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# *MONITORING BACTERIAL RESERVOIRS TO PREVENT CHRONIC LUNG INFECTION IN CYSTIC FIBROSIS*

S.S.D. MED/07

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Monitoring bacterial reservoirs to prevent chronic lung infection in cystic fibrosis
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## ABSTRACT

Cystic fibrosis (CF) is a disease caused by mutations in the CFTR (CF transmembrane conductance regulator) gene and associated with severe damage to the lungs and the gastrointestinal system. One of the most important challenges in CF is treating and solving chronic lung infections which are the major cause of morbidity and mortality in patients in that once bacteria are established in the lung; eradication is rarely successful.

The main objective of this study has been to evaluate the possible role of nasal/paranasal sinuses, saliva, and toothbrush as bacterial reservoirs in the development of chronic pulmonary infection and to compare the molecular profile of bacterial strains isolated from different sites in the body to confirm they circulate within the airways and oral cavity.

Differently from previous works, our study was not limited to *P. aeruginosa* and *S. aureus* (the most important CF pathogens) but also included other potentially pathogenic emerging bacterial species that are frequently isolated from the sputum of CF subjects but still lack a clear clinical role in CF. Moreover, in this study, we have considered not only the classical sputum sample but also nasal lavage, saliva, and toothbrushes and have monitored a significant number of patients longitudinally.

We evaluated the presence of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Serratia marcescens* in sputum and nasal lavage of 59 CF patients and also in saliva samples and used toothbrushes from 38 of them. At the end of the study, 190 sputum samples, 189 nasal lavage samples, 79 samples of saliva, and 87 toothbrushes were obtained and examined recovering and analyzing a total of about 1000 bacterial strains.

Patients were classified by age (adult and paediatric) and by stage of lung colonisation with *P. aeruginosa* at enrolment (chronic, occasional). Based on these criteria, patients were divided into four groups: adults with chronic *P. aeruginosa* lung infection (AC), paediatrics with occasional *P. aeruginosa* colonisation (PO), adults and paediatrics free from *P. aeruginosa* (AN and PN, respectively).

About 80% of the patients were positive for at least one of the bacterial species examined in nasal lavage and sputum. Among the subjects with positive sputum, 74%

presented the same species in the nasal lavage and saliva and 26% on their toothbrush. *S. aureus* was the most abundant species detected in all samples.

Clonal identity (≥80% similarity) of the strains isolated among the different samples from each patient was confirmed in almost all cases indicating that most of them belong to single clones circulating in the upper and lower airways, the oral cavity, and in some cases, in the toothbrush too.

We concluded that nasal and oral sites act as bacterial reservoirs favoring transmission of pathogenic and potentially pathogenic microorganisms to the lower airways and vice-versa. It can be deduced that only the eradication of the microorganism from the reservoirs might avoid a lung chronic infection.

Based on the results obtained, it has been established an understanding with the clinician from the Cystic Fibrosis Centre of Verona of the need of eradicating bacterial reservoirs. A specific protocol for the monitoring of the analyzed bacterial reservoirs and the eradication of the microorganisms present to prevent lung infection, was proposed, and accepted by them.

# TABLE OF CONTENTS

## 1. ABBREVIATIONS

CF=Cystic Fibrosis

CFTR= transmembrane conductance regulator

cAMP=cyclic AMP regulated chloride channel

MSDs=membrane-spanning domains

NBD1 and NBD2=nucleotide-binding domains

RD=regulatory domain

TMDs=transmembrane domains

PKA=protein kinase A

DF508=position 508 of CFTR protein

ENaC=sodium epithelial channels

ATPase= triphosphatase

mRNA=messenger RNA

NBS=newborn screening

IRT= immunoreactive trypsinogen

BMI=body mass index

FEV1=forced expiratory volume in the first second

$FEF_{25-75\%}$=midexpiratory phase

$FEV_{0.75}$=forced expiratory volume in 0.75 second

FCV=forced vital capacity

IL=interleukin

TNFα=tumor necrosis factor-alpha

PMNs=polymorphonuclear cell family

QS=quorum-sensing

AIs=molecules autoinducers

T3SS=type III secretion systems

ExoU=exoenzyme U

ROS=reactive oxygen species

LAW=lower airways

UAW=upper airways

PRPs=potential respiratory pathogens

MALDI-TOF=matrix-assisted laser desorption ionization-time of flight

MS=mass spectrometry

PFGE=pulsed field gel electrophoresis

CHEF=contour-clamped homogeneous electric field electrophoresis

PAE=*Pseudomonas aeruginosa*

SAU=*Staphylococcus aureus*

SMA=*Serratia marcescens*

PMA=*Stenotrophomonas maltophilia*

AXY=*Achromobacter xylosoxidans*

CA=*Candida albicans*

AC=adult patients with chronic infection by PAE

PO=paediatric patients with occasional PAE

BALF=bronchoalveolar lavage fluid

MRSA=methicillin-resistant *S. aureus*

SCVs=small-colony variants of *S. aureus*

NFB=non glucose fermenter

BCSA=*Burkholderia cepacia* selective agar

LPS=lipopolysaccharide

AN=adult free from PAE lung colonisation

PN=paediatric patients free from PAE lung colonisation

BHI=Brain Heart Infusion

CHCA=cyano-4-hydroxycinnamic acid matrix

ASC=advanced spectrum classifier

## 2. RATIONAL AND OBJECTIVE OF THE STUDY

Aim of this study was:

- To evaluate the prevalence of pathogenic/potentially pathogenic bacterial species in the upper airways and lower airways of patients with cystic fibrosis (CF).
- To evaluate the prevalence of pathogenic/potentially pathogenic bacterial species in other possible infection reservoirs such as saliva and toothbrushes of the same CF patients.
- To compare the molecular profile (pulsed-field gel electrophoresis) of bacterial strains isolated from different sites to confirm their circulate within the airways an oral cavity and possible cross-colonisation
- To confirm the possibility that saliva and toothbrushes may be sources of oral contamination and act as reservoirs favoring the transmission of potentially pathogenic microorganisms from the environment to the oral cavity and eventually to the upper and lower airways.
- To evaluate the clinical importance of individuating, monitoring, and eradicating bacterial reservoirs in CF.

# 3. INTRODUCTION

## 3.1 Cystic Fibrosis: History, overview, and epidemiology

Cystic fibrosis (CF) is the most common lethal genetic disorder amongst Caucasians with a current life expectancy of about 40 years. CF is an autosomal recessive hereditary disease associated with a gene located in the long arm of chromosome 7, locus q31. The gene consists of 250 Kb, presents 27 coding sequences (exons), and transcribes a long ribonucleic acid (mRNA) with 6.5 Kb and an ion transmembrane transport regulatory protein, or chloride channel regulator known as the CF transmembrane conductance regulator (CFTR)[1, 2] (Figure 1) .

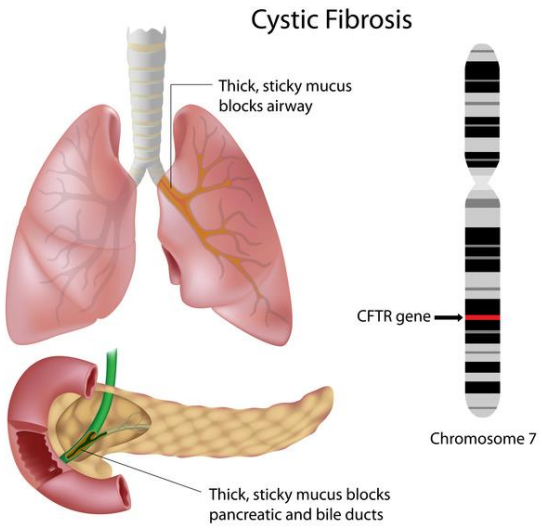


Figure 1. Location of the *CFTR gene* on chromosome 7 [3]

The term CF applies to patients with a defect in the CFTR protein (a central role in regulating ionic transport across the cell membrane), which is expressed in many epithelial cells, including sweat ducts, airways, pancreatic duct, intestine, gallbladder, and vas deferens, which can give rise to high concentration of sweat chloride, lung disease characterized by repeated bacterial infections and bronchiectasis, pancreatic insufficiency, intestinal obstruction, biliary cirrhosis, and congenital bilateral absence of the vas deferens in the reproductive tract, often in combination [4]. Improvements in care resulting from a greater understanding of the clinical manifestations and

pathophysiology of the disease resulted in solid improvements in survival. Striking discoveries, such as the identification of the *CFTR* gene, led to the development of therapies targeting specific areas of the pathophysiological pathway.

When CF was first described in the 1930s, patients rarely survive beyond the first months of life and early childhood [5]. Although the CF Registry data 2018 showed that the life expectancy of people with CF who are born between 2014 and 2018 is predicted to be 44 years. Data also show that of the babies who are born in 2018, half are predicted to live to be 47 years or older [6]

The incidence in Europe is 1 in 2000-3000 newborns are affected with CF. Even where populations appear relatively homogeneous, there may be marked local and regional variations. In France, for example, there is a very high incidence of CF in Northwest Brittany and a lower incidence in the South [7]. In Italy Veneto, there is the most prevalence of CF patients (9.6 out of 100.000 patients) [8]. Although CF is usually diagnosed in childhood (in the first year of life in 70% of cases), the number of CF cases diagnosed in adolescence and adulthood increased with the result of a greater level of clinical suspicion and availability of diagnostic techniques [9]. In 2018, there were 30,775 individuals with CF in the Registry [6]

The establishment of CF as a disease dates from medieval times, according to a centuries-old European legend, children that tasted salty when kissed on the forehead were considered “enchanted” or “bewitched” and would soon die. Over the centuries, reported cases describe patients with clinical features of CF. However, the first complete pathological description of the CF is attributed to Dorothy Andersen, in 1938, referring to it as “CF of the pancreas” [10]. In mid-1945, the cause of the disease was believed to be a "generalized state of thickening of secretions", thus taking the term mucoviscidosis [4]. In 1946, they discovered that the disease had a genetic origin and resulted from an autosomal recessive mutation and in 1948, Paul di Sant’Agnese observed that many newborns and children presented a high incidence of “prostration” and these children had abnormal sweat, with high concentrations of sodium and chloride [11]. The sweat chloride test was standardized by Gibson & Cooke in 1959 and is still considered the gold standard for diagnosing the disease [12]. Finally, in the 1980s, the CF gene (cftr) was discovered and described as encoding the CFTR protein, a cyclic AMP regulated chloride channel (cAMP) [13].

Currently, the term CF applies to patients with a defect in the CFTR protein, a phosphorylation-dependent epithelial $Cl^-$ channel. It is located primarily in the apical membrane, where it provides a pathway for $Cl^-$ movement across epithelia and regulates the rate of $Cl^-$ flow. Thus, CFTR is central in determining transepithelial salt transport, fluid flow, and ion concentrations. In the intestine, pancreas, and sweat gland secretory coil, CFTR plays a key role in fluid and electrolyte secretion, and in sweat gland duct and airway epithelia, it participates in fluid and electrolyte absorption. Dysfunction of CFTR $Cl^-$ channels in the genetic disease CF disrupts transepithelial ion transport and hence the function of a variety of organs lined by epithelia[14]. This leads to the wide-ranging manifestations of the disease, which can include airway disease, pancreatic failure, meconium ileus, male infertility, and elevated levels of salt in sweat.

## 3.2 Protein CFTR

CFTR is a member of a large family of membrane transport proteins, the ATP-binding cassette (ABC) transporters, composed of 1480 amino acids and has a molecular weight of 168,173 Da is a cAMP-activated chloride (Cl-) channel located on the apical membranes of epithelial cells. In many tissues, such as the airways, sweat glands, pancreas cells, bile ducts, and genital ducts, the CFTR protein contributes to the regulation of normal physiological function (175,176).

In the lungs, hydration of mucus is maintained by chloride secretion and sodium absorption that reveal a structural homology to a family of transport proteins termed the transport ATPases. It is expressed in various tissues of the human organism, where it plays a key role in controlling ionic transport across cell membranes on mucous surfaces [15].

The CFTR structure is comprised of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBD1 and NBD2), and a regulatory domain (RD) [16] (Figure 1). The NBDs and RD are situated in the cytoplasm, while the MSDs form the main pore of the chloride channel. Both the RD and NBDs are involved in regulating the channel activity. The first stage consists of

phosphorylation of the RD by a cAMP-dependent protein kinase. This phosphorylation allows the binding of ATP to NBD1. When ATP is hydrolyzed by NBD1 the channel opens and the anions pass according to the electrochemical gradient, through the pore formed by the transmembrane domains (TMDs). When domain R is completely phosphorylated, NBD2 can bind ATP. This connection allows the stabilization of the open channel. When ATP is hydrolyzed by NBD2 and ADP + Pi is released from both NBDs, the channel will close again [16, 17].

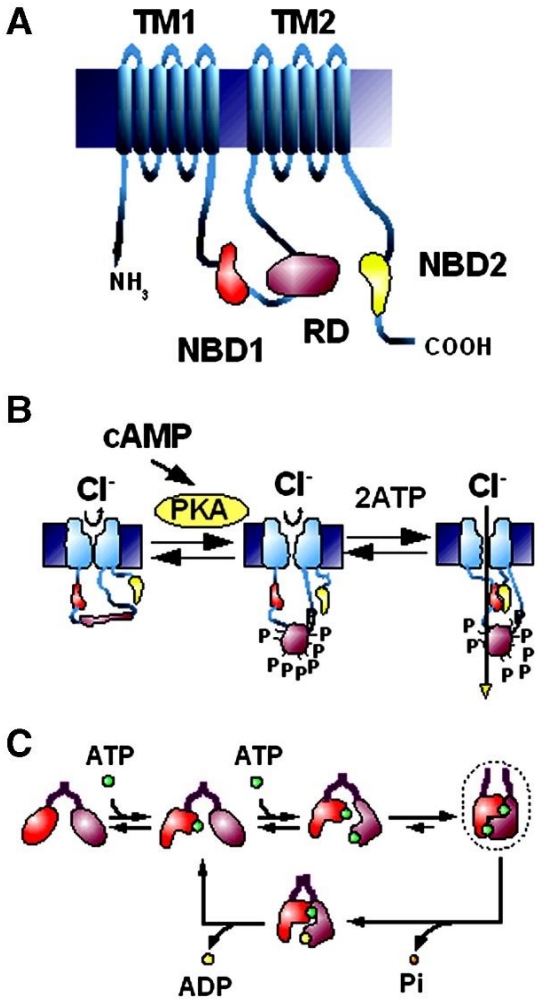


Figure 2. Topology of the CFTR inserted in the cell membrane. The two transmembrane domains, TM1 and TM2, are composed of 6 membrane spanning helices each; the two nucleotide binding domains, NBD1 and NBD2, are located in the c-terminus of each TMD, in the cytoplasm, the regulatory domain, RD, joins NBD1 to TMD2. B: The functional cycle of CFTR. Protein kinase A (PKA) is activated by the cytoplasmic cAMP, and catalyzes the phosphorylation of the RD of inactive CFTR, promoting a conformational transition that leads to active CFTR. The binding of ATP to the NBDs induces a second conformational change, that gates the channel to the open – chloride permeable – conformation. C: The gating cycle of the NBDs of CFTR initiates with the successive binding of two ATP molecules, which induces the dimerization of these domains, by a quasi-irreversible transition, allowing the channel to open. The ATPase activity of the NBDs brings on the hydrolysis of one ATP, and the consequent destabilization of the NBD1–NBD2 interaction, that causes the closure of the channel and the release of an ADP [18]

CFTR is essential for the transport of ions across the cell membrane, being involved in regulating the flow of Cl, Na, and water. A few thousand mutations have been described in the CF gene, but the most frequent is due to a deletion of three base

pairs, causing the loss of an amino acid (phenylalanine) at position 508 (DF508) of the CFTR protein, which prevents its functioning appropriate. Approximately 70% of the CF chromosomes in northern Europe have the ΔF508 mutation [19].

The cells of the ciliated epithelium express the sodium epithelial channels (ENaC) and Na + / K + - triphosphatase (ATPase) pumps to mediate the transcellular absorption of Na +. Ciliated cells also express chloride channels in the apical portion (CFTR and calcium-activated chloride channels) and Na + - K + - 2Cl- co-transporters in the basolateral portion, to secrete chloride when ENaCs are blocked and chloride secretory forces are formed. Physiologically, there is passive reabsorption of sodium and chloride ions from the light of the exocrine glands, decreasing the loss of electrolytes in the secretions. The key molecule in the epithelial transport of chloride ions is CFTR. The main function of CFTR in the channels of the sweat glands is to reabsorb the luminous chloride ions and increase the reabsorption of sodium via sodium channels. Therefore, in the sweat channels, the loss or reduction of CFTR function induces a decrease in sodium chloride absorption, consequently a decrease in sodium absorption, and hypertonic sweat production [18, 20, 21].

In the respiratory and intestinal epithelia, the CFTR channel forms one of the most important pathways for active luminal chloride secretion. In these places, CFTR mutations result in the loss or reduction of chlorine secretion into the fire. The active absorption of luminal sodium is also increased (due to the loss of sodium channel inhibiting activity-ENaC) and both of these ion changes increase the passive absorption of water by the fire, lowering the water content of the superficial fluid layer that lines mucosal cells. Therefore, differently from what happens in the sweat channels, there is no difference in the salt concentration of the superficial liquid layer of the respiratory and intestinal mucous cells in normal individuals versus those with CF [22, 23] (Figure 3).

In this scenario, the result is dehydration of mucous secretions and an increase in their viscosity, favoring the obstruction of the ducts, accompanied by an inflammatory reaction and subsequent fibrosis process. Thus, cells from various organs are affected, although not all individuals express similar clinical responses, with a wide range of symptoms from patient to patient.

