage of lung diseases [203]. Firstly, we proposed an early use of NIV in stable patients with CF to evaluate a favourable relationship with clinical outcomes. Infectious exacerbations of lung diseases in patients with CF must be recognised early and treated promptly in order to counteract lung failure. In addition to its physiological effects, NIV treatment, facilitating airway drainage all through the day, has been considered as a useful adjunct to other airway clearance techniques [204]. So far, only a late treatment with NIV has been used in hypercapnic patients and associated with the slowing progression of respiratory failure, herein, we firstly proposed an early treatment with NIV and showed that, after one year of treatment, all enrolled patients reported a significant decrease in number of exacerbations/year. In parallel, polygraphic respiratory events during sleep in patients treated with home NIV appeared significantly improved; an overall decrease in respiratory events and normalization of respiratory gases were recorded. Although the positive NIV effects on nocturnal cardio-respiratory polygraphy are well known [193-195], to the best of our knowledge, no trials evaluated diagnostic polysomnography results after long-term NIV use. Herein, we firstly reported significant improvement in polysomnography results after 12 months NIV use and also revealing as this tool can

further support the long-term NIV use in stable CF patients. Treatment with NIV in severe CF patients has been shown to slow the lung failure in terms of FEV1 [194, 195]. Accordingly, we found that the FEV1 was unchanged after one year of nocturnal NIV even in stable CF patients. Although the FEV1 has been the main outcome of most studies for many years, recently the LCI has been also used for the functional evaluation of patients with CF. LCI reflects the degree of inhomogeneity of ventilation distribution and provides informations complimentary to FEV1, and it can be an alternative measure to high resolution computed tomography (CT) for detecting early pulmonary abnormalities. Firstly, our study provided the evidence that a long-term NIV did not influence LCI values. The mechanism underlying the effect of NIV in CF is unclear. In patients with chronic obstructive lung disease NIV treatment reduces distal airway resistance, improves the ventilation/perfusion ratio (VQ), and recruits collapsed alveolar units via expiratory positive pressure; thus, it is reasonable to hypothesize that similar mechanisms can occur even in patients with CF favouring an improvement both in the ventilation distribution and VQ ratio. Moreover, we noted that NIV treatment significantly reduced the nocturnal respiratory rate in our population, although maintaining stable nocturnal levels of TcCO2 . Even if the association between lung function and quality of life is widely described in patients with CF [203], the available studies are unable to provide data on the impact of nocturnal NIV treatment on quality of life in stable CF patients [204-206]. In this regard, our study showed that nocturnal NIV treatment significantly improved quality of life and it appeared well tolerated despite the use of relatively high inspiratory pressures for a long time (12 months). We suggest that the observed improvement in health status of all enrolled patients is related to a decrease of infectious exacerbations. Our study has some limitations. First, the sample size is small and not entirely representative of the wide spectrum of the disease in relationship to the clinical presentation and respiratory impairment, even in the presence of identical genetic mutation. It is also noteworthy that the MBW test to derive LCI, is an emerging technique to monitor the disease progression and standardized protocols in CF are still unavailable. In our studies LCI values were decreasing but not significantly, probably, a further study with a larger sample size could be useful to achieve to more sensitive information on this issue and define if LCI could as a reliable tool for an useful clinical assessment of CF. In conclusion, we believe that our preliminary data may provide the base to other larger, multicenter studies. We base this assumption on the following novel findings: 1) the use of preventive nocturnal NIV

in normoxemic and normocapnic awake CF has never been explored; 2) the nocturnal NIV seems to be beneficial because it has been able to prevent the number of exacerbations/year and the effect on nocturnal cardiorespiratory polygraphic values is a further element explaining usefulness of NIV treatment in CF; 3) the nocturnal NIV treatment significantly improved quality of life and it appeared well tolerated despite the use of relatively high inspiratory pressures for a long time.

# CHAPTER 7. RESEARCH#5: LCI FOR THE EVALUATION OF LONG TERM SEQUELAE OF PEDIATRIC COVID-19

## 7.1 Introduction

In March 2020, the World Health Organization (WHO) declared the Coronavirus disease 2019 (COVID-19) pandemic. The novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a top threat to global health, emerged in Wuhan (China) in December last year and rapidly spread worldwide [207]. It is now known that many patients who recover from the acute phase of the COVID-19 continue to have clinical manifestations or develop new ones. This finding has alerted the scientific community, and researchers immediately began investigating these alterations and the possible correlation with SARS-CoV-2 infection. The term "long-COVID" was coined to indicate these manifestations.

To better define the discussion in this study, we referred to the guidelines produced by the National Institute for Health and Care Excellence (NICE), published on 18 December 2020 [208].

These guidelines use several clinical definitions:

- Acute COVID-19: signs and symptoms of COVID-19 up to four weeks after the onset of the disease.

- Ongoing symptomatic COVID-19: signs and symptoms of COVID-19 four to 12 weeks after the onset of the disease.

- Post-COVID-19 syndrome: signs and symptoms that continue or develop after an infection compatible with COVID-19, persist for more than 12 weeks from the onset of the disease, and are not explained by alternative diagnoses [208].

The NICE guidelines report that in addition to the previous clinical definitions, the term long-COVID is commonly used to describe the signs and symptoms that continue or develop after the acute stage of COVID-19, thus including both COVID-19 that continues to be symptomatic and the post-COVID-19 syndrome as defined above [208].

As for adult patients, much scientific evidence is already available. In this sense, the systematic review and meta-analysis by Lopez-Leon et al. highlights how 80% of patients who contracted COVID-19 developed at least one long-term symptom of which

the five most common were fatigue (58%), headache (44%), attention disturbance (27%), hair loss (25%), and dyspnea (24%) [209].

As for children, the situation is slightly different. In fact, it is known that the course of COVID-19 in children is much less severe than in adults, and more serious complications, such as pneumonia, are less frequent [210-212]. However, recent evidence seems to show that long-Covid symptoms also affect younger patients [213-215].

## 7.2 Matherials and Methods

The pediatric pulmonology unit of the San Marco hospital in Catania, directed by professor Salvatore Leonardi, since March 2021 has activated an outpatient service dedicated to children (age range 0-18 years) recovered from COVID-19. The catchment area is that of central-eastern Sicily (provinces of Catania, Syracuse, Ragusa, Messina, Enna, Agrigento, Caltanissetta). The clinical/instrumental evaluation is multidisciplinary as several professional figures are involved: pediatrician, cardiologist, physiotherapist, psychologist, nurse. The child who underwent our Day-Hospital is subjected to the following investigations: anamnesis, assessment of the persistence of symptoms attributable to COVID-19, physical examination, blood sampling, skin prick tests, conventional spirometry, multiple breath washout with study of the LCI, lung ultrasound, echocardiography, quality of life questionnaires, 6-minute walk test. In relation to the outcome of this assessment, the patient is taken in charge and a personalized therapeutic path is identified in relation to the critical issues encountered.

## 7.3 Results

### *7.3.1 General results*

To date, 63 patients have been evaluated. From the analysis of the data it emerged that 30% of patients had microcytic anemia, mild platelet disease, a reduction in the CD4 / CD8 ratio and an increase in total IgE. The respiratory function tests documented, in 20% of patients, a reduction in FEV1 and FVC, an obstructive spirometric picture and a slight increase in LCI values. On pulmonary ultrasound, 30% of patients showed thickening and/or discontinuity of the pleural line and isolated B lines.

Physiotherapy evaluation in 20% of cases documented a reduced exercise tolerance and reduced expiratory capacity.

### *7.3.2 Focus on LCI*

Most children recovered from COVID-19 maintain normal LCI levels (mean 6.75 ± 1.10 s.d.). However, about 25% of patients had values above normal (**figure 18**).

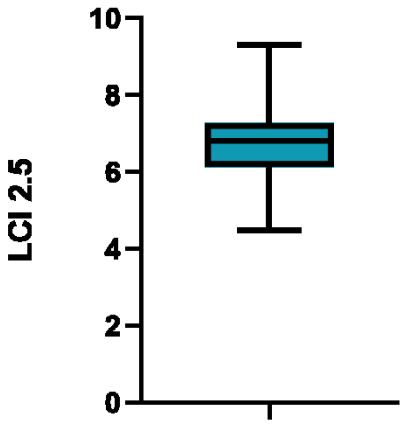


**FIGURE 18. LCI VALUES IN CHILDREN HEALED FROM COVID-19**

## 7.4 Discussion

The data derived from the patients followed in our outpatient clinic contribute to the description and quantification of long-Covid in the pediatric population, a phenomenon for which there is still little evidence in the literature on the actual ocurrence. The first fact that stands out is that unlike what happens in adults in whom the percentage of patients who complain of the persistence of symptoms after recovery from COVID-19 is 80% [209], in our pediatric cases, this persistence does not seem to exceed 20%. Regarding the most reported symptoms, in first place fatigue has been reported followed by headache and gastrointestinal disorders. Taste and smell disturbances and other symptoms seem to have little relevance in this population.