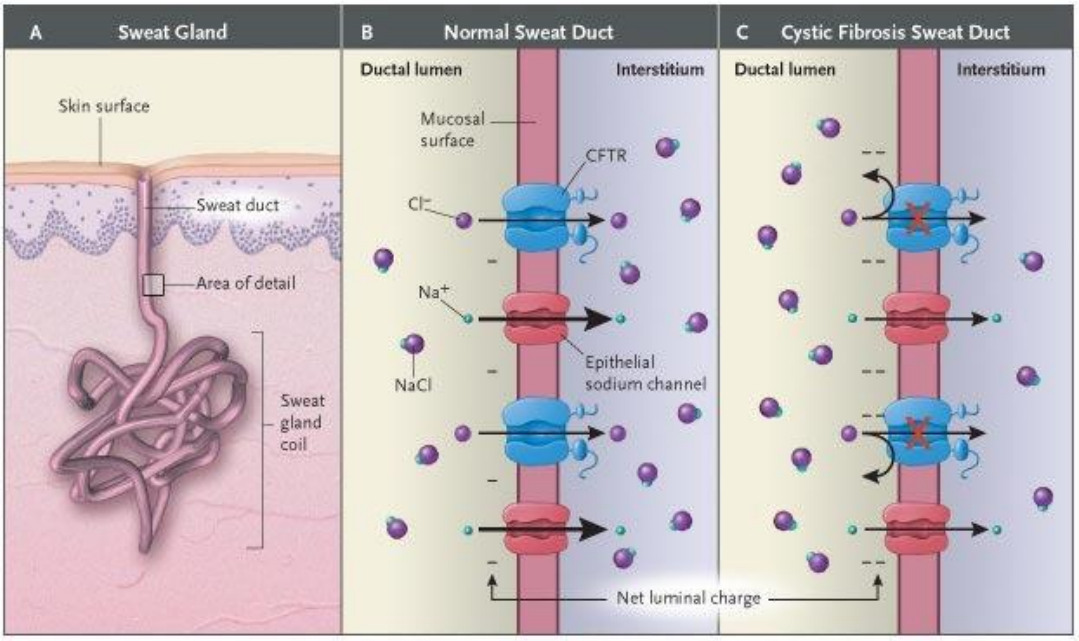


Figure 3. Mechanism Underlying Elevated Sodium Chloride Levels in the Sweat of Patients with CF. Sweat ducts (Panel A) in patients with CF differ from those in people without the disease in the ability to reabsorb chloride before the emergence of sweat on the surface of the skin. A major pathway for $Cl^-$ absorption is through CFTR, situated within luminal plasma membranes of cells lining the duct (i.e., on the apical, or mucosal, cell surface) (Panel B). Diminished chloride reabsorption in the setting of continued sodium uptake leads to an elevated transepithelial potential difference across the wall of the sweat duct, and the lumen becomes more negatively charged because of a failure to reabsorb chloride (Panel C). The result is that total sodium chloride flux is markedly decreased, leading to increased salt content. The thickness of the arrows corresponds to the degree of movement of ions[16]

### 3.3 Prevalence of the Most common CFTR Mutations

Under normal circumstances, extracellular signals lead to the expression of the *CFTR gene* to transcription to generate messenger RNA (mRNA), then migrates through nuclear pores to interact with ribosomes in the cytoplasm or the endoplasmic reticulum. In combination with transfer RNA, the *CFTR gene* is translated into nascent chains of amino acids. The resulting polypeptides are assembled into the immature CFTR protein product, which is subsequently folded within the lipid bilayer of the endoplasmic reticulum. Continuing the maturation, CFTR protein is then transferred to the Golgi apparatus for post-translational modification and packaging into transport

vesicles. Finally, the channel is transported or “trafficked” to the cell surface for final expression on the apical membrane of epithelial cells [24].

In 2018, more than 1,800 mutations have been found in the *CFTR gene*. Some mutations result in virtually no CFTR function and others are associated with some residual function. Various strategies have been used to categorize mutations with the goal of grouping individuals with CF with a similar prognosis. The first classification of CF mutations into four classes according to their primary biological defect was proposed by Welsh and Smith in a landmark paper [25]. Currently, six major classes are distinguished [16] [26]. This classification system helps understand the impact of mutations on the biosynthesis and function of the CFTR protein. However, it is increasingly recognized that this classification schema is an oversimplification. The figure below (Figure 4) illustrates the complexity in grouping mutations into distinct classes. Note that many mutations lead to more than one defect in the CFTR function. [6].

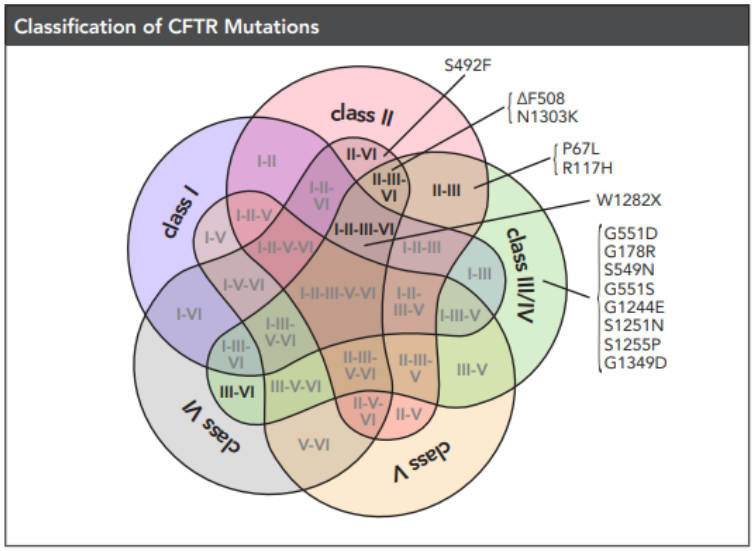


Figure 4. This diagram indicates all combinations of mutation classes with selected examples. Possible combinations without identified mutation are indicated in gray [5].

The variants in the *CFTR gene* were divided into six (with subdivision of class 1 into A and B) or seven classes according to the following criteria (Figure 5): (a) association with the phenotype of greater severity of the disease, (b) alterations in the DNA, (c) impact on the alteration of the CFTR protein, (d) structural and functional

alterations of the CFTR protein and (e) availability and type of drugs available for precision medicine. These are the most recent classification criteria for CFTR variants [27-30]. The classes are listed below:

Class I mutations, such as G542X, R553x, W1282X, and R1162X, result in the near or complete absence of CFTR at the epithelial surface [6, 31]. Class I mutations are divided in:

- Class IA mutation or class VII according to the new classification by De Boeck and Amaral [28] (no mRNA transcription): keeping mRNA from being synthesized. If the CFTR promoter contains a mutation, it could cause the RNA polymerase to not bind to DNA and therefore not copy the message into the mRNA. The result is that no CFTR proteins are being produced.
- Class IB mutation (no protein). In this class of mutations, the CFTR mRNA is produced but is damaged and cannot be made into protein. Sometimes, because of a mutation, one of these stop sequences appears too early in the mRNA. This results in the production of a shortened version of the CFTR protein, which is then degraded by the cell.
- Class II mutation (no traffic): is associated with defective processing due to misfolding of the protein. In this class of mutations, the CFTR protein is made but fails to reach the cell membrane. The misfolded protein is retained in the endoplasmic reticulum and re-translocated to the cytoplasm, where it is degraded by the ubiquitin/proteasome pathway. This class includes the most common mutation, p.Phe508del [32, 33]. To correct the misfolded proteins and help them reach the cell membrane, treatments called CFTR correctors can be used [31].
- Class III mutation (impaired gating): another type of mutation can result in the production of a CFTR protein that makes it to the cell membrane but does not open correctly. This is often referred to as a "gating defect." an example is G551D which accounts for 2–3% of CFTR mutations worldwide. Treatments called CFTR potentiators can be used to open the channels and/or keep them open for longer [31, 34].
- Class IV mutation (decreased conductance); This mutation results in a CFTR protein that makes it to the cell membrane and reacts to cell signaling are able to open and close; however, chloride and bicarbonate ions are unable to freely pass through

the channel due to conductance defects. CFTR potentiators can also be helpful for these mutations to keep the channels open for longer to allow more chloride ions to flow through [33, 34].

- Class V mutation (less protein). Sometimes a mutation can lead to CFTR protein being produced but just not in sufficient amounts, because of the process affected by pre-mRNA splicing in which correct versions of the protein can lead to the production but more often incorrect versions are produced, results in a reduced quantity of the protein being produced. Possible treatments for this type of mutation include CFTR corrector, potentiators, CFTR amplifiers or antisense oligonucleotides [33, 34].
- Class VI mutation (less stable protein). The final type of mutation is missense mutations which result in decreased CFTR stability at the membrane leading to decreased CFTR function[35].

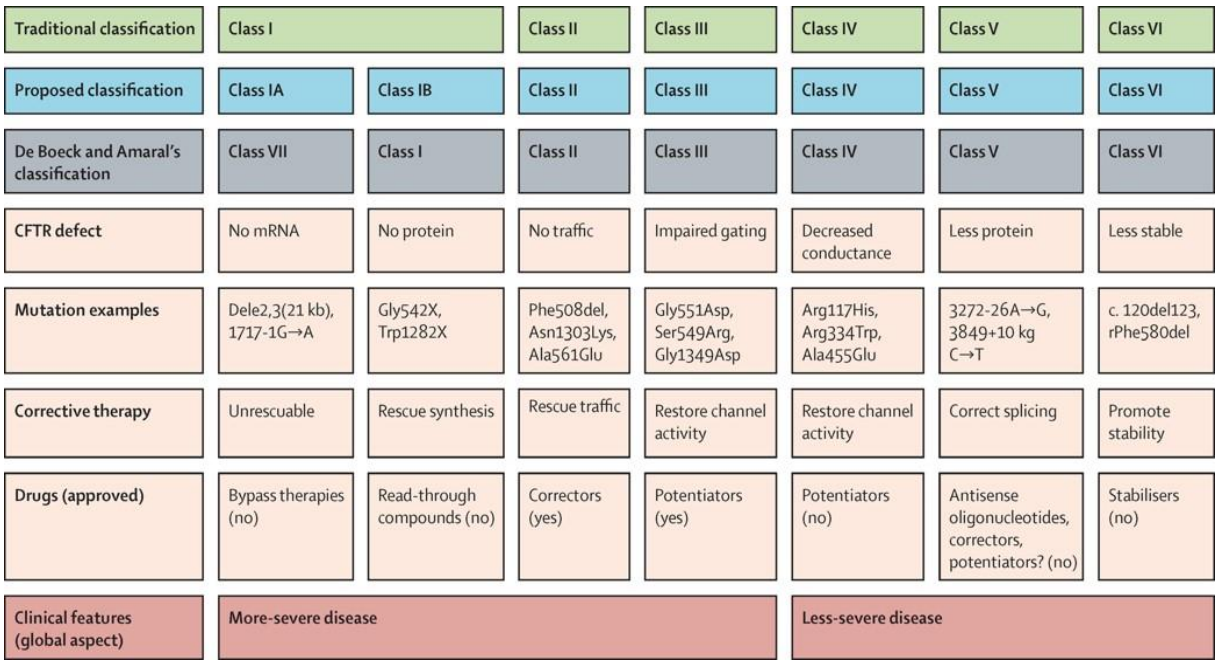


Figure 5. CFTR mutations and therapeutic strategies in the traditional classification system, our proposed classification, and De Boeck and Amaral's classification [28].

### 3.4 Diagnosis

The diagnosis of CF is based on clinical findings and is supported by laboratory tests. Clinical Presentation of CF includes positive NBS (newborn screening), signs and/or symptoms and family history.

The symptoms most common are respiratory symptoms, failure to thrive, steatorrhea, and meconium ileus [36].

The NBS algorithm for CF is based on the quantification of the levels of immunoreactive trypsinogen (IRT), a test that is used as part of newborn screening (NBS) programs, in two dosages, the second being done within 30 days of life. The laboratories are based on two consecutive measurements of the IRT or even on mutation tests of the *CFTR gene* (IRT / DNA). NBS is not a definitive test but can indicate the need for additional tests to determine whether the patient has in fact CF [36, 37].

Given two positive dosages, the sweat test is performed to confirm or exclude CF. The measurement of chloride by quantitative methods in sweat ≥ 60 mmol /L, in two samples, confirms the diagnosis. The standard method for the sweat test consists of stimulating the production of sweat by polycarpine, which is placed on the skin or directly in the sweat glands, using a potential gradient (iontophoresis) and analysis of the concentration of Na and Cl ions [38].

Even in the presence of a positive sweat test, the identification of 2 CF-causing mutations should be confirmed in a clinical genetics laboratory capable of performing in-depth genetic analysis when required to further define CF risk. Diagnosis of CF in the no screened population can be challenging because the age of onset and severity of symptoms can differ greatly as a result of highly variable levels of CFTR dysfunction. Presenting manifestations can include pancreatitis, respiratory symptoms, chronic sinusitis, and male infertility. A sweat chloride value <30 mmol/L makes the diagnosis of CF unlikely. However, specific CF causing mutations can be associated with a sweat test below 30 mmol/L. Individuals with sweat chloride values in the borderline range (30–59 mmol/L) should undergo a repeat sweat test and further evaluation in a specialist CF Centre, including detailed clinical assessment extensive *CFTR gene* mutation analysis, and are recommends additional CFTR physiological

testing that directly measures CFTR function, such as NPD ( Nasal potential difference) and ICM (intestinal current measurement)[36, 37, 39] (Figure 6).

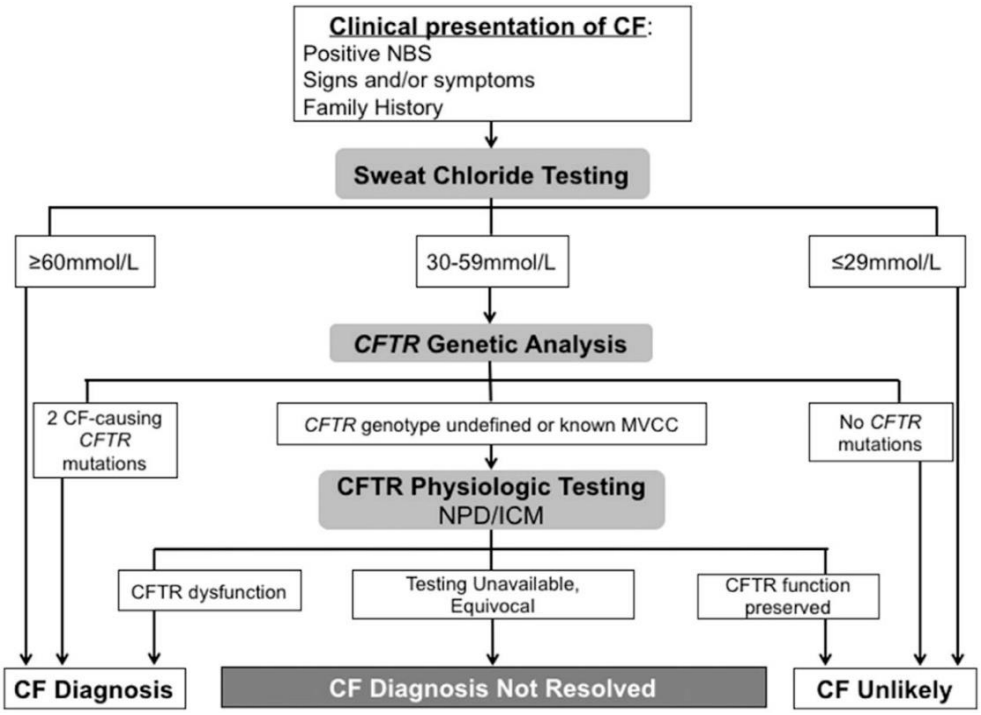


Figure 6. Management of cases with positive neonatal screening for CF. BNS: newborn screening; IRT: immunoreactive trypsinogen; MVCC: Mutations of varying clinical consequence; NPS: Nasal potential difference; and ICM: intestinal current measurement [37]

Clinically, CF-patients with chronic bacterial lung infections lean to have a lower quality of life, lower body mass index (BMI), and declining lung function measured by forced expiratory volume in the first second (FEV1) compared to a reference population (FEV1 % predicted), forced expiratory flow; mid expiratory phase ($FEF_{25–75\%}$), forced expiratory volume in 0.75 seconds ($FEV_{0.75}$) and forced vital capacity (FCV) [40, 41]. Pulmonary and nutritional goals are for children a FEV 1 percent predicted greater than or equal to 100 and a BMI percentile equal or exceeding the 50th percentile. For adults, the FEV1 percent predicted should be greater than or equal to 75, and BMI value greater than or equal to 22 for women and 23 for men [42]. The major purposes of treating patients with CF are to prevent or delay chronic lung infections and keep the lung function at a fixed state. This goal is difficult to achieve,

consequently, CF is the second largest group of lung transplanted recipients in Europe [43].

## 3.5 Clinical manifestations

### 3.5.1 Pulmonary manifestation

Lung disease is the dominant manifestation in CF, being the major cause of morbidity and mortality. The lungs, including mucus glands, appear histologically normal at birth, subtle abnormalities in mucus secretion appear very early and may represent the first turning point in pathogenesis [44].

CF alters the electrophysiological properties across airway epithelia, a widely held hypothesis is that CFTR inhibits ENaC, and loss of CFTR function causes amiloride-inhibitable sodium hyperabsorption, which dehydrates airways, reduces the height of the periciliary liquid, and disrupts mucociliary clearance. Mucociliary clearance is a primary innate airway defense but is reduced in CF because there is abnormal regulation of the periciliary liquid volume that contributes to it. Altered viscosity and regulation of submucosal gland secretion may also impair host defense. In addition, the reduced periciliary liquid volume promotes interactions between gel mucins in the mucus layer with cell-surface mucins that hinder clearance of particles from the airways, that in a normal situation the mucociliary clearance can require up to 6 hours, and this can be significantly prolonged in CF airways. The reduction of mucociliary clearance, with an elevated sodium chloride concentration in CF may also contribute to overloading innate antimicrobial peptides (Endogenous antimicrobial peptides can suppress bacterial growth for 3 to 6 hours) and thus promote initial endobronchial infection in young children with CF [45-47].

After birth, its common infection with bacterial pathogens commences and relates to an intense neutrophilic response localized to the peribronchial and endobronchial spaces. In adults the neutrophils are prevalent in the airway inflammation with elevated interleukin (IL) -8 and elastase, the consequence there is airway dilation (bronchiectasis), followed by protolysis and chondrolysis of airway support tissue. In

advanced stages, the lung parenchyma is affected by atelectasis (the airways or air sacs in the lungs do not fully expand or collapse), pneumonia [46]. Airway epithelial cells, macrophages, and neutrophils are all capable of producing cytokines, which suggested that the airway inflammation in CF is associated with increased production of pro-inflammatory cytokines in the lung, with elevated concentrations of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and tumor necrosis factor-alpha (TNFα) in the sputum and bronchoalveolar lavage fluid (BALF) [48].

The crucial mediators for the neutrophil influx in the CF lung include IL-8, tumor necrosis factor–α and IL-1, complement derived chemoattractants, and leukotriene B4. IL-8 is produced by macrophages, epithelial cells, airway smooth muscle cells, endothelial cells, and appears to be the predominant and sentinel neutrophil chemoattractant in the CF airway. IL-1β, tumor necrosis factor–α, neutrophil elastase, LPS, and *P. aeruginosa* antigens can all stimulate further IL-8 production to sustain the neutrophilic influx. Tumor necrosis factor–α stimulates neutrophil secretory and oxidative processes, and both tumor necrosis factor–α and IL-1 can prime neutrophils for a heightened response to chemoattractants. The activated neutrophils are the main effector cells for the pathogenesis of CF lung disease. Neutrophils release an enormous quantity of elastase and other proteases that overwhelm the local host defenses including α-1 antitrypsin and secretory leukocyte protease inhibitor. Since the neutrophils disintegrated, they release a lot of high molecular weight DNA that increases the viscosity of the endobronchial secretions that provide reduced mucociliary clearance, either the CFTR dysfunction in neutrophils compromises the phagocytic innate immunity, that leads a reduced bacterial function including delayed apoptosis (Figure 7). The airway epithelial cells (AECs) are one of the crucial sources of chemokines including IL-8 in the airway during the inflammatory response. Many studies show that airway inflammation is found at the early age of CF patients in the absence of considerable bacterial colonisation. This event supports the argument that the intrinsic deficiency of CFTR functions might be the primary factor that cause the CF airway susceptible to inflammation [46, 49-51].

A study made by Gangell et al (2011) demonstrated that children infected with *P. aeruginosa*, S. aureus, and Aspergillus species had significantly higher levels of neutrophilic inflammation and proinflammatory cytokines than did uninfected

children and Ramsey et al (2014) saw that this situation was associated with reductions in lung function, lower $FEV_{0.75}$ compared with healthy patients[51, 52].

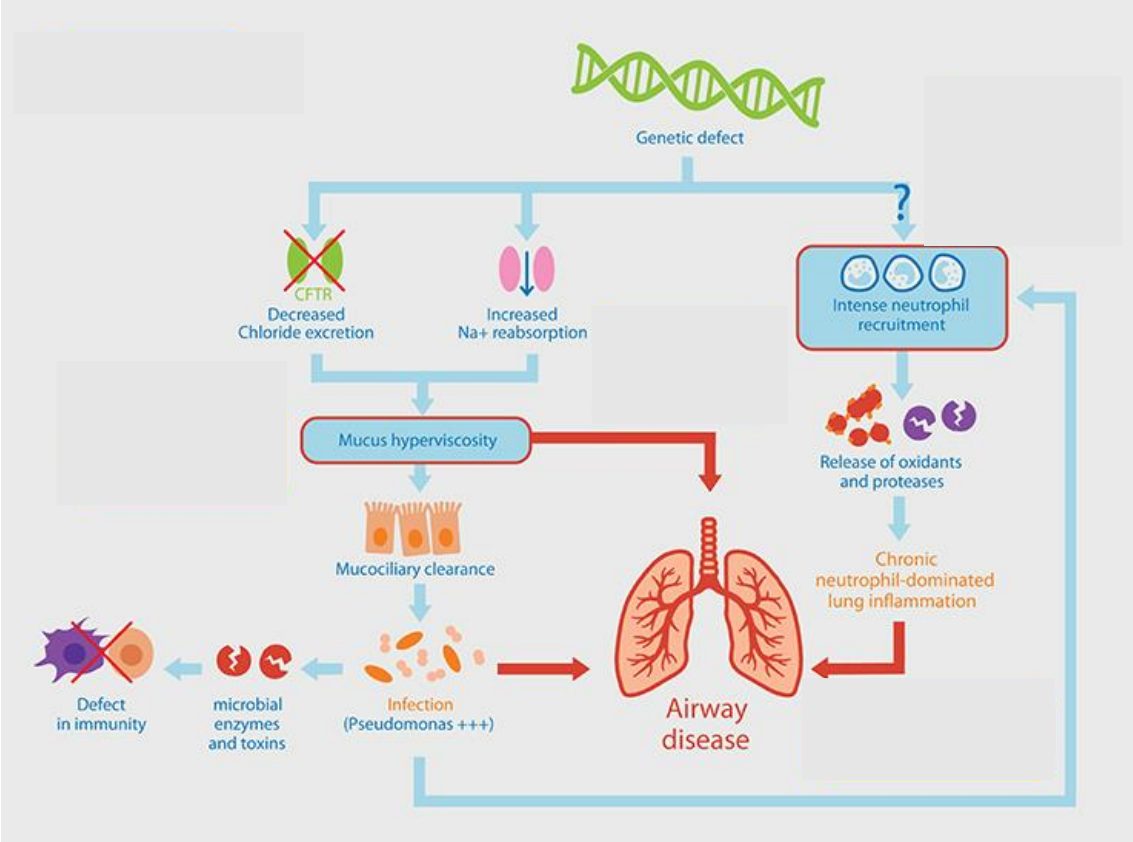


Figure 7. Defected or no CFTR channel leads to thick mucus in the respiratory airways, that decrease the mucociliary clearance, inducing an increase the susceptibility to microorganisms infections, like *P.aeruginosa*, this bacterial infection recruit intensive neutrophil and persistent airway inflammation. The defect in innate immunity, with prominent neutrophil infiltration, release of oxygen radicals and elastase, which leads to damage to the air cells and increased mucus viscosity, leading to high airway obstruction. Late apoptosis of neutrophils explains the persistence of the inflammatory state (adapted of Marteyn et al, 2017) [53].

## 3.6 Microbiology of respiratory disease

Lung infection can perennially reduce lung function in CF patients [54]. The prevalence of airways microorganisms that colonize CF people from infancy, through childhood and into adult life, change [55]. Between the microorganisms that cause

infections, *S.aureus* is the only one that may be pathogenic in immunocompetent person, all the others like *P.aeruginosa, Burkoldheria cepacea, Haemophilus influenza, Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* they are contemplated opportunistic pathogens, either *Aspergillus,* nontuberculous mycobacteria, and *Candida albicans*, that is a common commensal in the oropharynx and upper respiratory tract but chronic colonisation of CF lower airways has been associated with worsening of lung function and greater rate of pulmonary exacerbation[46, 56, 57].

In children, the most common respiratory infections are *Staphylococcus aureus* and *Haemophilus influenzae*, the latter is most commonly isolated in the lower respiratory tract of children around one year old [46, 55].

In older children, intermittent infection with *Pseudomonas aeruginosa* becomes more prevalent, had the point that 80% of individuals by age eighteen are infected. During the time, many CF individuals become chronically infected with *P.aeruginosa,* a mucoid phenotype of this bacteria very often gets the prevalent form. *P. aeruginosa* eradication is possible during early colonisation as long as colonies do not evolve to mucoid phenotypes, formation of biofilm, development of antibiotic resistance, and total bacterial abundance but when the infection becomes chronic there are few opportunities for a successful therapeutic intervention [46, 55, 58]. The prevalence of respiratory microorganisms by age is reported in Figure 8.

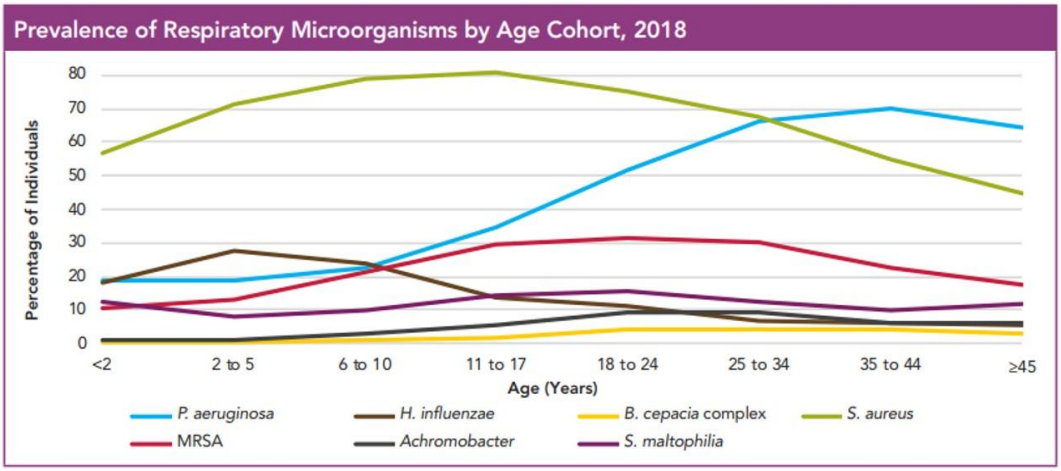


Figure 8. Microorganisms observed in bacterial cultures change as individuals age, 2018 [6].

*i. Staphylococcus aureus*

The Staphylococcus genus has 33 species, 17 of which can be isolated from human biological samples, are cocci grouped in the form of positive grape, Gram, and catalase clusters, with approximately 0.5 to 1.5 µm in diameter, immobile, not porous, and generally non-encapsulated. This genus is usually part of the microbiota of normal human skin and other anatomical sites. The species of greatest medical interest, mainly in the nosocomial environment, the species *S. aureus*, which is frequently related to several infections in humans. The strains of *S. aureus* grow in common media, broth, or simple agar, pH = 7, at an optimum temperature of 37ºC. On blood agar plates, a halo of hemolysis (beta hemolysis) develops around the colonies formed (Figure 9). Another important means for the identification of *S. aureus* is the mannitol-salt agar, selective for this species, with a high concentration of NaCl (6.5 to 7.5%) and an indicator phenol red pH - red in alkaline and neutral pH; the fermentation of mannitol by bacteria leads to the production of lactic acid, which determines the color change from red to yellow. Most *S. aureus* has a coagulase bound on the surface of the cell wall that reacts with plasma fibrinogen causing coagulation (coagulase test) [59-61].

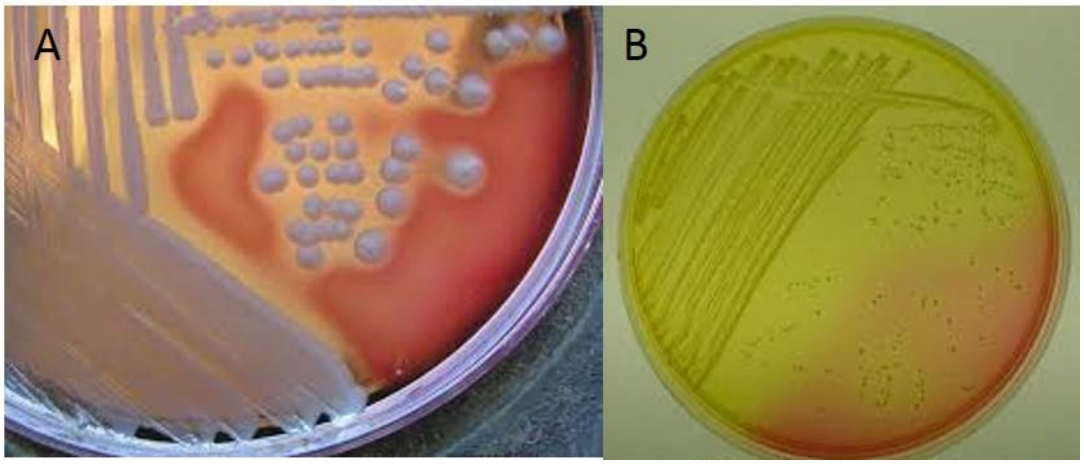


Figure 9. Colonies of *S.aureus*; A: form β hemolysis in blood agar and B: turning Mannitol salt agar to yellow upon release of acid.

*S. aureus* is known to be one of the first pathogens to colonize CF lungs with an 80% prevalence rate, peaking at ages 11 to 17 [6]. In addition to the high prevalence

of *S. aureus* in children, it has been associated with greater inflammation of the airways, reduced pulmonary function, and even greater later mortality when detected in association with *P. aeruginosa* [62]. Such tragic as the overall increase in S. aureus infection in CF is the increase in the prevalence of specific subtypes like methicillin-resistant S. aureus (MRSA) and small-colony variants of *S. aureus* (SCVs) may be associated with even worse outcomes in these patients [62, 63].

### *ii. Stenotrophomonas maltophilia*

First classified within the genera *Pseudomonas* and *Xanthomonas*. Gram-negative bacillus, non-glucose fermenter (NFB), aerobic, with ubiquitous distribution and low virulence. In the last decades, it has stood out as an emerging nosocomial pathogen, mainly in immunocompromised patients, with high morbidity and mortality, being, in many centers, the third most important NFB in-hospital infections. The colonies are rough 3 to 5 mm in diameter, yellow in MacConkey, and greenish-brown in blood agar, generally non-hemolytic and with a characteristic ammonia odor [59, 61, 64].

The incidence of *S. maltophilia* infection in CF has also increased in recent years, it is the fourth most common CF pathogen, found to be infecting approximately 12% of all monitored patients in 2018 [6].

*S. maltophilia*-associated infection is difficult because presents several innate antibiotic resistance mechanisms, like *P.aeruginosa*. Two β-lactamase (L1 and L2), are regulated by AmpR (genes that confer resistance to ampicillin), a system analogous to that observed in *P. aeruginosa*. This gives resistance against all β-lactams take in account of carbapenems. More than ten different chromosomally encoded antibiotic efflux pumps of various classes have been found in *S. maltophilia* whose presence and overexpression are key modulators of β-lactam, quinolone, aminoglycoside, trimethoprim/sulfamethoxazole, chloramphenicol, and tetracycline resistance [65]. *S. maltophilia* is able of adhering to and forming biofilm on CF bronchial epithelial cells, indicating that biofilm formation could be an important step in colonisation of CF lung [66]. Studies made by Hansen elucidated that pathogenic effects of *S. maltophilia* could not be found, although CF patients presented a significant decline in lung

function following exacerbation, so the presence of chronic *S. maltophilia* could be a marker of more pronounced lung disease in affected patients [67].

### *iii. Achromobacter xylosoxidans*

*Achromobacter* spp. are emergent pathogens in CF patients. Its taxonomic position was considered inserted for decades, the genus was named *Achromobacter* by Yabuuchi and Oyama in 1971, then Alcaligenes, and then again *Achromobacter* [68].

*Achromobacter* species are nonfermenting gram-negative bacilli found in soil and water. It grows well in standard microbiological media, such as MacConkey agar. It produces flat, scattered, and rough colonies and has peritritic flagella. Most strains also grow on *Burkholderia cepacia* selective agar (BCSA). The organisms are oxidase and catalase positive and oxidize glucose to produce acid, but are negative for urease and indole [69]. The species is detected in two subspecies, named *A. xylosoxidans subsp. xylosoxidans* and *A. xylosoxidans subsp. denitrificans* [68]. *A. xylosoxidans*, oxidizes xylose, distinguishing it from other species [69].

Distinguishing organisms and confirming identification by conventional phenotypic and biochemical tests is difficult, so molecular methods have been used as the MALDI-TOF mass spectrometry [70].

*A. xylosoxidans* is a multidrug-resistant microorganism, the treatment with antibiotics is limited, in studies done by dinarmaquees, they noticed that despite the early and aggressive treatment, the patients developed a speedy chronic infection by *A. xylosoxidans*; detected by the rise in antibody titers after some positive sputum cultures and this is maybe responsible for a greater decline in lung function [71]. *A. xylosoxidans* from CF showed a higher affinity for binding to mucin, collagen and a tendency to a higher affinity for binding to fibronectin, characteristics that may favor the colonisation of CF patients [72].

In studies carried out on lung cells, it was observed that culture supernatants of *A. xylosoxidans* express a cytotoxic factor able to induce lung cell alterations, such as vacuoles in the cytoplasm and cell death, the main effect of this cytotoxic factor was the stimulation of proinflammatory cytokine release (IL-6 and IL-8) [73], which is

involved in the inflammatory processes observed in CF patients and attracts a large number of neutrophils, which cannot be cleared from the lungs because of the physical and chemical changes of the mucus, leading to airway obstruction and tissue damage and worsening the CF disease [74].

### *iv. Serratia marcescens*

*S. marcescens*, a gram-negative bacillus classified as a belongs to the family of the Enterobacteriaceae, facultative anaerobic, oxidase negative, forms negative lactose colonies on McConkey agar and can be β hemolytic on blood agar, producing pigmented colonies, a very characteristic red pigment called prodigiosin [75].

Lipopolysaccharide (LPS) and extracellular products, enzymes lipase, gelatinase, and DNase, adherence and capability of biofilm formation are important factors of their pathogenicity [61]. It is showing resistance properties conferred by extended-spectrum beta-lactamase and a new cephalosporinase AmpC [76].

*Serratia* organisms are now identified as important causes of hospital-acquired infections, comprising pneumonia, bacteremia, urinary tract infections, and surgical wound infections. Most are linked to intravenous, intraperitoneal or urinary, or respiratory tract catheterization and instrumentation [77].

Over the last 30 years, however, this species has emerged as an important pathogen and a common cause of nosocomial infections[78]. Risk factors associated with these infections include prolonged immunosuppressive therapy, previous antimicrobial agents, indwelling catheterization, and underlying diseases such as chronic pulmonary disease and diabetes mellitus[79].