The few reports in the literature on this particular subject are not always in agreement but obviously they are difficult to compare due to the different methods of evaluation and quantification. Buonsenso et al. examined a cohort of 129 children younger than 18 years who had been diagnosed with COVID-19 and were evaluated

during the first and second pandemic waves. Most of them had mild COVID-19 symptoms at the time of diagnosis, and 33 were asymptomatic. Of the initial group, 68 were evaluated even after 120 days, and 51% still reported at least one symptom. The most frequent symptoms were fatigue, muscle or joint pain, headache, sleep disturbances, chest pain, dyspnea, and palpitations. On average, five months after diagnosis, only 42% had fully recovered and about one in three children still had at least one symptom [214]. Ludvigsson evaluated five children over time who had COVID-19, demonstrating that four out of five continued to have headaches, concentration disorders, and/or muscle weakness even six months after recovering from COVID-19 [213]. These data seem to have been confirmed by the same Swedish author who described a larger case series involving 35 children [215]. According to a Dutch survey involving 78% of national pediatric departments, 89 children had clinical characteristics attributable to long-covid. Specifically, the main complaints were fatigue, dyspnea and difficulty concentrating [216].

Focusing on our data, it would therefore seem that the phenomenon of long-covid in pediatric age is overestimated. This finding confirms that children do not undergo an acute course of the disease that is much more attenuated than adults but are also be more protected against long-term complications. Regarding the symptoms most frequently encountered, fatigue is in first place. Manifestations of long COVID mimic those of chronic fatigue syndrome (CFS), which includes the presence of severe incapacitating asthenia, pain, neurocognitive impairment, sleep disturbances, autonomic dysfunction, and worsening of overall symptoms following even mild physical or mental exertion [217, 218].

CFS is currently a complex and controversial clinical entity with no well-defined causal factors [219, 220]. Possible causes include viruses, immune dysfunction, metabolic endocrinological changes, and neuropsychiatric factors [219]. Infectious agents that have been related to CFS include Epstein-Barr virus, cytomegalovirus, enterovirus, herpesvirus [221], and SARS-CoV-1 [222, 223] (which is very similar to SARS-CoV- 2). It is certainly tempting to speculate that SARS-CoV-2 could be added to the list of viral agents causing CFS. Lidbury et al. proposed a hypothesis to explain the possible mechanisms underlying chronic post-viral fatigue syndrome; inflammatory cytokines abundantly produced during acute disease would impact on the functionality of mechanistic target of rapamycin (mTOR), a serine/threonine kinase that regulates cellular homeostasis by

influencing transcription, protein synthesis, autophagy, metabolism, biogenesis, and maintenance of organelles through its signaling pathways [223]. Among its many functions, mTOR has an important role in the regulation of mitochondrial activity, oxygen consumption, and oxidative capacity, which are also based on the nutritional status and energy needs of the cell [224, 225]. Therefore, the altered functionality of mTOR caused by inflammatory cytokines would impact mitochondrial function and cellular energy regulation possibly leading to chronic fatigue.

Finally, although the phenomenon of long-COVID in the pediatric population would seem to have little relevance, it is interesting to highlight that nearly 25% of our patients have mild changes in LCI, which could indicate that the infection causes minimal damage to the small airways.

# CHAPTER 8. CONCLUSIONS

Epidemiologic changes in pediatric pulmonary diseases such as CF are ongoing due to new screening, novel efficacy therapies, and improved knowledge of disease pathophysiology. Considering this perspective and considering that spirometry sensitivity will not be sufficient to track early disease, LCI use will increase in the future. LCI standardization appears to have a tangible future because Europe, North America, and Australia have agreed on an $N_2$ measurement standardization using the Exhalyzer-D to avoid variability in measurements among centers.

The majority of scientific papers emphasized the role of LCI in pre-school children because few tools are helpful in this age range, and an extensive amount of data is available for this population. Considering these epidemiological changes, additional data from adolescents are needed. The LCI value acquires an essential role in the longitudinal evaluation of patients, especially in those who have early and mild disease, and because there are forecasts for increases in these disease stages, we must be prepared to offer the best surveillance to our patients.

In this sense, the work of these three years of doctorate has contributed to increase the scientific evidence on the MBW test in childhood respiratory diseases since it can also be performed on preschool patients who are unable to perform forced expiratory manoeuvres, unlike conventional spirometry. Based on this, we can certainly affirm that LCI can be considered to all intents and purposes a biomarker of ventilatory inhomogeneity in children with respiratory diseases.

# REFERENCES


1- Zar HJ, Ferkol TW. The global burden of respiratory disease-impact on child health. Pediatr Pulmonol. 2014 May;49(5):430-4. doi: 10.1002/ppul.23030. Epub 2014 Mar 9. PMID: 24610581.

2- https://www.europeanlung.org/en/lung-disease-and-information/lung-diseases/lung-disease-in-children. Accessed on the first of April, 2021

3- Parisi GF and Leonardi S. Upper and Lower Airways Diseases in Childhood. Current Respiratory Medicine Reviews 2019; 15(3):161-162. DOI: 10.2174/1573398X1503191125160355

4- Parisi GF, Papale M, Tardino L, Nenna R, Midulla F, Leonardi S. Biomarkers in Pediatric Lung Diseases Including Cystic Fibrosis. Current Respiratory Medicine Reviews 2019; 15(3):163-173. DOI: 10.2174/1573398X15666190521112824

5- De Rose V, Molloy K, Gohy S, Pilette C, Greene CM. Airway Epithelium Dysfunction in Cystic Fibrosis and COPD. Mediators Inflamm. 2018 Apr 8;2018:1309746. doi: 10.1155/2018/1309746. PMID: 29849481; PMCID: PMC5911336.

6- de Jong W, Kaptein AA, van der Schans CP, Mannes GP, van Aalderen WM, Grevink RG, Koëter GH. Quality of life in patients with cystic fibrosis. Pediatr Pulmonol. 1997 Feb;23(2):95-100. doi: 10.1002/(sici)1099-0496(199702)23:2<95::aid-ppul4>3.0.co;2-n. PMID: 9065946.

7- Spicuzza L, Parisi GF, Tardino L, Ciancio N, Nenna R, Midulla F, Leonardi S. Exhaled markers of antioxidant activity and oxidative stress in stable cystic fibrosis patients with moderate lung disease. J Breath Res 2018. Feb 20;12(2):026010. doi: 10.1088/1752-7163/aa9b39.

8- Sanders DB, Bittner RCL, Rosenfeld M, Redding GJ, Goss CH. Pulmonary exacerbations are associated with subsequent FEV 1 decline in both adults and children with cystic fibrosis: Pulmonary Exacerbations and FEV 1 Decline. Pediatr Pulmonol. 2011;46(4):393-400. doi:10.1002/ppul.21374

9- Leonardi S, Parisi GF, Capizzi A, Manti S, Cuppari C, Scuderi MG, Rotolo N, Lanzafame A, Musumeci M, Salpietro C. YKL-40 as marker of severe lung disease in cystic fibrosis. J Cyst Fibros 2016; 15:583-586.

10- Goetz D, Ren CL. Review of Cystic Fibrosis. Pediatr Ann. 2019 Apr 1;48(4):e154-e161. doi: 10.3928/19382359-20190327-01. PMID: 30986316.

11- Fuchs SI, Gappa M. Lung clearance index: clinical and research applications in children. Paediatr Respir Rev. 2011;12(4):264-270. doi:10.1016/j.prrv.2011.05.001

12- Horsley AR, Gustafsson PM, Macleod KA, et al. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. Thorax 2008;63:135-140.

13- Usemann J, Yammine S, Singer F, Latzin P. Inert gas washout: Background and application in various lung diseases. Swiss Med Wkly. 2017;16:147

14- Aurora P, Bush A, Gustafsson P, et al. Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. Am J Respir Crit Care Med 2005; 171: 249–256.

15- Aurora P, Gustafsson P, Bush A, et al. Multiple breath inert gas washout as a measure of ventilation distribution in children with cystic fibrosis. Thorax 2004; 59: 1068–1073.

16- Califf RM. Biomarker definitions and their applications. Exp Biol Med (Maywood). 2018 Feb;243(3):213-221. doi: 10.1177/1535370217750088. PMID: 29405771; PMCID: PMC5813875.