It is known that the presence of a biofilm in the catheter can act as a reservoir [80].

*S. marcescens* are identified as a frequent organism from sputum culture in patients with CF, but the relationship at the pulmonary level with the development of chronic infection in CF patients is not clear yet [81].

### *v. Candida albicans*

The genus *Candida* comprises pleomorphic fungi, *Candida albicans* is part of the human commensal flora, however, these yeasts can become pathogenic in the event of an imbalance in their relationship with the host, so they are considered opportunistic [82].

*C. albicans* present several virulence factors that can contribute to pathogenesis, like adhesiveness, phenotypic, morphological changes (the most virulent forms of the fungus happen with yeast turns into hyphae and/or pseudo hyphae), secreted aspartyl proteases, phospholipases, and biofilm formation (as a consequence antifungal resistance) [83-85].

Studies have demonstrated that CF patients with airways colonized by *C. albicans* showed a decline in $FEV_1$ and increased hospital-treated exacerbations, mainly in adults who have co-infection with another microorganism. Bacteria-yeast interaction shows that the *C.albicans* mycofilmes can support the adhesion and colonisation by *S.aureus*, increasing the bacterial biofilm formation [86-88].

### *vi. Pseudomonas aeruginosa*

*P. aeruginosa* is the most significant pathogen in CF, and the first positive lung culture can be identified in the first few months of life until the patient acquires a chronic infection [46]. Due to its characteristic facultative anaerobe, in the absence of oxygen the production of energy by aerobiosis, with the reduction of nitrate or nitrite, is important to colonize lung mucus or hypoxic environment [89].

*P. aeruginosa* is a gram-negative bacillus, asporogenous, monoflagellated, oxidase, motility reduction from nitrate to nitrite, malonate, acetamide, citrate positive, lysine descaboxylase, indole and DNAse negative, ß-hemolysis in blood agar, characteristic odor and color, growth at 42º C, oxidative acid formation from glucose and mannitol, inability to oxidize maltose and lactose, sensitivity to polymyxin ß [59, 90]. This bacteria is part of ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter*

*spp*), a group of bacteria that cause nosocomial infections and high resistance to antibiotics and virulence, it lives in the environment and can cause diseases in plants and animals, but in humans it can cause serious infections in immunocompromised patients [90, 91].

In the chronic lung infected by *P. aeruginosa*, its conduct to immune complex-mediated chronic inflammation, which is control by PMNs (polymorphonuclear cell family) and is a principal cause of lung tissue damage and decreased lung function, in addition to the damage caused by bacteria [92].

A series of striking physical-chemical, genetic and nutritional changes occur when *P. aeruginosa* passes from the environment to the CF patient's airways. It encodes several virulence factors that allow the development of infections, with a complex network to control these factors, to obtain the maximum benefit for bacterial proliferation [90, 93]

*P. aeruginosa* produces pigments, like pyocyanin (blue-green) which it is a redox-active pigment that is necessary for virulence and is detected in patients' airway secretions, pyorubin (red-brown), and siderophores pyoverdine (yellow-green and fluorescent) which works to eliminate iron [90, 94, 105, 109] .

Quorum-sensing (QS) is a communication between the cells through chemical signals, by expression of a set of genes in response to a signaling molecule known as autoinducers (AIs), that play an important role in survival and colonisation, for physiological adaptation, coordinating phenotypic changes in bacteria. These genes are linked to the production of various virulence factors, resistance to antibiotics, biofilm formation, and others, until now, 4 pathways (Las, Rhl, Pqs e IQS) have been understood to mediate QS responses [95] .

Due to the continued use of antibiotics in CF, *P. aeruginosa* uses different resistance mechanisms to survive, which is attributable to both intrinsic resistance and acquired resistance. The latter mainly refers the acquisition of resistance genes on mobile genetic elements, while the intrinsic resistance primarily refers to chromosomally encoded resistance mechanisms. The three most generally studied resistance mechanisms in *P. aeruginosa* are the resistance genes (ampC-Mediated Resistance toβ-Lactams, genes Mediating Resistance to Aminoglycoside, efflux

pumps (MexAB-OprM, MexXY/OprM(OprA), MexCD-OprJ, e MexEF-OprN) and membrane impermeability [90, 96].

Adhesion of *P. aeruginosa* to the host tissues is a crucial early step in infection and pathogenesis, there are at least three different adherence factors or adhesins: type IV pili, mediating adhesion to epithelial host cells; flagella, binding to mucin on epithelial cells; and the core oligosaccharide of LPS, mediating adhesion to the CFTR of epithelial cells [90].

One of the most striking features of *P. aeruginosa* adaptation to the airways of patients with CF is the frequent conversion to a mucoid phenotype owing to excessive and constitutive production of the extracellular polysaccharide alginate, which forms a glycocalyx that covers the surface of the bacterium. Alginate overexpression is generally interpreted as a sign of biofilm production in the CF airways. Alginate production appears to be part of the overall envelope stress in response, protecting bacteria from unfavorable environmental stresses, such as desiccation and hydrophobic agents. [93, 97]. The most frequent mutations responsible for the mucoid changed are found in mucA, which encodes an inner-membrane-associated anti-σ-factor MucA normally limits the expression of the algD operon, which encodes the enzymes required for alginate synthesis, by sequestering the alternative RNA polymerase σ-factor σ22, encoded by algU59–63. In addition to the algD cluster, σ22 regulates a large number of stress response and virulence-associated genes and is involved, directly and indirectly, in the regulation of virulence and motility in *P. aeruginosa* [96, 98-107].

Products of type III secretion systems (T3SS) are expressed commonly in acute infection, often in the setting of hospital-acquired pneumonia, by causing cytotoxicity and facilitating invasion. The T3SS effector proteins include exoenzyme U (ExoU), a potent phospholipase A2 that requires host superoxide dismutase for activation and often is associated with severe pneumonia [108, 109].

Biofilm formation. During acute infection, a variety of cytotoxic components are produced by the bacteria, compromising the host's cellular processes. On the other hand, the bacterium is fought by mechanisms of the immune system, such as the production of microbial components, phagocytosis by neutrophils and macrophages, and production of ROS (Reactive Oxygen Species). In the acute phase of an infection,

the bacterium is highly mobile and, in the case of *P. aeruginosa*, several motility mechanisms, such as swimming or crawling involving flagella, and spasms using type IV pili, are associated with greater virulence. In addition, these structures mediate the recognition and induction of signaling pathways that trigger bacterial phagocytosis and the consequent inflammatory responses. To escape such stressful conditions, the change to a sessile lifestyle at the expense of furniture can confer an advantage for the survival of the bacteria, which occurs through the formation of so called biofilms (microcolonies which are embedded in extracellular polymeric substances (EPS)) to protect bacteria from the surrounding environment [110-113]. The formation of mucoid biofilm by *P. aeruginosa* is a sign of chronic infections and indicates the progression of the disease and long-term persistence. As a result, *P. aeruginosa* dominates the microbial community of the lungs with CF in patients older than 24 years [114].

The natural course of the disease can be improved by preventing or postponing chronic *P. aeruginosa* colonisation, the eradication is possible in early colonisation as long as colonies do not evolve to mucoid phenotypes [115].

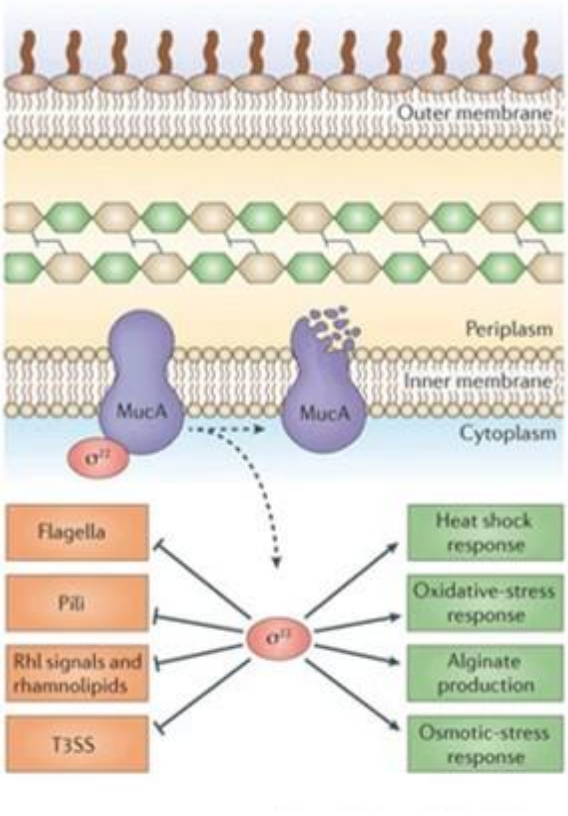


Figure 10. The function of the RNA polymerase σ-factor $\sigma^{22}$ is antagonized through protein-protein binding by the anti-σ-factor MucA. Mucoid *P. aeruginosa* isolates from patients with CF most often carry knockout mutations in mucA, leaving $\sigma^{22}$ free to activate transcription of many genes, such as those involved in alginate production and the responses to heat shock, osmotic stress, and oxidative stress $\sigma^{22}$ also negatively regulates several virulence factors, including flagella, pili, the type III secretion system (T3SS) and Rhl quorum-sensing signals, as well as rhamnolipids that are controlled by Rhl3 [93].

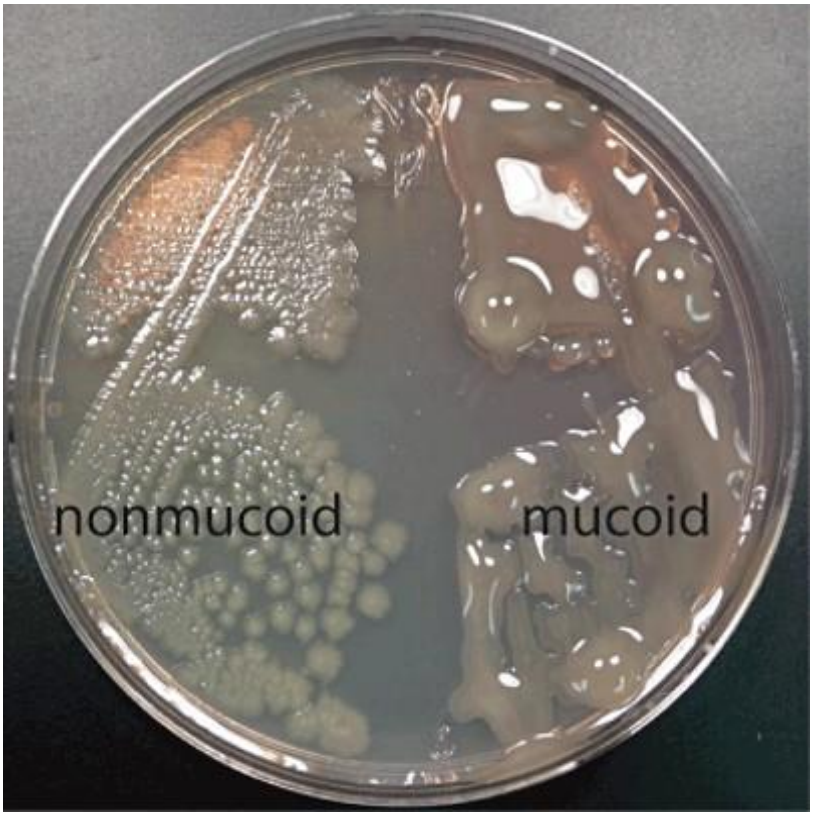


Figure 11. *P. aeruginosa* mucoid and non-mucoid phenotypes [116].

### 3.7 Microbiology in possible reservoirs of microorganisms in Cystic Fibrosis

Analogous to the lower airways (LAW), mucociliary clearance of the upper airways (UAW) is reduced by the causative CFTR defect. Therefore, mucus retention, chronic inflammation, and colonisation of the UAW with opportunistic bacteria are typical in CF [115]. The main source of chronic lung infection by opportunistic bacteria has been linked to sinus involvement [117].

It has been suggested that the paranasal sinuses could be a bacterial reservoir for pulmonary infections in patients with CF [118]. The paranasal sinuses are aerated cavities within the skull that connect to the nasal cavity. There are four sets of paired sinuses: the maxillary sinuses surrounding the nasal cavity, the frontal sinuses placed in the forehead above the eyes, the ethmoidal sinuses are many small sinuses between the orbits, and the sphenoid sinuses are deep and posterior to the ethmoids. The sinuses are lined by mucosa and produce mucus. However, the high mucus viscosity impairs normal cilia motility in CF patients, resulting in poor mucociliary clearance,

obstruction of the sinus ostia, and chronic sinusitis. Common findings are nasal congestion, polyposis, mucoceles, mucopurulent material, medial bulging of the maxillary walls, ostitis, and hypoplasia or aplasia of the paranasal sinuses especially of the frontal sinus, furthermore, polyp formation accompanies chronic sinusitis in CF in 6–48%, but its mechanism is not fully understood [119-123].

The CFTR defect affects the upper airway mucosa like the lower airways so that the paranasal sinuses (PS) are almost universally involved in CF [117, 124, 125]. Rhinosinusitis (RS) symptoms with or without nasal polyps are therefore a hallmark of the disease. Predominant clinical signs are chronic nasal congestion, rhinorrhoea with anterior or postnasal drip, mouth breathing, anosmia, facial pain, and sleep disorders [126, 127]. The bacteria persist in the sinuses for years and can be a focus for initial lung colonisation and maintain the infection and changes in CT exams that indicate sinonasal diseases and symptoms appear soon after birth, which may suggest a careful examination of the upper airways in children with CF [128, 129]. Performing nasal and paranasal endoscopy, Sakano *et al*. (2007) also a showed that only 1/50 of the patients did not present nasal and paranasal sinuses affections [130].

Interestingly, Mainz et al. (2009) with a non-invasive method to sample the UAW (nasal lavage and deep nasal swabs) and LAW ( sputum and deep throat swabs) for bacteriological analysis, verified in 50% of cases, the same microorganism was also recovered from the lungs if it had been cultured from the UAW, and the best method to recover microorganism from the UAW is nasal lavage [131]. Moreover, Goerke *et al*. (2000) reported that the prevalence of *S. aureus* in the nares among patients with CF is significantly higher in those who had not received antibiotics and higher than in healthy controls, this suggests that the altered physiology due to the primary mutation in the *CFTR gene* leads to a higher susceptibility for *S. aureus* colonisation in the nose, and the higher susceptibility in the nose may be explained by mechanisms proposed for a chronic lung infection in CF: The altered mucus composition and viscosity in the airways of CF patients leads to reduced mucociliary clearance [132, 133].

Interestingly, the studies addressing this issue have shown that *P. aeruginosa* strains isolated from the nasal sinus and the sputum of the same patient have a common genomic profile, indicating that they belong to the same clone circulating within the airways this has been proven through the analysis of patients who underwent lung

transplantation, where they presented the same *P.aeruginosa* clone present before the procedure [134, 135]. CF patients with UAW colonisation with *P. aeruginosa* and *S. aureus* reveal a 4000 and 25-fold increased likelihood for pulmonary *P. aeruginosa* and *S. aureus* colonisation [136]. Another study revealed that most *S aureus* carriers and almost all *P aeruginosa* carriers are harboring the same clones in the upper airways and the lungs; 86% of the *S aureus* strain pairs and 95% of the *P aeruginosa* strain pairs were genotypically identical [131]

Muhlebach *et al.* (2006) studied 45 paired sinus-bronchoalveolar lavage fluid cultures of 31 CF patients, 83% of the samples analyzed positive *for P. aeruginosa*, they showed identical genotypes in UAW and LAW cultures [137].

On the other hand, other possible bacterial reservoirs could be considered. As an example, the passage of bacteria from the oral cavity to the lungs may occur by aspiration of pathogens released in saliva or may be facilitated by the passage of medical devices such as bronchoscopes and endotracheal tubes [138].

The oral cavity is a complex anatomical region comprising different tissues. It consists of a maxilla and mandible where the teeth are found, tooth supporting tissue called the periodontal tissues. The inner cheeks come from either side of the jaw and in the center is the tongue. These tissues are bathed in saliva [139].

Hundreds of distinct microorganisms colonized the mouth on different surfaces: teeth, dorsum of the tongue, and oral mucosa that constitute oral biofilm [139]. The dental plaque has many bacterial species and serves as a reservoir for microorganisms, such as potential respiratory pathogens (PRPs) [139, 140]. In healthy adults, the organism that predominates in the oral cavity is *Streptococcus viridans*, but the oral flora in patients in a critical state of health changes to predominantly gram-negative organisms, constituting a more aggressive flora, for example, S. *aureus*, *S. pneumoniae*, *Acinetobacter baumannii*, *H. influenzae* and *P. aeruginosa* become predominant and constitute a more aggressive flora in critically ill patients [141]. In addition, the use of antibiotics can induce modifications in the oral microbiota and favor oral colonisation with other bacterial species [154]. Caldas *et al* (2015) made a systematic review and noted a lot of authors demonstrated that the upper aero-digestive tract is the first location of colonisation by *P. aeruginosa* and its presence appears to be predictive of subsequent broncho-pulmonary colonisation[139].

Bensel and colleagues (2010) found identical facultative anaerobes (*P. aeruginosa, S. aureus* and *Burkholderia cepacia*) in periodontal pockets and sputum in 44.4% of the studied CF patients CF patients [142].

*S. aureus, P. aeruginosa, Acinetobacter* species, and enteric species were isolated from plaque and bronchoalveolar lavage samples of patients who receive mechanical ventilation in a study made by Heo et al (2008), they showed that respiratory pathogens from the lung and oral cavity had >95% similarity of pulse-field gel electrophoresis patterns; this provides convincing evidence that dental plaque serves as an important reservoir for respiratory pathogens in these patients [143].

These observations suggest that the mouth environment could favor the entry of respiratory pathogens and their arrival in the airways. Thus, the presence of respiratory pathogens in the oral cavity might constitute a risk of airways contamination and contribute to pathogens translocation and colonisation of the lower airways. In this scenario, toothbrushes may also play a role as vehicles for respiratory pathogens towards the oral cavity, and subsequently to the airways, increasing the risk of respiratory infections. Indeed, they can get easily contaminated from various sources, like the environment, hands, aerosol, the oral cavity itself, and can serve as reservoirs of potentially pathogenic microorganisms [144-147] .

Toothbrushes can also become contaminated by the oral cavity itself. Retention and survival of bacteria on toothbrushes after brushing represent a possible cause of re-contamination of the mouth. Several studies [146, 148, 149] have shown that prolonged use of the toothbrush facilitates contamination by various microorganisms such as species of *Streptococcus, Staphylococcus, Lactobacillus, Pseudomonas, Klebsiella, Candida,* and herpes simplex virus, and by *Escherichia coli*. In CF children, *P. aeruginosa* and *S. aureus* nasopharyngeal carriage is up to 14%. In a pilot study, it has been found that *P. aeruginosa* and *S. aureus* are present on 15% and 22% of CF patients' toothbrushes, respectively and in two patients the same *P. aeruginosa* clone was identified on the toothbrush and sputum [146].

The actual impact of toothbrush contamination on bronchial primary colonisation by *S. aureus* or *P. aeruginosa* seems to be very difficult to assess because bronchial colonisation can occur from different sources and is highly dependent on the child's pulmonary status. Currently, CF patients should follow the general recommendations

of the health authorities including the Centers for Disease Control and Prevention, suggesting patients not share toothbrushes, rinse their toothbrush thoroughly with tap water following brushing, not routinely cover the toothbrush or store it in closed containers allowing it to air dry, and renew the toothbrush every 3 or 4 months, sooner if the bristles appear worn or splayed (www.cdc.gov/oralhealth/infectioncontrol/questions/toothbrushhandling.html).

However, bases on the increasingly frequent detection of emergent pathogens colonising the CF lung, it would be interesting to evaluate whether other bacterial species could also be present in the paranasal sinus and oral cavity and constitute a reservoir for colonisation of the lung.

## 3.8 Automated phenotypic identification

Distinguishing among species of microorganisms using manual and automated commercial identification systems is often challenging due to limited biochemical reactivity [150]. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) offers the potential for routine identification of microorganisms that is faster, more accurate, and less expensive than conventional biochemical methods [151, 152]. Diagnostic automation is increasingly required by microbiologists due to the huge variety of microorganisms nowadays and the low efficiency of identification of the phenotypic manual methods for identification of all. It is recommended that the accuracy of the automated system exceeds 90% in its ability to identify microorganisms in the clinical environment and that it be able to identify commonly isolated organisms with at least 95% accuracy compared to the accuracy of conventional methods [153].

### 3.8.1 MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry)

Mass spectrometry is a microanalytical technique used to obtain the molecular weight and structural characteristics of the sample. Mass spectrometry is one of the most important analytical tools capable of providing information on the basic composition of samples; the molecular structure; the qualitative and quantitative composition of complex mixtures; the structure and the composition of solid surfaces and the isotopic proportions of atoms in samples. In mass spectrometry, the energy, supplied by the laser, is transferred to the sample to cause its ionization. The basic requirement for analysis by mass spectrometry is the formation of free ions in the gas phase. The scope and usefulness of the method are dictated by the ionization process. The appearance of the spectrum of 16 mass of a molecular species is highly dependent on the ionization method used [154, 155].

The ionizing agents used in mass spectrometry are divided into two categories: those that require the gas phase sample and the agents that cause desorption in solid or liquid samples. The advantage of the latter applies to non-volatile and thermally unstable samples. This is the case of the mass spectrometry technique used to identify bacteria [156, 157]. MALDI-TOF is composed of 4 main components: the ionization of the molecules, desorption, laser, and matrix. The matrix consists of an organic molecule that will contribute to the supply of electrical charge to the molecules of the bacterium. The laser will be responsible for generating this energy so that ionization occurs and then the desorption, i.e. separation, of these molecules. After ionization, the molecules become attached to an electric field, preventing them from getting lost in space. Right after the electric field, there is the vacuum tube, where the molecules will move due to negative pressure that will suck them up. This displacement, together with the electrical charge and mass of the molecule, will be fundamental components for the elaboration of the spectrum mass, which will be formed through a receiver located at the end of the vacuum tube, connected to a signal transducer connected to a computer. This mass spectrum will compose the identity of each bacterium since each microorganism has a set of species-specific proteins. The molecules interpreted by the

device are the ribosomal proteins, due to the large amount available in bacteria and conservation within the same species [156-158].

The method analyzes the profiles of bacterial macromolecules that are obtained from whole bacteria. This new proteomic approach allows rapid and accurate identification of bacteria as well as yeast and fungi. Mass spectrometers are instruments capable of producing and separating ions according to their mass-to-charge ratio (m/z)of gas-phase ions and consisting of the following components: ionization source, mass analyzer, and ion detector [159]. In the first stage, the sample is mixed with the matrix, usually an organic acid (solution with alpha-cyano-4-hydroxycinnamic acid) with strong absorption of ultraviolet radiation, resulting in the crystallization of the sample and matrix molecules. This mixture is pumped by a laser (electrospray ionization) and the matrix absorbs the energy from the laser, transferring it to the sample molecules resulting in the desorption and soft ionization of the sample/matrix molecules, forming a “cloud” of protonated ions in the gas phase. Small molecules generally produce mono-charged ions (with a charge). However, large molecules such as proteins acquire multiple charges in the ionization process. The ions are accelerated to a TOF tube through the action of an electrostatic field and separated according to the time of flight; the separation of ions is carried out under vacuum, to avoid collision with air molecules. The dimension of the ions is directly proportional to the flight time, which comprises the time elapsed between the initial laser signal and ion detection. Therefore, the flight time required for the ions to reach the detector depends on the mass (m) and charge (z) of the proteins. As a result, a spectrum (profile) is produced that associates the m / z ratio and the signal intensity, and this profile is compared with a database, which allows the identification of the bacteria (Figure 13). Thus, the MALDI-TOF MS analysis produces a unique and characteristic protein profile of a given bacterium [160-164].

Due to their robust fungal cell wall, comprised of glucans and chitins, yeasts require formic acid protein extraction before MALDI-TOF MS analysis [152]. This extraction process extends the turnaround time to results by 10 to 15 min. A more time-efficient approach is to apply formic acid directly on yeast analytes smeared onto the MALDI-TOF MS target plate before the addition of the matrix [165].

Ribosomal proteins are very abundant in microorganisms and, therefore, constitute the main components of evaluation by the mass spectrum. Based on the profile of ribosomal proteins, ranging from 2 to 20 kDa, which is unique for each microorganism species, the mass spectrum obtained is compared to a reference spectrum bank as a whole. The microorganism with the most related mass spectrum is identified and the measure of confidence in this analysis is indicated by a value, which differs from one system to another. The identification of species and genus is evaluated based on these values of trust [161, 162].

A percent probability, or confidence value, which represents the similarity in terms of presence/absence of specific peaks between the generated spectrum and the database spectra, was calculated by the algorithm. A perfect match between the spectrum and the unique spectrum of a single organism or organism group provided a confidence value of 99.9% (“good ID”). When a perfect match was not obtained, it was still possible for the spectrum to be sufficiently close to that of a reference spectrum such that a clear decision was provided about the organism ID (“good ID,” confidence value of >60 to 99.8%). If a unique ID pattern was not recognized, a list of possible organisms was given (“low discrimination” [LD], confidence value of >60%) or the strain was determined to be outside the scope of the database (“no ID”). The range of percent probabilities in the single-choice case was 60 to 99%. Values closer to 99.9% indicate a closer match to the typical pattern for the given organism. When the confidence value obtained was below 60, the organism was considered nonidentified [166].

Escherichia coli ATCC 8739 was used for every acquisition group on the target slide to calibrate the mass spectrometer. Matrix solution (a-cyano-4-hydroxycinnamic acid) without micro-organisms was used as the negative control [167].

### 3.8.2 Pulsed-field gel electrophoresis (PFGE)

DNA macrorestriction analysis of microorganisms with the use of pulsed-field gel electrophoresis (PFGE typing, pulse electrophoresis) is used in molecular biology for the study of the clonal structure and the typing of the causative agents of infectious diseases. The comparison of DNA restriction patterns is used to determine the degree

of relationship, find the epidemiological correlations between studied isolates, and examine the evolutionary history of pathogens [168].

Agarose gel electrophoresis is the method of choice to resolve DNA restriction fragments provided the fragments are between 1000 and 23 000 bp in size. For larger fragments, Schwartz and Cantor developed the technique of pulsed-field gel electrophoresis (PFGE) in 1984 [169]. Slices of agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands in the gel by an apparatus that switches the direction of current according to a predetermined pattern [170]. In PFGE DNA fragments greater than 23 kbp are forced to change their structure during electrophoresis by pulsing the applied field so causing the molecules to relax and expand regularly and thereby interact with the gel pores. DNA molecules up to the size of chromosomes can be electrophoresed in this manner, but the separation can take many hours or even days.

To discriminate bacterial strains of the same species, the total bacterial DNA, incorporated into an agarose block, is digested with restriction enzymes that cleave the chromosome into large fragments. In PFGE, 30 to 40U of the enzyme are needed for each agarose block and the incubation time can vary from 18 to 24 hours. The generated fragments are then separated by pulsed-field electrophoresis [171].

The restriction enzymes are naturally produced in bacteria to protect against the introduction of viral genetic material. The bacterium recognizes the viral DNA as foreign and destroys the molecule using restriction enzymes, which cut it into several fragments, restricting viral replication. These enzymes are specific to certain restriction sites, so they do not digest the DNA of the bacteria itself. On the other hand, when present in the bacterium's DNA, these restriction sites are protected by a methyl group (CH3), which is added to the DNA molecule by the action of the methylase enzyme. Often, the genes encoding the restriction enzyme and methylase are found adjacent to the genome. Approximately 900 restriction enzymes are known, isolated from more than 230 species. These enzymes are designated according to the microorganism from which they were extracted. The first three letters refer to the microorganism's scientific name, so they must be written in italics. The next letter refers to the lineage of the bacterium, and the Roman number designates the order of

discovery of the enzyme. For example, EcoRI is the enzyme extracted from *Escherichia coli*, strain R, being the first restriction enzyme identified in this bacterium[172]. With the possibility of cutting DNA fragments from the same or different species and being linked in a second step, recombinant DNA appears.

Many instrumentation approaches to PFGE have been described, but the contour-clamped homogeneous electric field electrophoresis (CHEF) have been used in the majority of laboratories which has now become essentially synonymous with the term PFGE [173]

The CHEF system is characterized by offering an array of 24 electrodes (Figure 14) producing a highly uniform electrophoresis gradient, forcing DNA fragments to change direction continuously, using pulses with an angle of 120°. This configuration produces an equidistant DNA migration to the left and right of the agarose gel center resulting in a straight migration along with the gel. As in all electrophoresis methods, the CHEF is influenced by factors such as the concentration and thickness of the agarose gel, the composition of the buffer, and the strength of the electric field (V / cm) [174].

The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness. The first interpretation procedure defined by Tenover et al. (1995) showed that the interpretation of the number of band differences between a pair of isolates is based on the minimum number of genetic mutational events that would result in the observed number of band differences. For example, two isolates that differ by two to three bands would be considered closely related since a single genetic event can explain this difference. It is considered that a minimum of 10 DNA fragments, consequently 10 bands in the gel, must be used by bacteria so that the technique has relevant discriminatory power. One strain is considered similar to another when a single genetic event occurs, such as a mutation, an insertion, or deletion, which alters the pattern of bands [170].

# 4. MATERIAL AND METHODS

## 4.1 Patients

A total number of sixty patients followed at the CF Centre in Verona (Italy), from 2017 to 2019, were recruited in this study, aged from 7 to 26 years old. They were classified into 4 different groups based on their situation regarding *P. aeruginosa* in that it is the most frequent and most important pathogen in CF. The four groups were: adult patients with chronic infection by *P. aeruginosa* (AC), paediatric patients with occasional *P. aeruginosa* colonisation (PO), adult free from *P. aeruginosa* lung colonisation (AN) and paediatric patients free from *P. aeruginosa* lung colonisation (PN), subdivided with and without nasal polyposis, (Table 1). Informed consent was obtained (projects CRCFC-CEPPO026 and CRCFC-CEPPO031) by the local Ethical Committee.

## 4.2 Study design

4.2.1 Patients were selected according to the following criteria:

- Diagnosis after neonatal screening and followed since birth in the center or diagnosis after symptoms.
- Age > 6 years old
- Complete clinical history available in the computerized database.
- At least two visits yearly since diagnosis
- Availability of at least two-yearly sputum cultures for microbiological ascertainment.
- At least one chest X-ray/year.
- At least one respiratory function test/year after the age of 10 years.

4.2.2 Clinical/microbiological status: Patients were subdivided into two categories:

- Lung uncolonized by *P. aeruginosa* (all available sputum samples negative for the bacteria with at least 6 samples/year during the last 2 years)
- Chronic lung colonisation by *P. aeruginosa* (at least three consecutive cultures positive for *P. aeruginosa* in the previous 6 months based on at least three positive cultures with at least one month in by intervals between them or more than 50% of the sputum samples positive for *P. aeruginosa* in the previous year) [175].

4.2.3 Pharmacological treatment

*P. aeruginosa* chronically infected patients were enrolled only if had been treated with tobramycin or tobramycin+ colistin and not with other antibiotics.

4.2.4 Exclusion criteria

- Infection with species of the *Burkholderia cepacia complex Bcc* (Colonisation and subsequent infection with Bcc has been associated with poor outcomes, including an accelerated decline in pulmonary function and fatal disease [176, 177]). Moreover, the identification of this bacterial species is done by Mass Spectrophotometry (MALDI-TOF MS), which often does not distinguish the species belonging to the *B. cepacea* complex.
- In the list for lung transplantation
- In immunosuppressive therapy

Patients and parents were informed on the purposes of the study and written informed consent will be obtained according to the rules approved by the ethical committee of the Azienda Ospedaliera Universitaria Integrata, Verona. Patients free

from colonisation/infection of *P. aeruginosa* that became colonized/infected by this bacterial species in the lung were treated and continued to participate in the study.

### 4.3 Visits

During routine visits to CF Centre of Verona, approximately every three months, we collected nasal lavage and sputum samples from all patients, saliva and toothbrushes from 40 patients (the group included 13 adults, and 27 pediatric patients, diagnosed as never or occasionally colonized by *P. aeruginosa*).

The sequence of samples collections were: i) sputum, ii) nasal lavage, iii) saliva, and iv) the toothbrushes, carried out on the same day at the time of the visit.

| | **Study patients** | | | |
|---|---|---|---|---|
| **Group** | AC | AN | PN | PO |
| **Number of patients** | 10 | 15 | 25 | 10 |
| **Age (in years)** | 21 (18-23) | 22.5 (18-26) | 12.5 (7-17) | 12.5 (7-17) |
| **Male** | 4 (40%) | 10 (66.7%) | 9 (36%) | 4 (40%) |
| **Female** | 6 (64%) | 5 (33.3%) | 16 (64%) | 6 (60%) |

Table 1. Characteristics of study patients, including classification based on the status of *P. aeruginosa* lung colonisation at the beginning of the study (AC = adults with chronic *P. aeruginosa* infection; AN = adults free from *P. aeruginosa* lung colonisation; PN = paediatric patients free from *P. aeruginosa* lung colonisation; PO = paediatric patients with occasional *P. aeruginosa* infection), number of patients per group, average age and sex.

### 4.4 Collecting samples

NL was performed by inserting 10 ml of sterile isotonic saline (0.9%) into each nostril with a 10 ml syringe. This was performed following the standard diagnostic procedure for nasal lavage [131, 178]; head in a slightly reclined position and the soft palate occluded. The material was collected in a transparent universal collector for laboratory exams. The saliva was recovered by spitting in a sterile tube, while the sputum was collected by natural expectoration or by tracheal cannula when the patient

was unable to expectorate. At every visit, a new toothbrush with soft bristles, along with a commercial toothpaste containing sodium fluoride was given to the patients, together with a written protocol explaining how to use and store toothbrushes at home. During the visit, a dental hygienist also explained to the CF patient (and the parents, in the case of pediatric patients) to rinse the toothbrush only with water, not to treat it with disinfectants, and to store it without cap and with the head turned upward. During the control visits, the used toothbrushes were recovered, placed in disposable plastic bags, and immediately transported to the laboratory.

## 4.5 Procedure with collected material

Saliva and sputum samples were treated with 0.5 ml (for saliva) and 1ml (for sputum) of Sputolysin (Calbiochem) and incubated for 40 minutes at 37°C with shaking. The head of the toothbrush was detached with sterile scissors, immerse in a 5 ml BHI (Brain Heart Infusion) medium, and underwent five cycles of sonication (Branson 1210 Ultrasonic Cleaner) by 30 seconds each, to release the microorganisms present in the bristles. After this procedure, the tubes with the toothbrush heads were incubated for 24 hours at 37°C with shaking.

Figure 12. Collected material

## 4.6 Isolation and identification of bacterial strains

### 4.6.1 Phenotype manual identification

An aliquot of each sample (100 µl) was plated on not select medium Columbia blood agar (5% sheep blood), select medium for: Gram negative bacteria McConkey agar, gram positive bacteria Mannitol Salt agar, and yeasts Sabouraud agar plates (BD Difco), and incubated at 37°C for 48 hours, for phenotypic identification.

All plates were kept at room temperature (RT) for five additional days to recover small variant colonies and slow-growing strains.

### 4.6.2 Phenotype identification automated by MALDI-TOF-MS (VITEK® MS-Biomerieux).

After the initial manual phenotypic screening, it was necessary to identify the genera/species of the isolates, so isolated colonies were identified using MALDI-TOF-MS (VITEK® MS-Biomerieux).