17- FOWLER WS, COMROE JH Jr. Lung function studies; the rate of increase of arterial oxygen saturation during the inhalation of 100 per cent O2. J Clin Invest. 1948;27(3 Pt 1):327-334.

18- FOWLER WS. Lung function studies; the respiratory dead space. Am J Physiol. 1948;154(3):405-416. doi:10.1152/ajplegacy.1948.154.3.405

19- FOWLER WS. Lung function studies; uneven pulmonary ventilation in normal subjects and in patients with pulmonary disease. J Appl Physiol. 1949;2(6):283-299. doi:10.1152/jappl.1949.2.6.283

20- Fowler WS. LUNG FUNCTION STUDIES. IV. POSTURAL CHANGES IN RESPIRATORY DEAD SPACE AND FUNCTIONAL RESIDUAL CAPACITY. J Clin Invest. 1950;29(11):1437-1438. doi:10.1172/JCI102382

21- FOWLER WS. Lung function studies. V. Respiratory dead space in old age and in pulmonary emphysema. J Clin Invest. 1950;29(11):1439-1444. doi:10.1172/JCI102383

22- COMROE JH Jr, FOWLER WS. Lung function studies. VI. Detection of uneven alveolar ventilation during a single breath of oxygen. Am J Med. 1951;10(4):408-413. doi:10.1016/0002-9343(51)90285-9

23- FOWLER WS, BLAKEMORE WS. Lung function studies. VII. The effect of pneumonectomy on respiratory dead space. J Thorac Surg. 1951;21(5):433-437.

24- FOWLER WS, CORNISH ER Jr, KETY SS. Lung function studies. VIII. Analysis of alveolar ventilation by pulmonary N2 clearance curves. J Clin Invest. 1952;31(1):40-50. doi:10.1172/JCI102575

25- Kraemer R, Meister B. Fast real-time moment-ratio analysis of multibreath nitrogen washout in children. J Appl Physiol (1985). 1985;59(4):1137–44.

26- Lombardi E, Gambazza S, Pradal U, Braggion C. Lung clearance index in subjects with cystic fibrosis in Italy. Ital J Pediatr. 2019;45(1):56. doi:10.1186/s13052-019-0647-5

27- Saunders C, Bayfield K, Irving S, Short C, Bush A, Davies JC. Developments in multiple breath washout testing in children with cystic fibrosis. Current Medical Research and Opinion. 2017;33(4):613-620. doi:10.1080/03007995.2016.1268999

28- Nuttall AGL, Velásquez W, Beardsmore CS, Gaillard EA. Lung clearance index: assessment and utility in children with asthma. Eur Respir Rev. 2019 Nov 20;28(154):190046. doi: 10.1183/16000617.0046-2019. PMID: 31748419.

29- Irving S, Dixon M, Fassad MR, Frost E, Hayward J, Kilpin K, Ollosson S, Onoufriadis A, Patel MP, Scully J, Carr SB, Mitchison HM, Loebinger MR, Hogg C, Shoemark A, Bush A. Primary Ciliary Dyskinesia Due to Microtubular Defects is Associated with Worse Lung Clearance Index. Lung. 2018 Apr;196(2):231-238. doi: 10.1007/s00408-018-0086-x. Epub 2018 Jan 24. PMID: 29368042; PMCID: PMC5854730.

30- Vogt B, Falkenberg C, Weiler N, Frerichs I. Pulmonary function testing in children and infants. Physiol Meas. 2014;35(3):R59-R90. doi:10.1088/0967-3334/35/3/R59

31- Beydon N, Davis SD, Lombardi E, et al. An Official American Thoracic Society/European Respiratory Society Statement: Pulmonary Function Testing in Preschool Children. Am J Respir Crit Care Med. 2007;175(12):1304-1345. doi:10.1164/rccm.200605-642ST

32- WEIBEL ER, GOMEZ DM. Architecture of the human lung. Use of quantitative methods establishes fundamental relations between size and number of lung structures. Science. 1962 Aug 24;137(3530):577-85. doi: 10.1126/science.137.3530.577. PMID: 14005590.

33- Horsfield K. Diameters, generations, and orders of branches in the bronchial tree. J Appl Physiol (1985). 1990 Feb;68(2):457-61. doi: 10.1152/jappl.1990.68.2.457. PMID: 2318756.

34- Robinson PD, Goldman MD, Gustafsson PM. Inert Gas Washout: Theoretical Background and Clinical Utility in Respiratory Disease. Respiration. 2009;78(3):339-355. doi:10.1159/000225373

35- Patwa A, Shah A. Anatomy and physiology of respiratory system relevant to anaesthesia. Indian J Anaesth. 2015;59(9):533. doi:10.4103/0019-5049.165849

36- Macklem PT. The Physiology of Small Airways. Am J Respir Crit Care Med. 1998;157(5):S181-S183. doi:10.1164/ajrccm.157.5.rsaa-2

37- Guyton and hall (2005). Textbook of Medical Physiology (11 ed.). Philadelphia: Saunders. pp. 103g. ISBN 978-81-8147-920-4.

38- Tortora, Gerard J. (2016). Principles of anatomy & physiology. Derrickson, Bryan (15th ed.). Hoboken, NJ. p. 874. ISBN 978-1119447979. OCLC 1020568457.

39- Robinson PD, Latzin P, Verbanck S, et al. Consensus statement for inert gas washout measurement using multiple- and single- breath tests. Eur Respir J. 2013;41(3):507-522. doi:10.1183/09031936.00069712

40- Horsley A. Lung clearance index in the assessment of airways disease. Respiratory Medicine. 2009;103(6):793-799. doi:10.1016/j.rmed.2009.01.025

41- van der Wiel E, ten Hacken NHT, Postma DS, van den Berge M. Small-airways dysfunction associates with respiratory symptoms and clinical features of asthma: A systematic review. Journal of Allergy and Clinical Immunology. 2013;131(3):646-657. doi:10.1016/j.jaci.2012.12.1567

42- Shaw RJ, Djukanovic R, Tashkin DP, Millar AB, Du Bois RM, Corris PA. The role of small airways in lung disease. Respiratory Medicine. 2002;96(2):67-80. doi:10.1053/rmed.2001.1216

43- Jensen R, Stanojevic S, Gibney K, Salazar JG, Gustafsson P, Subbarao P, et al. Multiple breath nitrogen washout: a feasible alternative to mass spectrometry. PLoS One. 2013;8(2):e56868. doi: http://dx.doi.org/10.1371/journal.pone.0056868.

44- Poncin W, Singer F, Aubriot AS, Lebecque P. Agreement between multiple-breath nitrogen washout systems in children and adults. J Cyst Fibros. 2017;16(2):258–66.

45- Kraemer R, Meister B. Fast real-time moment-ratio analysis of multibreath nitrogen washout in children. J Appl Physiol (1985). 1985;59(4):1137–44.

46- Yammine S, Lenherr N, Nyilas S, Singer F, Latzin P. Using the same cut-off for sulfur hexafluoride and nitrogen multiple-breath washout may not be appropriate. J Appl Physiol (1985). 2015;119(12):1510–2. doi: http://dx.doi.org/10.1152/japplphysiol.00333.2015.

47- Anagnostopoulou P, Latzin P, Jensen R, et al. Normative data for multiple breath washout outcomes in school-aged Caucasian children. Eur Respir J. 2020;55(4):1901302. doi:10.1183/13993003.01302-2019

48- Yammine S, Lenherr N, Nyilas S, Singer F, Latzin P. Using the same cut-off for sulfur hexafluoride and nitrogen multiple-breath washout may not be appropriate. Journal of Applied Physiology. 2015;119(12):1510-1512. doi:10.1152/japplphysiol.00333.2015

49- Summermatter S, Singer F, Latzin P, Yammine S. Impact of Software Settings on Multiple-Breath Washout Outcomes. PLoS One. 2015;10(7):e0132250. doi:http://dx.doi.org/10.1371/journal.pone.0132250.