A. To perform MALDI-TOF in the VITEK-MS equipment (BioMérieux, France) a portion of the microbial colony ( 1 to 3 colonies ) from solid media is placed as a thin layer to a disposable target slide spot (which accommodates up to 48 simultaneous tests) [179];

B. For bacteria with the mucoid phenotype (such as alginate-producing *P. aeruginosa)* and yeasts, a portion of one colony isolated from a Sabouraud agar plate or Columbia blood agar is applied directly onto the Vitek-MS disposable target, and before placing the matrix, the sample needs to be lysed with 0.5µl of 25% formic acid [180];

C. After drying completely at room temperature;

D. 1µl of ready-to-use -cyano-4-hydroxycinnamic acid (CHCA) matrix for the ionization of proteins is applied to the spot;

E. An internal calibrator consisting of a fresh colony of *Escherichia coli* (ATCC) is added to the designated spot (in the middle of each quadrant) on the prepared slide in accordance with the standard Vitek®MS procedure;

F. After drying the samples, the slide is inserted ready to be analyzed by MALDI-TOF MS.

G. For each bacterial sample, mass fingerprints were processed by the compute engine and the advanced spectrum classifier (ASC) algorithm associated with the Vitek MS system, which then automatically identifies the organism by comparing the characteristics of the spectrum obtained (presence and absence of specific peaks) with those of the typical spectrum of each claimed species.

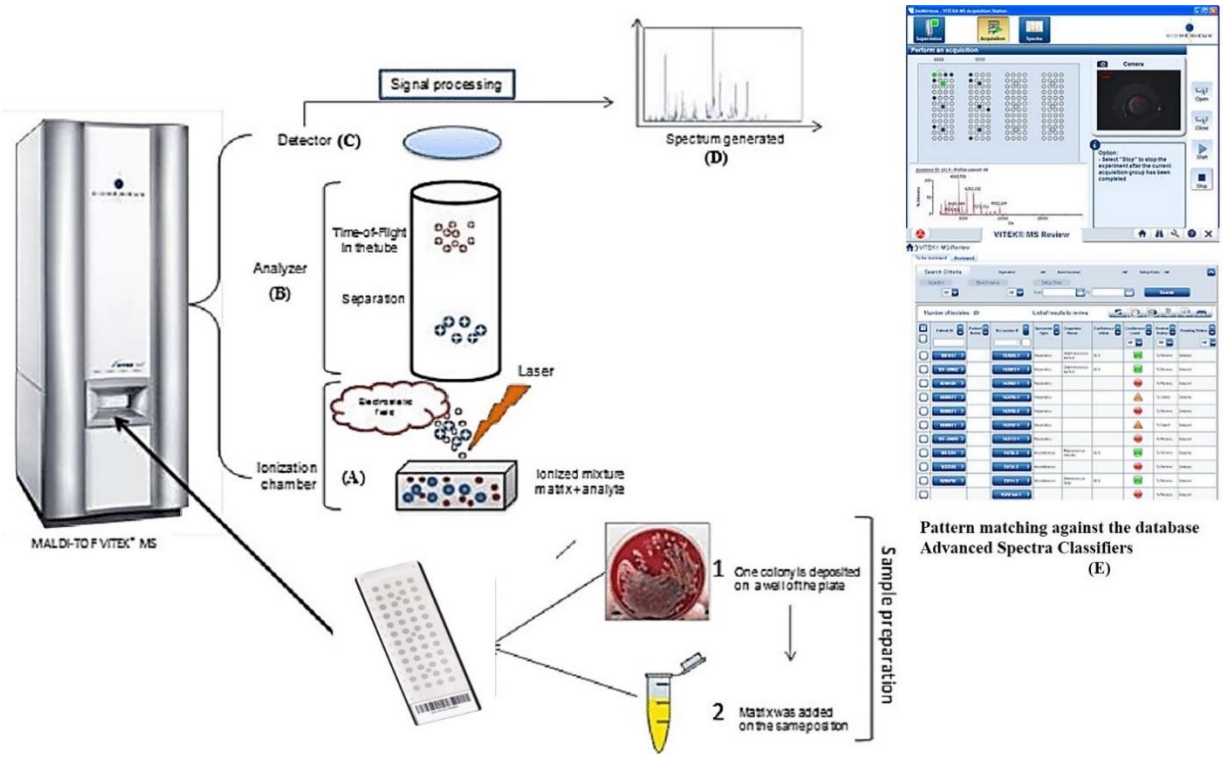


Figure 13. MALDI-TOF MS's operating principle and the sample preparation step for identification. The principle of this measurement is based on the ability of an electric and/or magnetic field to deflect a flow of ions, each with a mass and a charge proportional to their trajectories. Overall, mass spectrometry can be divided into three steps: the ionization chamber that produces ions in the gas phase (A), the analyzer which selects ions by the mass-to-charge ratio (m/z) (B), and the detector that converts the ionic current into electric current (C). Bombing with a laser beam generates ions in the ionization chamber. These ions are accelerated into an electric field which directs them to the analyzer that separates them according to their time-of-free flight (TOF: Time-Of-Flight). The smaller molecules grasp the detector first, followed by the biggest, according to the m/z ratio. Those which have the same

m/z ratio are then separated by an electrostatic mirror. The detector converts the received ions into electrical current which is amplified and digitized (D). Comparison with database reference spectra and species identification (E) [160].

### 4.6.3 Storage of bacterial and yeasts isolates

After identification, the species of microorganisms of interest in the study were stored. Isolates were frozen in Microbanks (ProLab Diagnostics) at -80°C.

## 4.7 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis, the "gold standard" fingerprinting method, is a laboratory technique used by scientists to produce a DNA fingerprint for a bacterial isolate. A bacterial isolate is a group of the same type of bacteria. The DNA fragments produce a DNA fingerprint with a specific pattern. Figure 14 shows an example of an agarose gel where each lane represents a DNA fingerprint or pattern. PFGE is different from conventional DNA electrophoresis because PFGE can separate very large fragments to generate a fingerprint by constantly changing the direction of the electric field [181].

Macro restriction analysis by PFGE of DNA was performed according to USA Centers for Disease Control and Prevention (CDC) highly standardized PFGE protocols with some minor modifications [181].

- ✓ Day 0

Grow bacterial strains on TSB (Tryptic Soy broth) agar plates for 24 to 48 hours, at 37ºC.

- ✓ Day 1

With a sterile polyester-fiber or cotton swab streak an isolated colony from Blood Columbia agar plates and in an Eppendorf resuspend the cells in 1ml of PIV buffer, centrifugate for 2 minutes in 12000rpm, discard the supernatant and resuspend again the pellet with PIV buffer adjusting the suspension at $10^9$ CFU/mL (colony-forming units per milliliter).

Prepare 2% Pulsed Field Certified Agarose (Bio-Rad) in TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) for PFGE plugs, dissolve the agarose by boiling, then cool at 70 °C (keep in thermoblock).

Mix the cells with an equal volume of molten (70 °C) agarose and pipet into the Eppendorf. Immediately, dispense part of the mixture into an appropriate well(s) of reusable plug mold without introducing bubbles. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4°C) for 5 minutes.

- Lysis of Cells in Agarose Plugs:

Label 2 ml Eppendorf with culture numbers and add 1 ml EC lysis solution with lysozyme and RNase (for all Gram-negative bacteria) or lysozyme, lysostaphin and RNase (for *S. aureus*). Trim excess agarose from top of plugs with a scalpel, razor blade, or similar instrument (optional). Open reusable plug mold and transfer plugs from mold with a spatula to an appropriately labeled tube. Gently shake the Eppendorf overnight at 37 ° C.

- ✓ Day 2

Remove the EC lysis solution from the Eppendorf, add 1ml TE 1x Buffer, and shake the tubes at 37°C incubator for 1 hour.

- DNA deproteinization of DNA in Agarose Plugs:

Add 1 ml per well ESP buffer with proteinase K (Sigma). Seal Eppendorf inside a heat-sealable bag. Place inside a shaking water bath, and shake gently overnight (or at least for 5 hours) at 55 °C.

- ✓ Day 3

Aspirate off the ESP buffer, plugs can be transferred to others tubes and add 1ml TE 1x Buffer, shake at 37°C for 15 min, the washes might be made 5 times. After removing the last wash, add 1 ml sterile TE to each well and store plugs at 4°C, if you don't use immediately.

- o Restriction Digestion of DNA in Agarose Plugs:

With a sterile spatula or pair of tweezers, carefully remove a DNA plug from its well and place it on a sheet of parafilm. Place the one plug inside a sterile Eppendorf (1,5ml) tube containing 300 µl fresh restriction enzyme buffer and enzyme ((SpeI, SmaI, XbaI, and Cfr9I) Thermo scientific). Use approximately 20 to 40 units of enzyme per reaction. The plugs should fit into the bottom of the tube. Place tubes at 37 °C incubator overnight.

- ✓ Day 4

Remove the Eppendorfs from 37°C incubator. Remove enzyme/buffer mixture and add 1mll 1X TBE. Incubate at 4°C for1 hour. Alternatively, digested plug slices can be kept in the refrigerator for up to three days if they are stored in 1X TBE.

- o Gel pouring:

Prepare a solution of 1% Pulsed Field Certified Agarose (Bio-Rad) in 0.5X TBE. Cool melted agarose in 55-60°C and carefully pour agarose into gel form (casting stand) fitted with a comb. Be sure there are no bubbles. Remove plug slices from tubes with the tapered end of the spatula and load into appropriate wells, including *S.aureus* genotype and Molecular weight (Chef DNA Size, std, Lambda Ladder 1703635-Bio-Rad) standards in outside lanes as needed . Gently push plugs to bottom and front of

wells with wide end of spatula. Manipulate position with the spatula and be sure that there are no bubbles. Fill all empty wells and, top off sample wells with molten 1% agarose (Certified Low Melting-Bio-Rad).

- Electrophoresis:

Pulsed field gel electrophoresis is run using the BioRad CHEF MAPPER TM system.

Fill gel chamber with 2L 0.5X TBE. Turn on the cooler and set it to 14 °C. Turn on the pump, set the pump speed to 40, and recirculate the buffer until it equilibrates to 14°C. Place gel inside the gel chamber and secure it with a clamp on each corner. Program run according to the instructions under two-state mode and start run. Run parameters will vary depending on the size of the fragments you are trying to resolve and the initial mAmps should be between 110-150 mAmps, angle 120°, and voltage 6 V/cm.

✓ Day 5

When electrophoresis run is over, turn off equipment; remove and place gel in a covered container with GelRed Nucleic Acid Stain 3X Water (Sigma). Stain gel for 20-30 min in a covered container.

Visualize gel under UV light and capture image on GenoSmart (VWR, USA).

### 4.7.1 Genotype analysis

DNA profiles were analyzed with InfoQuest FP 5.1 (Bio-Rad Laboratories, Hercules, USA), using the Dice correlation coefficient and Unweighted Pair Group Method with Arithmetic Mean (position tolerance and optimization between 1.0 and 1.1%). By applying the criteria of Tenover and colleagues (1995) [170], based on the differences in the numbers of bands: an isolate is considered unrelated to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with three or more independent genetic events (generally seven or more band differences),

we identified a cut-off value of 80% similarity to correctly cluster the PFGE profiles. Consequently, the strains showing ≥80% similarity were considered to be a single clone.

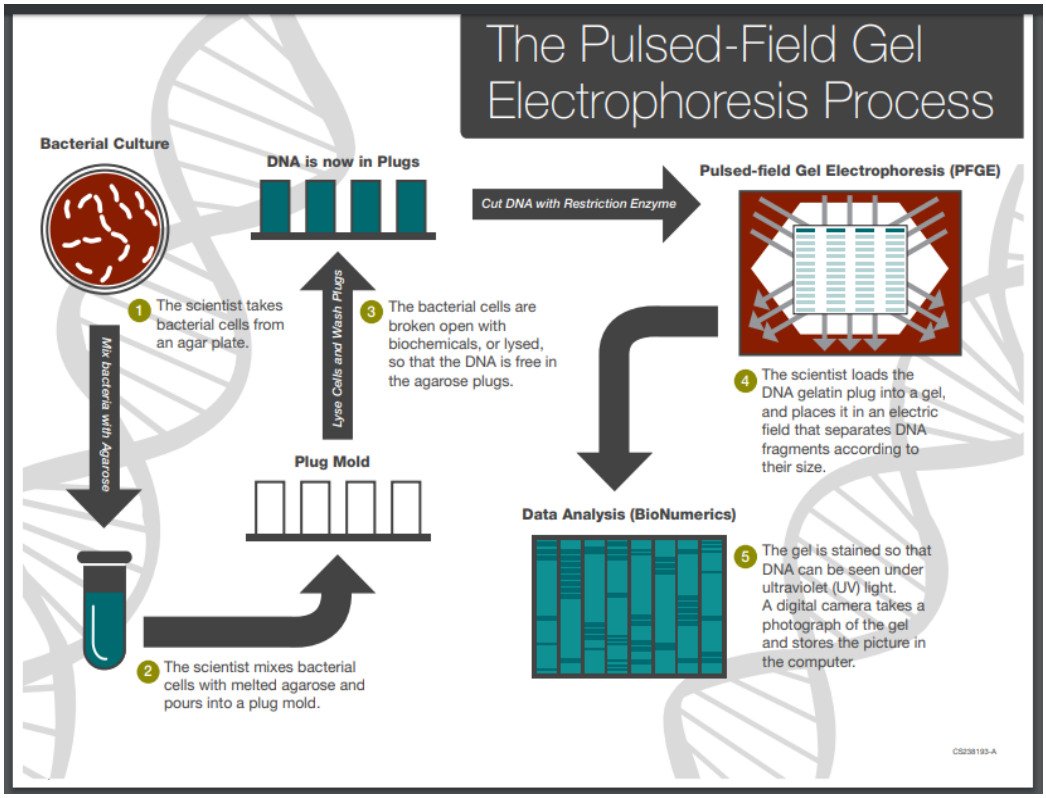


Figure 14. Schematic diagram of PFGE instrumentation. Contoured clamped homogeneous electric field (CHEF) systems use a hexagonal gel box that alters the angle of the fields relative to the agarose gel. After running the gel by PFGE, DNA fragments are visualized by staining with GelRed [182]

### 4.7.2 Solutions

| **PIV buffer:** |
|---|
| **1 M NaCl** |
| **10 mM Tris-HCl (pH 7.6)** |

| **EC lysis solution :** |
|---|
| **6 mM tris-HCl (pH 7.6)** |
| **1 M NaCl** |
| **100 mM EDTA** |
| **0.5% Brij-58** |
| **0.2% deoxycholate** |
| **0.5% Sarkosyl** |
| **1 mg/ml lysozyme** |
| **1mg/ml lysostaphine (only to *S. aureus*)** |
| **50 µg/ml RNase** |

| **ESP buffer:** |
|---|
| **0.5 M EDTA (pH 9.0 to 9.5)** |
| **1% Sarkosyl** |
| **50 µg/ml proteinase K** |

| **TE (pH 7.5):** |
|---|
| **10 mM Tris-HCl (pH 7.5)** |
| **1 mM EDTA** |

| **Recommended Protocol for Digestion** | | |
|---|---|---|
| Enzyme: | Restriction enzyme recognizes | Strains: |
| Sma I | 5'...C C C↓G G G…3'<br>3'...G G G↑C C C...5' | *S. aureus* |
| XbaI | 5'...T↓C T A G A…3'<br>3'...A G A T C↑T…5' | *A. xylosoxidans* |
| SpeI | 5'...A↓C T A G T...3'<br>3'...T G A T C↑A...5' | *P. aeruginosa*<br>*S. maltophilia*<br>*S. marcescens* |
| Cfr9I | 5'... C↓C C G G G...3'<br>3'...G G G C C ↑ C...5' | *S. aureus* |
| Nuclease-free water 268 µL | | |
| 10X Buffer Tango 30 µL | | |
| Enzyme 2-4 µL (20-40U) | | |

# 5. RESULTS

## 5.1 Population characteristics

A total of 59 patients were recruited for this study from the CF Centre of Verona (Italy). Among these, 38participated in the analysis of the oral cavity. The CF patients were classified into four groups based on their age (adult- pediatric) and the state of their colonisation by *P. aeruginosa*. The patient group showed an approximately balanced sex ratio (26m/33f) with a preponderance of females (56%) to male subjects compared to the European CF patient population (around 1.1 males for every female) [183]. The average age at inclusion was 16,5 years.