50- Verbanck S, Schuermans D, Van Muylem A, et al. Conductive and Acinar Lung-zone Contributions to Ventilation Inhomogeneity in COPD. Am J Respir Crit Care Med. 1998;157(5):1573-1577. doi:10.1164/ajrccm.157.5.9710042

51- Verbanck S, Schuermans D, Van Muylem A, Paiva M, Noppen M, Vincken W. Ventilation distribution during histamine provocation. Journal of Applied Physiology. 1997;83(6):1907-1916. doi:10.1152/jappl.1997.83.6.1907

52- Crawford AB, Makowska M, Paiva M, Engel LA. Convection- and diffusion-dependent ventilation maldistribution in normal subjects. Journal of Applied Physiology. 1985;59(3):838-846. doi:10.1152/jappl.1985.59.3.838

53- Sullivan L, Forno E, Pedersen K, Nielsen JG, Weiner DJ. Nitrogen back-diffusion during multiple-breath washout with 100% oxygen. Eur Respir J. 2017;50(3):1700679. doi:10.1183/13993003.00679-2017

54- Kane M, Rayment JH, Jensen R, McDonald R, Stanojevic S, Ratjen F. Correcting for tissue nitrogen excretion in multiple breath washout measurements. Singer F, ed. PLoS ONE. 2017;12(10):e0185553. doi:10.1371/journal.pone.0185553

55- Lundin G. Nitrogen elimination from the tissues during oxygen breathing and its relationship to the fat:muscle ratio and the localization of bends. The Journal of Physiology. 1960;152(1):167-175. doi:10.1113/jphysiol.1960.sp006478

56- Nielsen N, Nielsen JG, Horsley AR. Evaluation of the Impact of Alveolar Nitrogen Excretion on Indices Derived from Multiple Breath Nitrogen Washout. Rojas M, ed. PLoS ONE. 2013;8(9):e73335. doi:10.1371/journal.pone.0073335

57- Guglani L, Kasi A, Starks M, Pedersen KE, Nielsen JG, Weiner DJ. Difference between SF 6 and N 2 multiple breath washout kinetics is due to N 2 back diffusion and error in N 2 offset. Journal of Applied Physiology. 2018;125(4):1257-1265. doi:10.1152/japplphysiol.00326.2018

58- Kinghorn B, McNamara S, Genatossio A, et al. Comparison of Multiple Breath Washout and Spirometry in Children with Primary Ciliary Dyskinesia and Cystic Fibrosis and Healthy Controls. Annals ATS. Published online June 30, 2020:AnnalsATS.201905-375OC. doi:10.1513/AnnalsATS.201905-375OC

59- Robinson PD, Lum S, Moore C, et al. Comparison of facemask and mouthpiece interfaces for multiple breath washout measurements. Journal of Cystic Fibrosis. 2018;17(4):511-517. doi:10.1016/j.jcf.2018.01.003

60- Anagnostopoulou P, Kranz N, Wolfensberger J, et al. Comparison of different analysis algorithms to calculate multiple-breath washout outcomes. ERJ Open Res. 2018;4(3):00021-02017. doi:10.1183/23120541.00021-2017

61- Fuchs SI, Eder J, Ellemunter H, Gappa M. Lung clearance index: Normal values, repeatability, and reproducibility in healthy children and adolescents: Lung Clearance

Index: Variability and Normal Values. Pediatr Pulmonol. 2009;44(12):1180-1185. doi:10.1002/ppul.21093

62- Lum S, Stocks J, Stanojevic S, et al. Age and height dependence of lung clearance index and functional residual capacity. Eur Respir J. 2013;41(6):1371-1377. doi:10.1183/09031936.00005512

63- Houltz, Kent. Tidal N2 washout ventilation inhomogeneity indices in a reference population aged 7-70 years. In: ; 2012:2.

64- Ratjen F. Cystic fibrosis: the role of the small airways. J Aerosol Med Pulm Drug Deliv. 2012 Oct;25(5):261-4. doi: 10.1089/jamp.2011.0923. Epub 2012 Aug 2. PMID: 22857153.

65- Parisi GF, Cutello S, Di Dio G, Rotolo N, La Rosa M, Leonardi S. Phenotypic expression of the p:leu1077Pro CFTR mutation in Sicilian cystic fibrosis patients. BMC Res Notes 2013; 13;6:461

66- Parisi GF, Papale M, Rotolo N, Aloisio D, Tardino L, Scuderi MG, Di Benedetto V, Nenna R, Midulla F, Leonardi S. Severe disease in Cystic Fibrosis and fecal calprotectin levels. Immunobiology 2017; 222:582-586

67- Stephenson AL, Stanojevic S, Sykes J, Burgel P-R. The changing epidemiology and demography of cystic fibrosis. La Presse Médicale. 2017;46(6):e87-e95. doi:10.1016/j.lpm.2017.04.012

68- Parisi GF, Portale A, Papale M, Tardino L, Rotolo N, Licari A, Leonardi S. Successful treatment with omalizumab of allergic bronchopulmonary aspergillosis in patients with cystic fibrosis: Case reports and literature review. J Allergy Clin Immunol Pract. 2019 May-Jun;7(5):1636-1638. doi: 10.1016/j.jaip.2019.01.056. Epub 2019 Feb 15. PMID: 30772479.

69- Chmiel JF, Davis PB. State of the Art: Why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? Respir Res. 2003;4(1):8. doi:10.1186/1465-9921-4-8

70- Pier GB, Grout M, Zaidi TS. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung. Proceedings of the National Academy of Sciences. 1997;94(22):12088-12093. doi:10.1073/pnas.94.22.12088

71- Bergeron C, Cantin AM. Cystic Fibrosis: Pathophysiology of Lung Disease. Semin Respir Crit Care Med. 2019;40(06):715-726. doi:10.1055/s-0039-1694021

72- Teichgräber V, Ulrich M, Endlich N, et al. Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat Med. 2008;14(4):382-391. doi:10.1038/nm1748

73- Ranganathan SC, Hall GL, Sly PD, Stick SM, Douglas TA. Early Lung Disease in Infants and Preschool Children with Cystic Fibrosis. What Have We Learned and

What Should We Do about It? Am J Respir Crit Care Med. 2017;195(12):1567-1575. doi:10.1164/rccm.201606-1107CI

74- Nichols D, Chmiel J, Berger M. Chronic Inflammation in the Cystic Fibrosis Lung: Alterations in Inter- and Intracellular Signaling. Clinic Rev Allerg Immunol. 2008;34(2):146-162. doi:10.1007/s12016-007-8039-9

75- Esterly JR, Oppenheimer EH. Cystic fibrosis of the pancreas: structural changes in peripheral airways. Thorax. 1968;23(6):670-675. doi:10.1136/thx.23.6.670

76- Redondo M, Keyt H, Dhar R, Chalmers JD. Global impact of bronchiectasis and cystic fibrosis. Breathe (Sheff). 2016 Sep;12(3):222-235. doi: 10.1183/20734735.007516. PMID: 28210295; PMCID: PMC5298141.

77- Sly PD, Wainwright CE. Diagnosis and early life risk factors for bronchiectasis in cystic fibrosis: a review. Expert Review of Respiratory Medicine. 2016;10(9):1003-1010. doi:10.1080/17476348.2016.1204915

78- Bortoluzzi C-F, Volpi S, D'Orazio C, et al. Bronchiectases at early chest computed tomography in children with cystic fibrosis are associated with increased risk of subsequent pulmonary exacerbations and chronic pseudomonas infection. Journal of Cystic Fibrosis. 2014;13(5):564-571. doi:10.1016/j.jcf.2014.03.006

79- Chalmers JD. Cystic fibrosis lung disease and bronchiectasis. The Lancet Respiratory Medicine. 2020;8(1):12-14. doi:10.1016/S2213-2600(19)30335-2

80- Brennan S, Gangell C, Wainwright C, Sly PD. Disease surveillance using bronchoalveolar lavage. Paediatric Respiratory Reviews. 2008;9(3):151-159. doi:10.1016/j.prrv.2008.01.002

81- Salamon E, Lever S, Kuo W, Ciet P, Tiddens HA. Spirometer guided chest imaging in children: It is worth the effort! Pediatr Pulmonol. 2017 Jan;52(1):48-56. doi: 10.1002/ppul.23490. Epub 2016 Jun 6. PMID: 27273821.

82- Kuo W, Ciet P, Tiddens HA, Zhang W, Guillerman RP, van Straten M. Monitoring cystic fibrosis lung disease by computed tomography. Radiation risk in perspective. Am J Respir Crit Care Med. 2014 Jun 1;189(11):1328-36. doi: 10.1164/rccm.201311-2099CI. PMID: 24697683.

83- Bell SC, Mall MA, Gutierrez H, et al. The future of cystic fibrosis care: a global perspective. The Lancet Respiratory Medicine. 2020;8(1):65-124. doi:10.1016/S2213-2600(19)30337-6

84- Kraemer R, Blum A, Schibler A, Ammann RA, Gallati S. Ventilation Inhomogeneities in Relation to Standard Lung Function in Patients with Cystic Fibrosis. Am J Respir Crit Care Med. 2005;171(4):371-378. doi:10.1164/rccm.200407-948OC

85- Stanojevic S, Davis SD, Retsch-Bogart G, et al. Progression of Lung Disease in Preschool Patients with Cystic Fibrosis. Am J Respir Crit Care Med. 2017;195(9):1216-1225. doi:10.1164/rccm.201610-2158OC

86- Svedberg M, Gustafsson PM, Robinson PD, Rosberg M, Lindblad A. Variability of lung clearance index in clinically stable cystic fibrosis lung disease in school age children. Journal of Cystic Fibrosis. 2018;17(2):236-241. doi:10.1016/j.jcf.2017.08.004

87- Fuchs SI, Gappa M, Eder J, Unsinn KM, Steinkamp G, Ellemunter H. Tracking Lung Clearance Index and chest CT in mild cystic fibrosis lung disease over a period of three years. Respiratory Medicine. 2014;108(6):865-874. doi:10.1016/j.rmed.2014.03.011

88- fus KA, Rosenow T, Turkovic L, et al. Lung Clearance Index and Structural Lung Disease on Computed Tomography in Early Cystic Fibrosis. Am J Respir Crit Care Med. 2016;193(1):60-67. doi:10.1164/rccm.201507-1409OC

89- Hoo A-F, Thia LP, Nguyen TTD, et al. Lung function is abnormal in 3-month-old infants with cystic fibrosis diagnosed by newborn screening. Thorax. 2012;67(10):874-881. doi:10.1136/thoraxjnl-2012-201747

90- Belessis Y, Dixon B, Hawkins G, Pereira J, Peat J, MacDonald R, Field P, Numa A, Morton J, Lui K, Jaffe A. Early cystic fibrosis lung disease detected by bronchoalveolar lavage and lung clearance index. Am J Respir Crit Care Med. 2012 Apr 15;185(8):862-73. doi: 10.1164/rccm.201109-1631OC. Epub 2012 Feb 9. PMID: 22323305.

91- O'Neill K, Bradley JM, Reid A, Downey DG, Rendall J, McCaughan J, Moore JE, Tunney MM, Elborn JS. Airway infection, systemic inflammation and lung clearance index in children and adults with cystic fibrosis. Eur Respir J. 2018 Jan 31;51(2):1701704. doi: 10.1183/13993003.01704-2017. PMID: 29386349.

92- Voldby C, Green K, Kongstad T, et al. Lung clearance index-triggered intervention in children with cystic fibrosis – A randomised pilot study. Journal of Cystic Fibrosis. Published online June 2020:S1569199320307554. doi:10.1016/j.jcf.2020.06.010

93- Subbarao P, Stanojevic S, Brown M, et al. Lung Clearance Index as an Outcome Measure for Clinical Trials in Young Children with Cystic Fibrosis. A Pilot Study Using Inhaled Hypertonic Saline. Am J Respir Crit Care Med. 2013;188(4):456-460. doi:10.1164/rccm.201302-0219OC

94- Amin R, Subbarao P, Lou W, et al. The effect of dornase alfa on ventilation inhomogeneity in patients with cystic fibrosis. European Respiratory Journal. 2011;37(4):806-812. doi:10.1183/09031936.00072510

95- Ratjen F, Hug C, Marigowda G, et al. Efficacy and safety of lumacaftor and ivacaftor in patients aged 6–11 years with cystic fibrosis homozygous for F508del-CFTR : a randomised, placebo-controlled phase 3 trial. The Lancet Respiratory Medicine. 2017;5(7):557-567. doi:10.1016/S2213-2600(17)30215-1

96- Rosenow T, Mok LC, Turkovic L, et al. The cumulative effect of inflammation and infection on structural lung disease in early cystic fibrosis. Eur Respir J. 2019;54(1):1801771. doi:10.1183/13993003.01771-2018

97- Montgomery ST, Mall MA, Kicic A, Stick SM. Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. Eur Respir J. 2017;49(1):1600903. doi:10.1183/13993003.00903-2016

98- Vermeulen F, Proesmans M, Boon M, Havermans T, De Boeck K. Lung clearance index predicts pulmonary exacerbations in young patients with cystic fibrosis. Thorax. 2014;69(1):39-45. doi:10.1136/thoraxjnl-2013-203807

99- Rosenfeld M. An Overview of Endpoints for Cystic Fibrosis Clinical Trials: One Size Does Not Fit All. Proceedings of the American Thoracic Society. 2007;4(4):299-301. doi:10.1513/pats.200611-178HT

100- Papale M, Parisi GF, Spicuzza L, Licari A, Bongiovanni A, Mulè E, Rotolo N, Manti S, Leonardi S. Lung clearance index evaluation in detecting nocturnal hypoxemia in cystic fibrosis patients: Toward a new diagnostic tool. Respir Med. 2020 Apr;164:105906. doi: 10.1016/j.rmed.2020.105906. Epub 2020 Feb 19. PMID: 32217291.

101- Davies J, Sheridan H, Bell N, et al. Assessment of clinical response to ivacaftor with lung clearance index in cystic fibrosis patients with a G551D- CFTR mutation and preserved spirometry: a randomised controlled trial. The Lancet Respiratory Medicine. 2013;1(8):630-638. doi:10.1016/S2213-2600(13)70182-6

102- Stanojevic S, Ratjen F. Physiologic endpoints for clinical studies for cystic fibrosis. Journal of Cystic Fibrosis. 2016;15(4):416-423. doi:10.1016/j.jcf.2016.05.014

103- Leigh MW, Pittman JE, Carson JL, et al. Clinical and genetic aspects of primary ciliary dyskinesia/Kartagener syndrome. Genet Med. 2009;11(7):473-487. doi:10.1097/GIM.0b013e3181a53562

104- Davis SD, Rosenfeld M, Lee H-S, et al. Primary Ciliary Dyskinesia: Longitudinal Study of Lung Disease by Ultrastructure Defect and Genotype. Am J Respir Crit Care Med. 2019;199(2):190-198. doi:10.1164/rccm.201803-0548OC

105- Knowles MR, Zariwala M, Leigh M. Primary Ciliary Dyskinesia. Clinics in Chest Medicine. 2016;37(3):449-461. doi:10.1016/j.ccm.2016.04.008

106- Brown DE, Pittman JE, Leigh MW, Fordham L, Davis SD. Early lung disease in young children with primary ciliary dyskinesia. Pediatr Pulmonol. 2008;43(5):514-516. doi:10.1002/ppul.20792

107- Lindström M, Falk R, Hjelte L, Philipson K, Svartengren M. Long-term clearance from small airways in subjects with ciliary dysfunction. Respir Res. 2006;7(1):79. doi:10.1186/1465-9921-7-79

108- Green K, Buchvald FF, Marthin JK, Hanel B, Gustafsson PM, Nielsen KG. Ventilation inhomogeneity in children with primary ciliary dyskinesia. Thorax. 2012;67(1):49-53. doi:10.1136/thoraxjnl-2011-200726

109- Boon M, Vermeulen FL, Gysemans W, Proesmans M, Jorissen M, De Boeck K. Lung structure-function correlation in patients with primary ciliary dyskinesia. Thorax. 2015;70(4):339-345. doi:10.1136/thoraxjnl-2014-206578

110- Levy ML, Fleming L. Asthma reviews in children: what have we learned? Thorax. 2020 Feb;75(2):98-99. doi: 10.1136/thoraxjnl-2019-214143. Epub 2020 Jan 2. PMID: 31896734.

111- Leonardi S, Filippelli M, Lanzafame A, Parisi G, Mistrello G, Musumeci M, Torrisi V, Musumeci S, Cuppari C. Serum YKL-40 in children with asthma. J Biol Regul Homeost Agents 2015;29:114-9

112- Leonardi S, Cuppari C, Lanzafame A, Attardo D, Tardino L, Parisi G, Giacchi V, Manti S, Arrigo T. Exhaled breath temperature in asthmatic children. J Biol Regul Homeost Agents 2015: 29: 47-54

113- https://ginasthma.org/. Accessed on 1th, April 2021

114- James AL, Elliot JG, Jones RL, et al. Airway Smooth Muscle Hypertrophy and Hyperplasia in Asthma. Am J Respir Crit Care Med. 2012;185(10):1058-1064. doi:10.1164/rccm.201110-1849OC

115- Kiley J, Smith R, Noel P. Asthma phenotypes. Curr Opin Pulm Med. 2007 Jan;13(1):19-23. doi: 10.1097/MCP.0b013e328011b84b. PMID: 17133120.