Eleven over 59 (19%) patients were homozygous for the most common CFTR mutation delta F508 and 35/59 (59%) heterozygous for the CFTR mutation delta F508whereas 13/59 (22%) patients carried other genotypes. A percentage of 86% patients suffered from exocrine pancreas insufficiency. Lung function testing during the study period yielded the average of FEV1% of 89,3% (40,3% to 123,9%), with a lower average in adult chronic patients (74,3%). The patients average was underweight x BMI ≤21,6 kg/m2 (for adults) and BMI < 50th percentile (for children). Twenty-four patients at the end of the study developed chronic lung infection by *S. aureus* (not included AC patients), where the majority are pediatric patients, as expected. Two patients, not classified as chronic at the beginning of the study, developed during the study a chronic lung infection by *P. aeruginosa.*

| Group | Total of patients (n = 59) | AC (n=9) | AN (n=15) | PN/PNP (n=25) | PO (n=10) |
|---|---|---|---|---|---|
| **Mean age** | 16.5 (7-26) | 20.5 (18-23) | 22(18-26) | 12.5 (7-17) | 11.5 (7-16) |
| **No. (%) of males** | 26 (44) | 3 (40) | 10 (67) | 9 (36) | 4 (40) |
| **No. (%) of females** | 33 (56) | 6 (64) | 5 (33) | 16 (64) | 6 (60) |
| **No. (%)F508del homo**[a] | 11 (19) | 1 (11) | 4 (27) | 3 (12) | 3 (30) |
| **No. (%)F508del hetero**[b] | 35 (59) | 7 (78) | 4 (47) | 16 (64) | 5 (50) |
| **No. (%) others Genotypes** | 13 (22) | 1 (11) | 4 (27) | 6 (24) | 2 (20) |
| **BMI males average** | 19.3 | 19.9 | 20.7 | 42.9 (<50$^{th}$) | 35.8 (<50$^{th}$) |
| **BMI females average** | 19.2 | 20.7 | 21.6 | 49.6 (<50$^{th}$) | 34.0 (<50$^{th}$) |
| **No. (%) Ex.panc.insuff.**[c] | 51 (86) | 8 (89) | 14 (93) | 19 (76) | 10 (100) |
| **FEV1% average** | 89.3 (40.3-123.9) | 74.3 | 84.3 | 96.5 | 94.81 |
| **No. (%) of patients with chronic infection in the final of the study (3 positive samples)** | | | | | |
| ***P. aeruginosa*** | 11 (19) | 9 (100) | 0 | 1 (4) | 1 (10) |
| ***S. aureus*** | 25 (42) | 1 (11) | 4 (27) | 16 (64) | 4 (40) |

Table 2. Demographics of CF patients from CF Centre of Verona (Italy), including classification based on the status of *P. aeruginosa* lung colonisation at the beginning of the study (AC = adults with chronic *P. aeruginosa* infection; AN = adults free from *P. aeruginosa* lung colonisation; PN = paediatrics free from *P. aeruginosa* lung colonisation; PO = paediatrics with occasional *P. aeruginosa* infection), number of patients per group, age, sex, FEV1, BMI (body mass index) and BMI percentile for children, genotype, exocrine pancreatic insufficiency, chronic infection an exacerbation numbers are reported as average (range), respectively.
[a]F508del homozygous
[b]F508del heterozygous
[c]exocrine pancreatic insufficiency

At least 3 samples from each site were provided during the study of each patient, according to the beginning of the first collection, which was scheduled during the visit at the Verona Cystic Fibrosis Centre.

However, as shown in Table 3, the collection was heterogeneous, often due to the absence of patients on the day of the visit and/or patients not bringing the material, such as toothbrushes.

Two to five longitudinal samples of sputum and nasal lavage and one to four of saliva and toothbrush were collected from each subject: of most patients were collected three sputum (n=45 patients) and three nasal lavages (n=44 patients) samples, two

samples of saliva (n=30 patients) and two toothbrushes (n=21 samples). Only three patients collected single samples of saliva and toothbrush (Table 3).

At the end of the study was analyzed 190 sputum samples, 189 nasal lavage samples, 79 saliva, and 87 toothbrushes samples from 59 CF patients, with a total of around 1000 bacterial strains, 537 which belong to the bacterial species of *P. aeruginosa, S. maltophilia, A. xylosoxidans, S. marcescens* and *S. aureus*. At least four strains from each patient were genotyped by PFGE.

| Type of sample | 1 sample | 2 samples | 3 samples | 4 samples | 5 samples |
|---|---|---|---|---|---|
| **Nasal lavage** | 0 | 3 | 44 | 9 | 3 |
| **Sputum** | 0 | 2 | 45 | 9 | 3 |
| **Saliva** | 3 | 30 | 4 | 1 | 0 |
| **Toothbrush** | 3 | 22 | 11 | 2 | 0 |

Table 3. Number of patients that were sampled 1, 2, 3, 4 or 5 times for each type of sample collected during the study (nasal lavage, sputum, saliva, toothbrush).

## 5.2 Bacterial presence in samples from adult patients with chronic (AC) infection by *P. aeruginosa*

All AC individuals (n=9) showed the presence of *P. aeruginosa* in sputum and Nasal Lavage. Only in one of these patients (AC4), in addition to *P. aeruginosa* we isolated *S. aureus* from both samples in all visits (Table 4). In some cases, other opportunistic bacterial species appear, but specifically in a single visit and site.

| Patient ID | Sample | Visit 1 | Visit 2 | Visit 3 | N. samples |
|---|---|---|---|---|---|
| AC1 | Nasal lavage | PAE | PAE | PAE | 3 |
| | Sputum | PAE, PMA | PAE, ACX | PAE, ACX | 3 |
| AC2 | Nasal lavage | PAE | PAE | n | 3 |
| | Sputum | PAE | PAE, CA | PAE | 3 |
| AC3 | Nasal lavage | PAE | PAE | PAE | 3 |
| | Sputum | PAE | PAE | PAE, CA | 3 |
| AC4 | Nasal lavage | PAE, SAU | PAE, SAU | PAE, SAU | 3 |
| | Sputum | PAE, SAU | PAE, SAU, PMA | PAE, SAU | 3 |
| AC5 | Nasal lavage | PAE | PAE, | PAE, PMA | 3 |
| | Sputum | PAE | PAE | PAE | 3 |
| AC6 | Nasal lavage | PAE | PAE | PAE | 3 |
| | Sputum | PAE | PAE | PAE | 3 |
| AC7 | Nasal lavage | PAE | CA | PAE, SAU | 3 |
| | Sputum | PAE | CA | PAE, SAU, CA | 3 |
| AC8 | Nasal lavage | PAE | PAE | PAE | 3 |
| | Sputum | PAE | PAE, CA | CA | 3 |
| AC9 | Nasal lavage | PAE | PAE | PAE | 3 |
| | Sputum | PAE | PAE, CA | PAE | 3 |

Table 4. Examined bacterial species collected from nasal lavage, sputum, saliva, and toothbrush of adult patients with chronic (AC) infection by *P. aeruginosa* at different visits during the study. PAE = *P. aeruginosa*, SAU = *S. aureus*, PMA = *S. maltophilia*, AXY = *A. xylosoxidans*, SMA = *S. marcescens*, CA = *Candida spp.*, n= no isolation of the investigated species, - = sample not available. Empty rectangle = patient not included in the analysis of saliva and toothbrush. Number of samples for each patient is also indicated. Patients with no saliva samples nor toothbrushes were not included in the collection of these samples.

## 5.3 Bacterial presence in samples from adult patients free from *P. aeruginosa* lung colonisation

In the AN group (n=15), the selected bacterial species were isolated from the nasal and the sputum of 11 patients (Table 5). Seven patients (AN3, AN7, AN8, AN9, AN12, AN13, AN15) presented *S. aureus* in most of the samples, both nasal lavage and sputum, obtained in the three visits. Interestingly, one of these patients (AN9), free from *P. aeuruginosa* lung colonisation at the enrollment time, showed the acquisition of this bacterial species in both LAW and UAW. As regards the other investigated species, two patients (AN12 and AN15) resulted positive for *S. aureus* in all samples, also including saliva and toothbrush. Five patients were positive for *A. xylosoxidans*:

three patients (AN2, AN4, AN10) showed the presence of this bacterial species in both sputum and nasal lavage, while in two other patients (AN5 and AN9) it was isolated only from the sputum. Four of these five patients (AN2, AN5, AN9 and AN10) resulted positive for the presence of *A. xylosoxidans* also in saliva. Another patient (AN14) was positive for *S. maltophilia* in sputum, nasal lavage, and saliva samples obtained during four different visits. Based on the least frequent opportunistic bacteria evaluated, only three patients (AN5, AN6, and AN11) had S. *marcescens* and were mainly isolated from oral areas (table 5).

| Patient ID | Sample | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | N. samples |
|---|---|---|---|---|---|---|---|
| AN1 | Nasal lavage | n | n | n | n | - | 4 |
| | Sputum | n | n | CA | CA | - | 4 |
| | Saliva | n | n | n | - | - | 3 |
| | Toothbrush | n | n | AXY, CA | - | - | 3 |
| AN2 | Nasal lavage | AXY | PAE | n | - | - | 3 |
| | Sputum | AXY | AXY, SAU, CA | AXY | - | - | 3 |
| | Saliva | AXY | PAE, SAU | - | - | - | 2 |
| | Toothbrush | CA | - | - | - | - | 1 |
| AN3 | Nasal lavage | SAU | n | SAU | - | - | 3 |
| | Sputum | SAU | SAU, PMA | SAU | - | - | 3 |
| | Saliva | SAU, PMA | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| AN4 | Nasal lavage | AXY | n | - | - | - | 2 |
| | Sputum | AXY | SAU, PMA | - | - | - | 2 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| AN5 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | AXY | AXY | AXY, CA | - | - | 3 |
| | Saliva | AXY, SMA | AXY, PMA | - | - | - | 2 |
| | Toothbrush | n | SMA, CA | - | - | - | 2 |
| AN6 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | SMA | n | n | - | - | 3 |
| | Saliva | n | n | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| AN7 | Nasal lavage | n | n | SAU | - | - | 3 |
| | Sputum | SAU, PMA | SAU, CA | SAU | - | - | 3 |
| | Saliva | SAU | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| AN8 | Nasal lavage | n | SAU | SAU | - | - | 3 |
| | Sputum | PMA | SAU | SAU, PMA, CA | - | - | 3 |
| | Saliva | SMA, SAU, CA | CA | - | - | - | 2 |
| | Toothbrush | n | - | - | - | - | 1 |
| AN9 | Nasal lavage | PAE | PAE, SAU | PAE, SAU | PAE, SAU | PAE, SAU | 5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sputum | PAE, AXY | PAE, SAU, AXY | AXY | AXY | AXY, SAU | 5 |
| | Saliva | SAU, AXY, CA | - | - | - | - | 1 |
| | Toothbrush | n | n | - | - | - | 1 |
| AN10 | Nasal lavage | n | n | AXY | - | - | 3 |
| | Sputum | AXY | AXY | AXY | - | - | 3 |
| | Saliva | PMA, CA | AXY, PMA, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| AN11 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | SAU | SAU | n | - | - | 3 |
| | Saliva | SAU, CA | n | - | - | - | 2 |
| | Toothbrush | SMA | n | - | - | - | 2 |
| AN12 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | n | SAU | SAU | - | - | 3 |
| | Saliva | SAU, CA | SAU, CA | SAU, CA | - | - | 3 |
| | Toothbrush | n | SAU | SAU | - | - | 3 |
| AN13 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| AN14 | Nasal lavage | PMA | n | PMA | n | - | 4 |
| | Sputum | PMA | PMA | PMA | PMA | - | 4 |
| | Saliva | PMA | PMA, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| AN15 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU, PAE | - | - | 3 |
| | Saliva | SAU | - | SAU | - | - | 2 |
| | Toothbrush | SAU | CA | n | - | - | 3 |

Table 5. Examined bacterial species collected from nasal lavage, sputum, saliva and toothbrush of adult patients free from *P. aeruginosa* (AN) lung colonisation at different visits during the study. PAE = *P. aeruginosa*, SAU = *S. aureus*, PMA = *S. maltophilia*, AXY = *A. xylosoxidans*, SMA = *S. marcescens*, CA = *Candida spp.*, n= no isolation of the investigated species, - = sample not available. Empty rectangle= patient not included in the analysis of saliva and toothbrush. Number of samples for each patient is also indicated. Patients with no saliva samples nor toothbrushes were not included in the collection of these samples.

## 5.4 Bacterial presence in samples from paediatric patients with occasional *P. aeruginosa* colonisation

The Pediatric patients of the PO group (n=10), seven of them showed positive samples for the presence of the selected bacterial species, mainly *S. aureus* and *P. aeuruginosa*. Interestingly, three of the patients (PO1, PO5, and PO8) presented the same bacterial species (*S. aureus* alone or in combination with *P. aeruginosa*) in all four types of samples. One patient (PO4) presented in 3 sites (sputum, saliva, and toothbrushes) *S. marcescens*, an observation to consider, it first appeared in the saliva and toothbrush and then on another visit was also isolated in the sputum. *A.xylosoxidans* were found in the same patient (PO10) in all visits in three samples (nasal lavage, sputum, and saliva). Moreover, one of the patients (PO6) presented *P. aeruginosa* only in the nasal lavage during the first and second visits but at the third visit *P. aeruginosa* was isolated from the sputum too (Table 6).

| Patient ID | Sample | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | N. samples |
|---|---|---|---|---|---|---|---|
| PO1 | Nasal lavage | n | n | SAU | SAU, PAE | - | 4 |
| | Sputum | PAE | SAU | SAU, PAE | SAU, PAE | - | 4 |
| | Saliva | n | SAU, PAE | - | - | - | 2 |
| | Toothbrush | n | PAE | PAE | - | - | 3 |
| PO2 | Nasal lavage | n | n | SAU | - | - | 3 |
| | Sputum | n | SAU | n | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PO3 | Nasal lavage | n | n | - | - | - | 2 |
| | Sputum | PAE | PAE, CA | | - | - | 2 |
| | Saliva | CA | CA | - | - | - | 2 |
| | Toothbrush | CA | CA | - | - | - | 2 |
| PO4 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | SAU | SAU, PMA | SAU, SMA | - | - | 3 |
| | Saliva | SAU, SMA | SAU, SMA | - | - | - | 2 |
| | Toothbrush | SMA | SMA | - | - | - | 2 |
| PO5 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU, PAE | SAU, PAE | SAU | - | - | 3 |
| | Saliva | SAU, PAE | SAU, PMA | - | - | - | 2 |
| | Toothbrush | SAU, PAE | n | - | - | - | 2 |
| PO6 | Nasal lavage | PAE | n | CA | PAE | - | 4 |
| | Sputum | n | n | PAE | n | - | 4 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PO7 | Nasal lavage | PAE | n | n | - | - | 3 |
| | Sputum | PAE, PMA, SMA | PAE, SMA, CA | PAE, SMA, CA | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PO8 | Nasal lavage | n | SAU | n | SAU | SAU | 5 |
| | Sputum | SAU | SAU, SMA | SAU | SAU, PMA | SAU, CA | 5 |
| | Saliva | SAU, PMA, CA | SAU | SAU | - | - | 3 |
| | Toothbrush | PMA | SAU, CA | SMA | - | - | 3 |
| PO9 | Nasal lavage | n | SAU, PAE | PAE | - | - | 3 |
| | Sputum | n | SAU | SAU, PAE, CA | - | - | 3 |
| | Saliva | SAU, CA | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PO10 | Nasal lavage | AXY, PAE | AXY | AXY | - | - | 3 |
| | Sputum | AXY | AXY | AXY | - | - | 3 |
| | Saliva | AXY | AXY, CA | - | - | - | 2 |
| | Toothbrush | CA | CA | - | - | - | 2 |

Table 6. Examined bacterial species collected from nasal lavage, sputum, saliva and toothbrush of paediatric patients occasionally colonized by *P. aeruginosa* (PO) at different visits during the study. PAE = *P. aeruginosa,* SAU = *S. aureus,* PMA = *S. maltophilia,* AXY = *A. xylosoxidans*, SMA = *S. marcescens*, CA = *Candida spp.*, n= no isolation of the investigated species, - = sample not available. Empty rectangle = patient not included in the analysis of saliva and toothbrush. Number of samples for each patient is also indicated. Patients with no saliva samples nor toothbrushes were not included in the collection of these samples.

## 5.5 Bacterial presence in samples from paediatric patients free from *P. aeruginosa* lung colonisation

Within the PN group (n=25), several individuals (n=6) presented polyposis in their nasal/paranasal area. Although this is a condition that could favor the persistence of microbial cells, no difference in the prevalence of the investigated bacterial species was noticed between children with and without polyposis. Nineteen PN patients (PN1, PN2, PN4, PN5, PN6, PN7, PN8, PN9, PN10, PN11, PN12, PN13, PN14, PN15, PN17, PN18, PN21, PN22, PN24) presented *S. aureus* in most nasal lavages and sputum samples. Thirteen of them were also positive for this species in saliva, and four also in the toothbrush. Among the positive patients for *S. aureus*: i) three children were positive also for *S. maltophilia* in most samples: PN4 (3 sputum and 2 saliva samples), PN6 (3 sputum samples), and PN9 (2 nasal lavages, 4 sputum, and 2 saliva samples); 2) four patients were positive also for *A. xylosoxidans* in different types of samples: PN3 (3 nasal lavages, 4 sputum, and 3 saliva samples), PN5 (1 nasal lavage and 2 sputum), PN14 (1 nasal lavage, 3 sputum, and 2 saliva samples), PN17 (1 sputum, 2 saliva samples, and 1 toothbrush); 3) PN17 was positive also for *P. aeruginosa* in almost all the samples during four visits. Interestingly, patients PN1 were initially positive for *P. aeruginosa* only in the nasal lavage, then this species was isolated also from sputum at a later visit. Another interesting the case of the patient PN22, that during the first and second visits shown positive for *S. aureus* only in the nasal lavage while at the third visit was positive also in the sputum. A final observation to consider

is that the opportunistic bacteria *S. marcescens* was isolated only once in a sputum (PN4) (Table 7).