116- T, Oga T, Niimi A, et al. Relationship between Small Airway Function and Health Status, Dyspnea and Disease Control in Asthma. Respiration. 2010;80(2):120-126. doi:10.1159/000242113

117- de Gouveia Belinelo P, Nielsen A, Goddard B, et al. Clinical and lung function outcomes in a cohort of children with severe asthma. BMC Pulm Med. 2020;20(1):66. doi:10.1186/s12890-020-1101-6

118- van der Molen T, Postma DS, Martin RJ, et al. Effectiveness of initiating extrafine-particle versus fine-particle inhaled corticosteroids as asthma therapy in the Netherlands. BMC Pulm Med. 2016;16(1):80. doi:10.1186/s12890-016-0234-0

119- Nuttall AGL, Velásquez W, Beardsmore CS, Gaillard EA. Lung clearance index: assessment and utility in children with asthma. Eur Respir Rev. 2019;28(154):190046. doi:10.1183/16000617.0046-2019

120- Bongiovanni A, Parisi GF, Scuderi MG, Licari A, Brambilla I, Marseglia GL, Leonardi S. Gastroesophageal reflux and respiratory diseases: does a real link exist? Minerva Pediatr. 2019 Dec;71(6):515-523. doi: 10.23736/S0026-4946.19.05531-2. Epub 2019 May 23. PMID: 31129955.

121- Steinbacher M, Pfleger A, Schwantzer G, Jauk S, Weinhandl E, Eber E. Small airway function before and after cold dry air challenge in pediatric asthma patients during remission. Pediatr Pulmonol. 2017;52(7):873-879. doi:10.1002/ppul.23724

122- Vilmann L, Buchvald F, Green K, Nielsen KG. Fractional exhaled nitric oxide and multiple breath nitrogen washout in preschool healthy and asthmatic children. Respiratory Medicine. 2017;133:42-47. doi:10.1016/j.rmed.2017.10.027

123- Landier W, Bhatia S, Eshelman DA, et al. Development of Risk-Based Guidelines for Pediatric Cancer Survivors: The Children's Oncology Group Long-Term Follow-Up Guidelines From the Children's Oncology Group Late Effects Committee and Nursing Discipline. JCO. 2004;22(24):4979-4990. doi:10.1200/JCO.2004.11.032

124- Dietz AC, Chen Y, Ness KK, et al. Risk and impact of pulmonary complications in survivors of childhood cancer: A report from the Childhood Cancer Survivor Study (CCSS). JCO. 2014;32(15_suppl):10022-10022. doi:10.1200/jco.2014.32.15_suppl.10022

125- Landier W, Armenian SH, Lee J, et al. Yield of Screening for Long-Term Complications Using the Children's Oncology Group Long-Term Follow-Up Guidelines. JCO. 2012;30(35):4401-4408. doi:10.1200/JCO.2012.43.4951

126- Segura A, Yuste A, Cercos A, López-Tendero P, Gironés R, Pérez-Fidalgo JA, Herranz C. Pulmonary fibrosis induced by cyclophosphamide. Ann Pharmacother. 2001 Jul-Aug;35(7-8):894-7. doi: 10.1345/aph.10297. PMID: 11485142.

127- Cooper SL, Brown PA. Treatment of Pediatric Acute Lymphoblastic Leukemia. Pediatric Clinics of North America. 2015;62(1):61-73. doi:10.1016/j.pcl.2014.09.006

128- Parisi GF, Cannata E, Manti S, Papale M, Meli M, Russo G, Di Cataldo A, Leonardi S. Lung clearance index: A new measure of late lung complications of cancer therapy in children. Pediatr Pulmonol. 2020 Dec;55(12):3450-3456. doi: 10.1002/ppul.25071. Epub 2020 Sep 24. PMID: 32926567.

129- Verluys AB, Bresters D. Pulmonary complications of childhood cancer treatment. Paediatric Respiratory Reviews 2017; 17: 63-70.

130- Ewig S, Torres A, Riquelme R, El-Ebiary M, Rovira M, Carreras E, Raño A, Xaubet A. Pulmonary complications in patients with haematological malignancies treated at a respiratory ICU. Eur Respir J 1998; 12: 116-22.

131- Elbahlawan L, Rains KJ, Stokes DC. Respiratory care considerations in the childhood cancer patient. Respir Care 2017; 62: 765-775.

132- Landier W, Bhatia S, Eshelman DA, et al. Development of risk-based guidelines for pediatric cancer survivors: The Children's Oncology Group Long-Term Follow-Up Guidelines from the Children's Oncology Group Late Effects Committee and Nursing Discipline. J Clin Oncol 2004; 22: 4979-4990.

133- Segura A, Yuste A, Cercos A, et al. Pulmonary fibrosis induced by cyclophosphamide. Ann Pharmacother 2001; 35: 894–7.
134- Usemann J, Yammine S, Singer F, Latzin P. Inert gas washout: Background and application in various lung diseases. Swiss Med Wkly 2017; 16: 147.
135- Aurora P, Bush A, Gustafsson P, Oliver C, Wallis C, Price J, et al. London Cystic Fibrosis Collaboration. Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. Am J Respir Crit Care Med 2005; 171: 249–56
136- Hjalmarson O, Sandberg K. Abnormal lung function in healthy preterm infants. Am J Respir Crit Care Med 2002; 165: 83–7.
137- Fowler WS, Cornish ER, Jr, Kety SS. Lung function studies. VIII. Analysis of alveolar ventilation by pulmonary N2 clearance curves. J Clin Invest 1952; 31(1): 40–50.
138- Robinson PD, Latzin P, Verbanck S, Hall GL, Horsley A, Gappa M, et al. Consensus statement for inert gas washout measurement using multiple- and single-breath tests. Eur Respir J 2013; 41: 507–22.
139- Robinson PD, Latzin P, Ramsey KA, et al. Preschool Multiple-Breath Washout Testing. An Official American Thoracic Society Technical Statement. Am J Respir Crit Care Med 2018; 197: e1–e19.
140- Jensen R, Stanojevic S, Klingel M, et al. A systematic approach to multiple breath nitrogen washout test quality. PloS One 2016; 11: e0157523 10.1371/journal.pone.0157523
141- Lum S, Stocks J, Stanojevic S, et al. Age and height dependence of lung clearance index and functional residual capacity. Eur Respir J 2013; 41: 1371-1377.
142- Beydon N, Davis SD, Lombardi E, et al. An official American Thoracic Society/European Respiratory Society statement: Pulmonary function testing in preschool children. Am J Respir Crit Care Med 2007; 175: 1304-1345.
143- Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. Eur Respir J 2005; 26: 319-338.
144- Quanjer PH, Stanojevic S, Cole TJ, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: The global lung function 2012 equations. Eur Respir J 2012; 40: 1324-1343.
145- Matsuno O. Drug-induced interstitial lung disease: mechanisms and best diagnostic approaches. Respir Res 2012; 13: 39.
146- Dietz AC, Chen Y, Yasui Y, et al. Risk and impact of pulmonary complications in survivors of childhood cancer: A report from the Childhood Cancer Survivor Study. Cancer 2016; 122: 3687-3696.
147- Camus P, Fanton A, Bonniaud P, Camus C, Foucher P. Interstitial lung disease induced by drugs and radiation. Respiration 2004; 71: 301-26.
148- Myers JL, Limper AH, Swensen SJ. Drug-induced lung disease: A pragmatic classification incorporating HRCT appearances. Semin Respir Crit Care Med 2003; 24: 445-54.
149- Silva CI, Müller NL. Drug-induced lung diseases: most common reaction patterns and corresponding high-resolution CT manifestations. Semin Ultrasound CT MR 2006; 27: 111-6.

150- Pietra GG. Pathologic mechanisms of drug-induced lung disorders. J Thorac Imaging 1991; 6(1): 1-7.
151- Eigen H, Wyszomierski D. Bleomycin lung injury in children. Pathophysiology and guidelines for management. Am J Pediatr Hematol Oncol 1985; 7: 71–8.
152- Sleijfer S. Bleomycin-induced pneumonitis. Chest 2001; 120: 617–24.
153- Mulder RL, Thonissen NM, van der Pal HJ, et al. Pulmonary function impairment measured by pulmonary function tests in long-term survivors of childhood cancer. Thorax 2011; 66: 1065–1071.
154- Landier W, Armenian SH, Lee J, et al. Yield of screening for long-term complications using the children's oncology group long-term follow-up guidelines. J Clin Oncol 2012; 30: 4401-8.
155- Hudson MM, Ness KK, Gurney JG, et al. Clinical ascertainment of health outcomes among adults treated for childhood cancer. JAMA 2013; 309: 2371-2381.
156- Armenian SH, Landier W, Francisco L, et al. Long-term pulmonary function in survivors of childhood cancer. J Clin Oncol 2015; 33: 1592-600.
157- Dietz AC, Chen Y, Yasui Y, et al. Risk and impact of pulmonary complications in survivors of childhood cancer: A report from the Childhood Cancer Survivor Study. Cancer 2016; 122: 3687-3696.
158- Nysom K, Holm K, Olsen JH, Hertz H, Hesse B. Pulmonary function after treatment for acute lymphoblastic leukaemia in childhood. Br J Cancer 1998; 78: 21-27.
159- Miller RW , Fusner JE , Fink RJ, et al. Pulmonary function abnormalities in long-term survivors of childhood cancer. Med Pediatr Oncol 1986; 14: 202-207.
160- Jenney ME , Faragher EB , Jones PH , Woodcock A. Lung function and exercise capacity in survivors of childhood leukaemia. Med Pediatr Oncol 1995; 24: 222-230.
161- Lu Z, Foong RE, Kowalik K, et al. Ventilation inhomogeneity in infants with recurrent wheezing. Thorax. 2018;73(10):936-941. doi:10.1136/thoraxjnl-2017-211351
162- Racette C, Lu Z, Kowalik K, et al. Lung clearance index is elevated in young children with symptom-controlled asthma. Health Sci Rep. 2018;1(8):e58. Published 2018 Jun 19. doi:10.1002/hsr2.58
163- Kim YH, Shin HJ, Sol IS, et al. Lung Clearance Index and Quantitative Computed Tomography of Post-Infectious Bronchiolitis Obliterans in Infants. Sci Rep. 2017;7(1):15128. Published 2017 Nov 9. doi:10.1038/s41598-017-15330-8
164- Kapur N, Deegan S, Parakh A, Gauld L. Relationship between respiratory function and need for NIV in childhood SMA. Pediatr Pulmonol. 2019;54(11):1774-1780. doi:10.1002/ppul.24455
165- Yaacoby-Bianu K, Plonsky MT, Gur M, Bar-Yoseph R, Kugelman A, Bentur L. Effect of late preterm birth on lung clearance index and respiratory physiology in school-age children. Pediatr Pulmonol. 2019;54(8):1250-1256. doi:10.1002/ppul.24357

166- Singer F, Kieninger E, Abbas C, Yammine S, Fuchs O, Proietti E, Regamey N, Casaulta C, Frey U, Latzin P. Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. Pediatr Pulmonol. 2013 Aug;48(8):739-46. doi: 10.1002/ppul.22651. Epub 2012 Aug 8. PMID: 22888105.

167- Villa MP, Pagani J, Lucidi V, et al. Nocturnal oximetry in infants with cystic fibrosis. Arch Dis Child. 2001;84(1):50-4.

168- Urquhart DS, Montgomery H, Jaffé A. Assessment of hypoxia in children with cystic fibrosis. Arch Dis Child. 2005;90(11):1138-43.

169- Spicuzza L, Sciuto C, Leonardi S, et al. Early occurence of obstructive sleep apnea in infants and children with cystic fibrosis. Arch Pediatr Adolesc Med. 2012;166(12):1165-1169.

170- Milross MA, Piper AJ, Norman M, et al. Predicting sleep-disordered breathing in patients with cystic fibrosis. Chest. 2001;120:1239–1245

171- Isaiah A, Daher A, Sharma PB, et al. Predictors of sleep hypoxemia with cystic fibrosis. Pediatr Pulmonol. 2019;1-7.

172- Castro-Silva C de, Bruin VM de, Cavalcante AG, et al. Nocturnal hypoxia and sleep disturbances in cystic fibrosis. Pediatr Pulmonol. 2009;44(11):1143–1150.

173- Versteegh FG, Bogaard JM, Raatgever JW, et al. Relationship between airway obstruction, desaturation during exercise and nocturnal hypoxaemia in cystic fibrosis patients. Eur Respir J. 1990;3:68–73

174- Pleil JD. QQ-plots for assessing distributions of biomarker measurements and generating defensible summary statistics. J Breath Res. 2016 Aug 5;10(3):035001

175- Swets JA. Measuring the accuracy of diagnostic systems. Science 1998; 240: 1285-93.

176- Youden WJ. Index for rating diagnostic tests. Cancer. 1950;3:32–35

177- Montgomery ST, Mall MA, Kicic A, et al. Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. Eur Resp J. 2017;49:1600903

178- Schaible B, Taylor CT, Schaffer K. Hypoxia increases antibiotic resistance in Pseudomonas aeruginosa through altering the composition of multidrug efflux pumps. Antimicrob Agents Chemother. 2012;56(4):2114-8.

179- Uyan ZS, Ozdemir N, Ersu R, et al. Factors that correlate with sleep oxygenation in children with cystic fibrosis. Pediatr Pulmonol. 2007;42(8):716–722.

180- Perin C, Fagondes SC, Casarotto FC, et al. Sleep findings and predictors of sleep desaturation in adult cystic fibrosis patients. Sleep Breath. 2012;16(4):1041–1048.

181- Amin R, Bean J, Burklow K, et al.. The relationship between sleep disturbance and pulmonary function in stable pediatric cystic fibrosis patients. Chest. 2005;128(3):1357–1363.

182- Hart N, Polkey MI, Clément A, et al. Changes in pulmonary mechanics with increasing disease severity in children and young adults with cystic fibrosis. Am J Respir Crit Care Med. 2002;166(1):61-6

183- Sonneveld N, Stanojevic S, Amin R, et al. Lung clearance index in cystic fibrosis subjects treated for pulmonary exacerbations. Eur Respir J. 2015;46(4):1055-64

184- Fauroux B, Pepin JL, Boelle PY, et al. Sleep quality and nocturnal hypoxaemia

and hypercapnia in children and young adults with cystic fibrosis. Arch Dis Child. 2012;97(11):960-6.
185- Stahl M, Wielputz MO, Graeber SY, et al. Comparison of lung clearance index and magnetic resonance imaging for assessment of lung disease in children with cystic fibrosis. Am J Respir Crit Care Med 2017; 195:349–359.
186- Kanhere N, Couch MJ, Kowalik K, et al. Correlation of lung clearance index with hyperpolarized (129)Xe magnetic resonance imaging in pediatric subjects with cystic fibrosis. Am J Respir Crit Care MedV 196 2017; 1073–1075.
187- Smith L, Marshall H, Aldag I, et al. Longitudinal assessment of children with mild cystic fibrosis using hyperpolarized gas lung magnetic resonance imaging and lung clearance index. Am J Respir Crit Care Med 2018; 197: 397–400.
188- Korten I, Kieninger E, Yammine S, Respiratory rate in infants with cystic fibrosis throughout the first year of life and association with lung clearance index measured shortly after birth J Cyst Fibros. 2019 Jan;18(1):118-126
189- Davies J, Sheridan H, Bell N, et al. Assessment of clinical response to ivacaftor with lung clearance index in cystic fibrosis patients with a G551D-CFTR mutation and preserved spirometry: a randomised controlled trial. Lancet Respir Med. 2013 Oct;1(8):630-638. doi: 10.1016/S2213-2600(13)70182-6. Epub 2013 Sep 10
190- Kane M, Gonska T, Jensen R et al. Lung clearance index response in patients with CF with class III CFTR mutations. Thorax. 2016 May;71(5):476-7. doi: 10.1136/thoraxjnl-2015-207894. Epub 2016 Mar 4.
191- Ratjen F, Hug C, Marigowda G et al. Efficacy and safety of lumacaftor and ivacaftor in patients aged 6-11 years with cystic fibrosis homozygous for F508del-CFTR: a randomised, placebo-controlled phase 3 trial. Lancet Respir Med. 2017 Jul;5(7):557-567. doi: 10.1016/S2213-2600(17)30215-1. Epub 2017 Jun 9.
192- Ratjen F, Klingel M, Black P et al. Changes in Lung Clearance Index in Preschool-aged Patients with Cystic Fibrosis Treated with Ivacaftor (GOAL): A Clinical Trial. Am J Respir Crit Care Med. 2018 Aug 15;198(4):526-528. doi: 10.1164/rccm.201802-0243LE.
193- Moran F, Bradley JM, Piper AJ. Non-invasive ventilation for cystic fibrosis. Cochrane Database Syst Rev 2017;2:CD002769.
194- Fauroux B, Le Roux E, Ravilly S, Bellis G, Clément A. Long-term noninvasive ventilation in patients with cystic fibrosis. Respiration 2008;76(2):168-74.
195- Archangelidi O, Carr SB, Simmonds NJ, Bilton D, Banya W, Cullinan P; CF-EpiNet. Non-invasive ventilation and clinical outcomes in cystic fibrosis: Findings from the UK CF registry. J Cyst Fibros 2019;18(5):665-670
196- Farrell PM, White TB, Ren CL, Hempstead SE, Accurso F, Derichs N, Howenstine M, McColley SA, Rock M, Rosenfeld M, Sermet-Gaudelus I, Southern KW, Marshall BC, Sosnay PR. Diagnosis of Cystic Fibrosis: Consensus Guidelines from the Cystic Fibrosis Foundation. J Pediatr 2017;181S:S4-S15.e1
197- Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, Rosenstein BJ, Smith AL, Wohl ME; The Pulmozyme Study Group. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and

on pulmonary function in patients with cystic fibrosis. N Engl J Med. 1994;331:637–642

198- Kapur VK, Auckley DH, Chowdhuri S, Kuhlmann DC, Mehra R, Ramar K, Harrod CG. Clinical Practice Guideline for Diagnostic Testing for Adult Obstructive Sleep Apnea: An American Academy of Sleep Medicine Clinical Practice Guideline. J Clin Sleep Med 2017;(3):479-504

199- Buysse DJ, Reynolds CF 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res 1989;28(2):193-213.

200- Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, Hallstrand TS, Kaminsky DA, McCarthy K, McCormack MC, Oropez CE, Rosenfeld M, Stanojevic S, Swanney MP, Thompson BR. Standardization of Spirometry 2019 Update. An Official American Thoracic Society and European Respiratory Society Technical Statement. Am J Respir Crit Care Med 2019;200(8):e70-e88

201- Foong RE, Harper AJ, Skoric B. The clinical utility of lung clearance index in early cystic fibrosis lung disease is not impacted by the number of multiple-breath washout trials. ERJ Open Res 2018;4(1):00094-2017

202- Pleil JD. QQ-plots for assessing distributions of biomarker measurements and generating defensible summary statistics. J Breath Res 2016;10(3):035001

203- Madden BP, Kariyawasam H, Siddiqi AJ, Machin A, Pryor JA, Hodson ME. Noninvasive ventilation in cystic fibrosis patients with acute or chronic respiratory failure (published correction appears in Eur Respir J. 2002 Sep;20(3):790.). Eur Respir J 2002;19(2):310–313

204- Abbott J, Morton AM, Hurley MA, Conway SP. Longitudinal impact of demographic and clinical variables on health-related quality of life in cystic fibrosis. BMJ Open 2015;5(5):e007418.

205- Clini E, Sturani C, Porta R, et al. Outcome of COPD patients performing nocturnal non-invasive mechanical ventilation. Respir Med 1998;92(10):1215–1222

206- Flight WG, Shaw J, Johnson S, et al. Long-term non-invasive ventilation in cystic fibrosis - experience over two decades. J Cyst Fibros 2012;11(3):187–192

207- Nalbandian A, Sehgal K, Gupta A, Madhavan MV, McGroder C, Stevens JS, Cook JR, Nordvig AS, Shalev D, Sehrawat TS, Ahluwalia N, Bikdeli B, Dietz D, Der-Nigoghossian C, Liyanage-Don N, Rosner GF, Bernstein EJ, Mohan S, Beckley AA, Seres DS, Choueiri TK, Uriel N, Ausiello JC, Accili D, Freedberg DE, Baldwin M, Schwartz A, Brodie D, Garcia CK, Elkind MSV, Connors JM, Bilezikian JP, Landry DW, Wan EY. Post-acute COVID-19 syndrome. Nat Med. 2021 Apr;27(4):601-615. doi: 10.1038/s41591-021-01283-z. Epub 2021 Mar 22. PMID: 33753937.

208- National Institute for Health and Care Excellence, Practitioners RC of G, Scotland HI. COVID-19 rapid guideline: managing the longterm effects of COVID-19. NICE Guidelines; 2020:1-35.

209- Lopez-Leon S, Wegman-Ostrosky T, Perelman C, Sepulveda R, Rebolledo PA, Cuapio A, Villapol S. More than 50 Long-term effects of COVID-19: a systematic

review and meta-analysis. medRxiv [Preprint]. 2021 Jan 30:2021.01.27.21250617. doi: 10.1101/2021.01.27.21250617. PMID: 33532785; PMCID: PMC7852236.
210- Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. Acta Paediatr. 2020 Jun;109(6):1088-1095. doi: 10.1111/apa.15270. Epub 2020 Apr 14. PMID: 32202343; PMCID: PMC7228328.
211- Parisi GF, Brindisi G, Indolfi C, Diaferio L, Marchese G, Ghiglioni DG, Zicari AM, Miraglia Del Giudice M. Upper airway involvement in pediatric COVID-19. Pediatr Allergy Immunol. 2020 Nov;31 Suppl 26(Suppl 26):85-88. doi: 10.1111/pai.13356. PMID: 33236430; PMCID: PMC7753446.
212- Parisi GF, Indolfi C, Decimo F, Leonardi S, Miraglia Del Giudice M. COVID-19 Pneumonia in Children: From Etiology to Management. Front Pediatr. 2020 Dec 14;8:616622. doi: 10.3389/fped.2020.616622. PMID: 33381482; PMCID: PMC7767924.
213- Ludvigsson JF. Case report and systematic review suggest that children may experience similar long-term effects to adults after clinical COVID-19. Acta Paediatr. 2021 Mar;110(3):914-921. doi: 10.1111/apa.15673. Epub 2020 Dec 3. PMID: 33205450; PMCID: PMC7753397.
214- Buonsenso D, Munblit D, De Rose C, Sinatti D, Ricchiuto A, Carfi A, Valentini P. Preliminary evidence on long COVID in children. Acta Paediatr. 2021 Jul;110(7):2208-2211. doi: 10.1111/apa.15870. Epub 2021 Apr 18. PMID: 33835507.
215- Ludvigsson JF. Reporting suspicions of long COVID in children is justified during this global emergency. Acta Paediatr. 2021 Apr;110(4):1373. doi: 10.1111/apa.15762. Epub 2021 Jan 28. PMID: 33459404; PMCID: PMC8013867.
216- Brackel CLH, Lap CR, Buddingh EP, van Houten MA, van der Sande LJTM, Langereis EJ, Bannier MAGE, Pijnenburg MWH, Hashimoto S, Terheggen-Lagro SWJ. Pediatric long-COVID: An overlooked phenomenon? Pediatr Pulmonol. 2021 Jun 8. doi: 10.1002/ppul.25521. Epub ahead of print. PMID: 34102037.
217- Wostyn P. COVID-19 and chronic fatigue syndrome: Is the worst yet to come? Med Hypotheses. 2021 Jan;146:110469. doi: 10.1016/j.mehy.2020.110469. Epub 2021 Jan 2. PMID: 33401106; PMCID: PMC7836544.
218- Cortes Rivera M, Mastronardi C, Silva-Aldana CT, Arcos-Burgos M, Lidbury BA. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Comprehensive Review. Diagnostics (Basel). 2019 Aug 7;9(3):91. doi: 10.3390/diagnostics9030091. PMID: 31394725; PMCID: PMC6787585.
219- Clayton EW. Beyond myalgic encephalomyelitis/chronic fatigue syndrome: an IOM report on redefining an illness. JAMA. 2015 Mar 17;313(11):1101-2. doi: 10.1001/jama.2015.1346. PMID: 25668027.
220- Proal A, Marshall T. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome in the Era of the Human Microbiome: Persistent Pathogens Drive Chronic Symptoms by Interfering With Host Metabolism, Gene Expression, and Immunity. Front Pediatr. 2018 Dec 4;6:373. doi: 10.3389/fped.2018.00373. PMID: 30564562; PMCID: PMC6288442.

221- Lam MH, Wing YK, Yu MW, Leung CM, Ma RC, Kong AP, So WY, Fong SY, Lam SP. Mental morbidities and chronic fatigue in severe acute respiratory syndrome survivors: long-term follow-up. Arch Intern Med. 2009 Dec 14;169(22):2142-7. doi: 10.1001/archinternmed.2009.384. PMID: 20008700.
222- Moldofsky H, Patcai J. Chronic widespread musculoskeletal pain, fatigue, depression and disordered sleep in chronic post-SARS syndrome; a case-controlled study. BMC Neurol. 2011 Mar 24;11:37. doi: 10.1186/1471-2377-11-37. PMID: 21435231; PMCID: PMC3071317.
223- Lidbury BA. Ross River Virus Immune Evasion Strategies and the Relevance to Post-viral Fatigue, and Myalgic Encephalomyelitis Onset. Front Med (Lausanne). 2021 Mar 24;8:662513. doi: 10.3389/fmed.2021.662513. PMID: 33842517; PMCID: PMC8024622.
224- Lipton JO, Sahin M. The neurology of mTOR. Neuron. 2014 Oct 22;84(2):275-91. doi: 10.1016/j.neuron.2014.09.034. Epub 2014 Oct 22. PMID: 25374355; PMCID: PMC4223653.
225- Kennedy BK, Lamming DW. The Mechanistic Target of Rapamycin: The Grand ConducTOR of Metabolism and Aging. Cell Metab. 2016 Jun 14;23(6):990-1003. doi: 10.1016/j.cmet.2016.05.009. PMID: 27304501; PMCID: PMC4910876.