| Patient ID | Sample | Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | N. samples |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| PN1 | Nasal lavage | SAU, PAE, PMA | n | n | - | - | 3 |
| | Sputum | SAU | SAU, PAE | SAU, CA | - | - | 3 |
| | Saliva | SAU | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| PN2 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU, PAE | - | - | 3 |
| | Saliva | SAU, CA | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| PN3 | Nasal lavage | n | AXY | AXY | AXY | - | 4 |
| | Sputum | AXY | AXY, SAU | AXY, SAU | AXY, SAU, CA | - | 4 |
| | Saliva | AXY, CA | AXY, CA | AXY, CA | CA | - | 4 |
| | Toothbrush | n | n | CA | n | - | 4 |
| PN4 | Nasal lavage | n | SAU | SAU | - | - | 3 |
| | Sputum | SAU, SMA, PMA | SAU, PMA | SAU, PMA | - | - | 3 |
| | Saliva | SAU, PMA | SAU, PMA | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| PN5 | Nasal lavage | n | SAU | SAU, AXY | - | - | 3 |
| | Sputum | SAU | SAU, AXY, CA | SAU, AXY, CA | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN6 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU, PMA | SAU, PMA | SAU, PMA | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN7 | Nasal lavage | n | SAU | n | - | - | 3 |
| | Sputum | SAU | SAU | SAU, PAE | - | - | 3 |
| | Saliva | SAU, CA | SAU | - | - | - | 2 |
| | Toothbrush | n | n | - | - | - | 2 |
| PN8 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU, PMA | SAU, PMA | SAU, PAE | - | - | 3 |
| | Saliva | SAU, PMA | PAE | - | - | - | 2 |
| | Toothbrush | SAU | SAU | SAU | - | - | 3 |
| PN9 | Nasal lavage | SAU, PMA | SAU, PMA | SAU | n | - | 4 |
| | Sputum | SAU, PMA | SAU, PMA | PMA | PMA | - | 4 |
| | Saliva | SAU, PMA | PMA | - | - | - | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Toothbrush | n | n | - | - | - | 2 |
| PN10 | Nasal lavage | SAU | - | SAU | - | - | 2 |
| | Sputum | SAU | SAU, CA | SAU | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN11 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU, PAE | - | - | 3 |
| | Saliva | SAU | - | - | - | - | 1 |
| | Toothbrush | n | - | - | - | - | 1 |
| PN12 | Nasal lavage | SAU | SAU | SAU, AXY | SAU | SAU | 5 |
| | Sputum | SAU | SAU | CA | n | SAU | 5 |
| | Saliva | SAU | SAU | - | - | - | 2 |
| | Toothbrush | SAU | CA | - | - | - | 2 |
| PN13 | Nasal lavage | SAU | SAU, PMA | SAU | - | - | 3 |
| | Sputum | SAU | SAU, PMA, CA | SAU | - | - | 3 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN14 | Nasal lavage | SAU, PMA | n | AXY, PMA | - | - | 3 |
| | Sputum | SAU, AXY | SAU, AXY | SAU, AXY, PMA | - | - | 3 |
| | Saliva | SAU, AXY | SAU, AXY, CA | - | - | - | 2 |
| | Toothbrush | CA | n | - | - | - | 2 |
| PN15 | Nasal lavage | n | SAU | SAU | - | - | 3 |
| | Sputum | n | SAU, AXY | SAU | - | - | 3 |
| | Saliva | - | PAE | SAU | - | - | 2 |
| | Toothbrush | n | n | PAE | - | - | 3 |
| PN16 | Nasal lavage | n | EXO | PMA | - | - | 3 |
| | Sputum | n | n | PMA, EXO | - | - | 3 |
| | Saliva | n | EXO | - | - | - | 2 |
| | Toothbrush | CA | n | - | - | - | 2 |
| PN17 | Nasal lavage | PMA | SAU, PAE | SAU | SAU | - | 4 |
| | Sputum | SAU, PAE, AXY | SAU, PAE | SAU, PAE, CA | SAU, PAE, CA | - | 4 |
| | Saliva | SAU, PAE, AXY, CA | SAU, PAE, CA | SAU, PAE, AXY, CA | - | - | 3 |
| | Toothbrush | SAU, PAE | SAU, CA | SAU, AXY, CA | n | - | 4 |
| PN18 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU, CA | SAU, PMA, CA | - | - | 3 |
| | Saliva | SAU | SAU, CA | - | - | - | 2 |
| | Toothbrush | n | n | n | - | - | 3 |
| PN19 | Nasal lavage | n | n | n | n | - | 4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sputum | SAU | PMA | n | CA | - | 4 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN20 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | SAU, PAE | n | PAE | - | - | 3 |
| | Saliva | PMA | n | - | - | - | 2 |
| | Toothbrush | PMA | n | - | - | - | 2 |
| PN21 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU | - | - | 3 |
| | Saliva | SAU | SAU | - | - | - | 2 |
| | Toothbrush | SAU | SAU, PMA | SAU | - | - | 3 |
| PN22 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | n | n | SAU | - | - | 3 |
| | Saliva | SMA | n | - | - | - | 2 |
| | Toothbrush | n | n | n | - | - | 3 |
| PN23 | Nasal lavage | n | SAU | SAU | PAE | - | 4 |
| | Sputum | n | PMA | PMA | PMA, PAE | - | 4 |
| | Saliva | | | | | | 0 |
| | Toothbrush | | | | | | 0 |
| PN24 | Nasal lavage | SAU | SAU | SAU | - | - | 3 |
| | Sputum | SAU | SAU | SAU | - | - | 3 |
| | Saliva | - | - | - | - | - | 0 |
| | Toothbrush | - | - | - | - | - | 0 |
| PN25 | Nasal lavage | n | n | n | - | - | 3 |
| | Sputum | PMA | PAE | PMA | - | - | 3 |
| | Saliva | - | n | - | - | - | 1 |
| | Toothbrush | n | n | n | - | - | 3 |

Table 7. Examined bacterial species collected from nasal lavage, sputum, saliva and toothbrush of paediatric patients never colonized by *P. aeruginosa* (PN) at different visits during the study. PAE = *P. aeruginosa*, SAU = *S. aureus*, PMA = *S. maltophilia*, AXY = A. *xylosoxidans*, SMA = *S. marcescens*, CA = *Candida spp*., n = no isolation of the investigated species, - = sample not available. Empty rectangle = patient not included in the analysis of saliva and toothbrush. Number of samples for each patient is also indicated. Patients with no saliva samples nor toothbrushes were not included in the collection of these samples.

## 5.6 Prevalence of the different bacterial species in the patient's samples

### 5.6.1 Microbiological analysis

Numerous bacteria were frequently isolated from sinus, sputum, saliva, and toothbrushes cultures of CF patients including *P. aeruginosa*, *S.aureus*. *S. maltophilia*, *A. xylosoxidans*, *S. marcescens* interestingly, in all four groups of patients (AC, AN, PN, and PO) (Table 8).

Taking into account as positives all the samples with the presence of at least one of the selected species and considering all groups of patients (exception for AC, since we already know that they are chronic for *P. aeruginosa*), the percentages of positivity for each type of sample were 85% (138 of 163 samples) for sputum, 62% (100 of 162 samples) for nasal lavage, 80% (63 of 79 samples) for saliva and 30% (26 of 87 samples) for toothbrushes. We observed that *S. aureus* is the most abundant species in sputum 59% (n=96), nasal lavage 49% (n=79), saliva 58% (n=46), and toothbrush 17% (n=15), outdistancing the prevalence of the other investigated species. *S. maltophilia* and *A. xylosoxidans* were frequently present in LAW and oral cavity, being isolated from 21% (n=35) and 18% (n=29) of sputum samples, and 19% (n=15) and 18% (n=14) of saliva samples, respectively. *P. aeruginosa* showed high prevalence in sputum 17% (n=28), nasal lavage 10% (n=16) and saliva 10% (n=8). *S. maltophilia* was present in 3% of toothbrushes and never in nasal lavage. Interestingly, among other isolated species, we observed a high incidence of *Candida spp.*, which was isolated from 7% (n=12) of sputum, 1% (n=1) of nasal lavage, 43% (n=34) of saliva and 17% (n=15) of toothbrushes samples. The overall prevalence of each species among all samples is reported in Figure 15.

At the end of the study, chronic colonisation of the UAW with *S. aureus* and/or *P. aeruginosa* and/or *A. xylosoxidans* was present in 17/50 (34 %), 1/50 (2%) and 2/50 (4%) patients. In almost all of these patients (94%), chronic UAW colonisation with *S. aureus* or *P. aeruginosa* or *A. xylosoxidans* was accompanied by intermittent or persistent LAW and oral colonisation with the respective pathogen.

In the AC group, we noticed a co-infection of *P.aeruginosa* with other species, such as *S.aureus* and *S.maltophilia*, in 19% and 7% of sputum samples and 15% and 4% of nasal lavage, respectively. *A. xylosoxidans* was isolated from only one sputum

sample. *Candida spp.* again had a high incidence on the samples, 26% of sputum and 4% of nasal lavage.

| Bacterial strains | Sputum | Nasal Lavage | Saliva | Toothbrush |
|---|---|---|---|---|
| *P. aeruginosa* | 53 (28%) | 41 (22%) | 8 (10%) | 5 (6%) |
| *S. aureus* | 101 (53%) | 83 (44%) | 46 (58%) | 15 (17%) |
| *S. maltophilia* | 37 (19%) | 11 (6%) | 15 (19%) | 2 (2%) |
| *A. xylosoxidans* | 30 (16%) | 12 (6%) | 14(18%) | 3 (3%) |
| *S. marcescens* | 7 (4%) | 0 | 7 (9%) | 5 (6%) |
| *Candida spp.* | 32(17%) | 2(1%) | 34(43%) | 15(17%) |

Table 8. Prevalence of each species in the different types of samples considering all subjects enrolled (AC, AN, PN, and PO patients). Number and percentages (in brackets) of positive samples are reported.

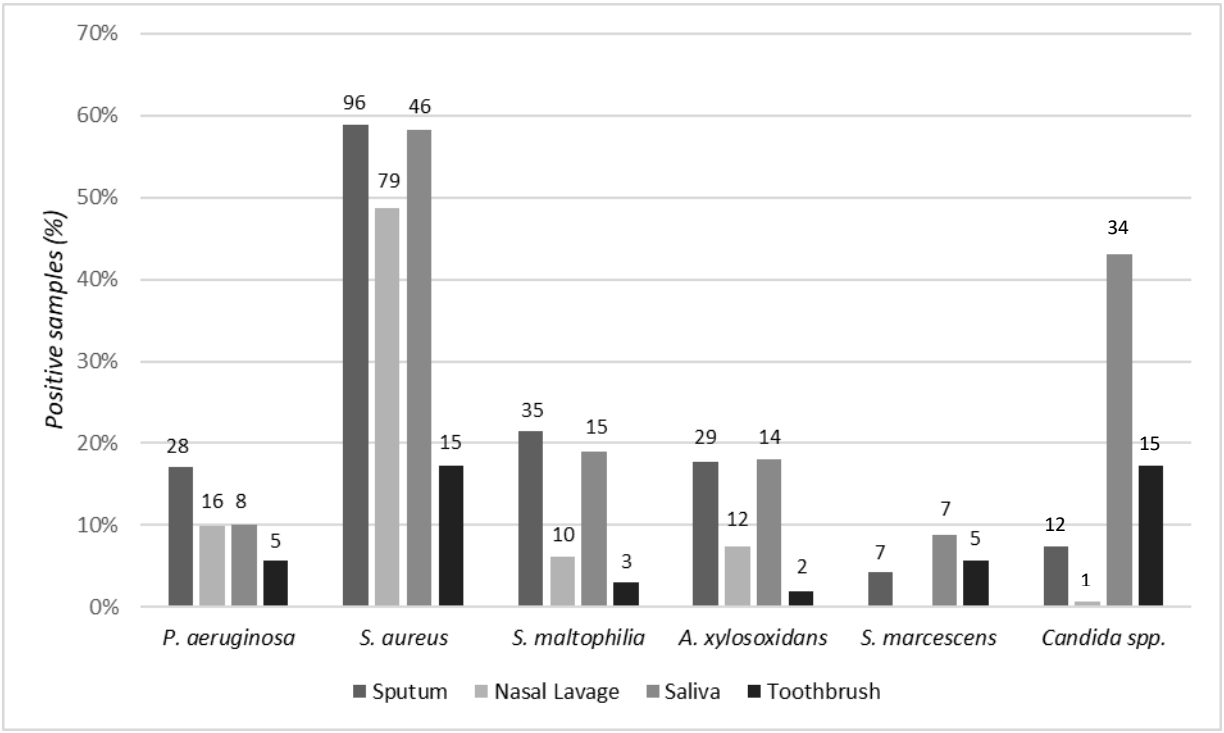


Figure 15. Prevalence of the examined species isolated from the sputum, nasal lavage, saliva, and toothbrush (considering AN, PN and PO patients). Percentage of positive samples is indicated on the X axis; number of positive samples is shown above bars.

## 5.7 Molecular typing of the bacterial strains isolated in the study

To verify the occurrence of transmission between the oral environment and airways, the clonal identity of the investigated species (*P. aeruginosa, S. aureus, S. maltophilia, A. xylosoxidans* and *S. marcescens*) isolated from nasal lavage, sputum, and/ or saliva and toothbrush of the same patients were assessed by molecular typing (PFGE). The reproducibility of the PFGE profiles was confirmed by the analysis *of S.aureus* (strain from San Rafaelle -Milan) in gel experiments, while the stability of the PFGE profiles was confirmed by multiple passages of the isolates and PFGE analysis. In Figure 16, Figure 17, Figure 18, Figure 19, and Figure 20 show some examples regarding the 5 bacterial species analyzed the isolates were defined as identical PFGE types if they were ≥80% identical.

To level of clarification, the first collection of sputum and nasal lavage there were no collection of saliva and toothbrushes because in the first visit the toothbrushes were delivered to the patient, thus, generally the first collection of toothbrushes and saliva correspond to the next visit of NL and sputum.

The genotypic analysis allowed us to investigate if (1) each patient was colonized by a unique genotype for a bacterial species (2) if the patient carried more than one genotype and (3) if the same genotype colonized both upper, lower airways and oral cavity in the patient.

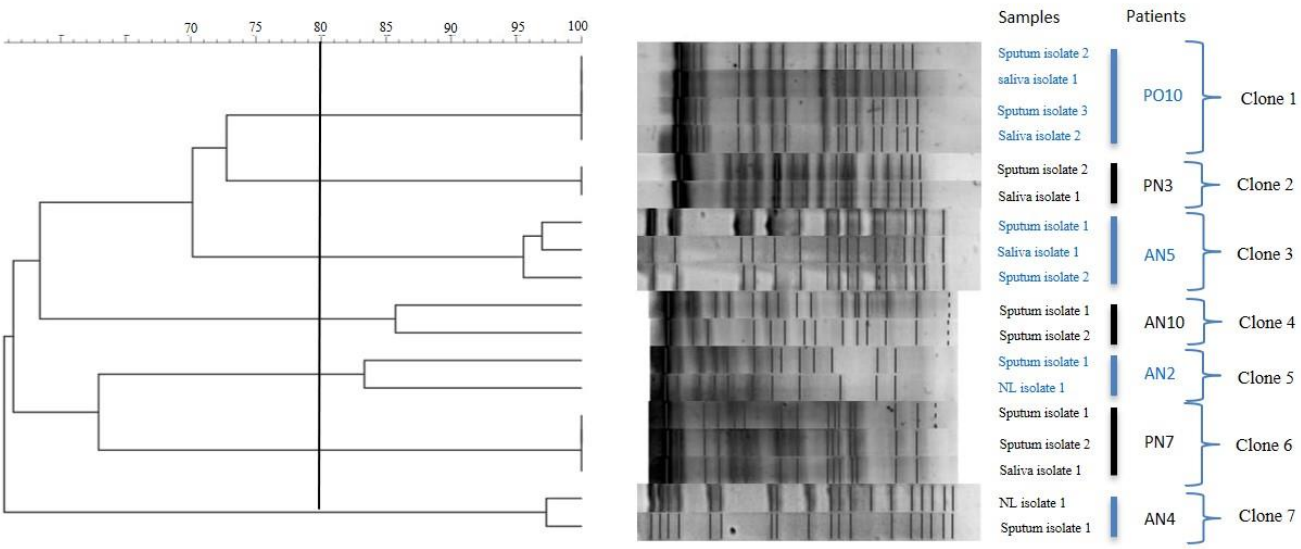


Figure 16. PFGE of salivary, pulmonary, and nasal lavage (NL) *A. xylosoxidans* isolates. Analysis of the genetic relationship with the XbaI restriction enzyme was performed with the 7 patients. Seven clonal groups were found. The threshold of 80% similarity is indicated by the vertical line.

For instance, in Figure 16 and Figure 17, sixteen strains of *A. xylosoxidans* and the twelve strains of *S. maltophilia* isolated restricted by XbaI and SpeI which belong to 7 (AN2, AN4, AN5, AN10, PO10, PN3, PN14) and 3 patients (AN14, PN4, PN9), each patient presented the same strain clones from different sites, similarity> 80%, that is, 7 groups of clones from *A. xylosoxidans* and 3 groups of clones from *S. maltophilia*, with no cluster of clone between the strains of different patients.

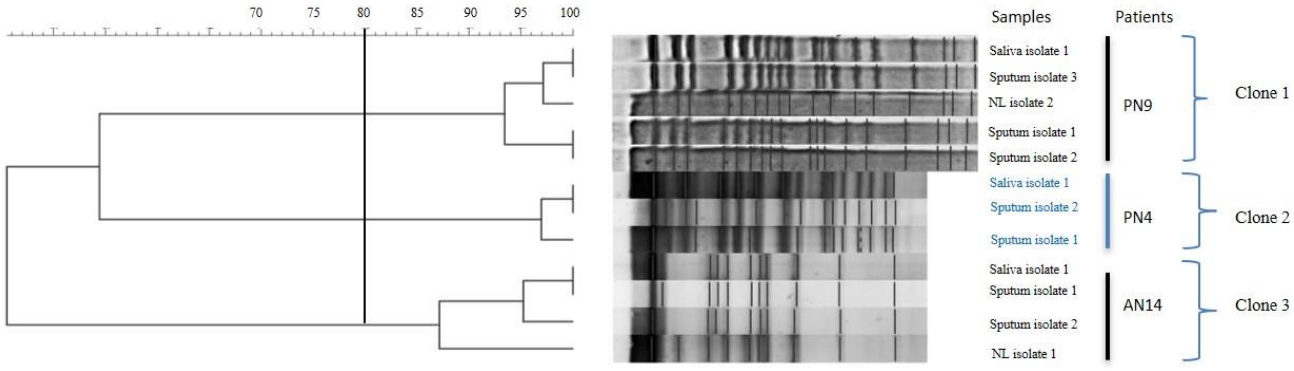


Figure 17. PFGE of salivary, pulmonary, and nasal lavage (NL) *S. maltophilia* isolates. Analysis of the genetic relationship with the SpeI restriction enzyme was performed with the 3 patients. Three clonal groups were found. The threshold of 80% similarity is indicated by the vertical line

Among the sputum samples, saliva and toothbrushes positive for *S. marcescens* in patient PO4, isolated on different visits, presented a single clone group, with strains with a high level of similarity ≥ 93% (Figure 18).

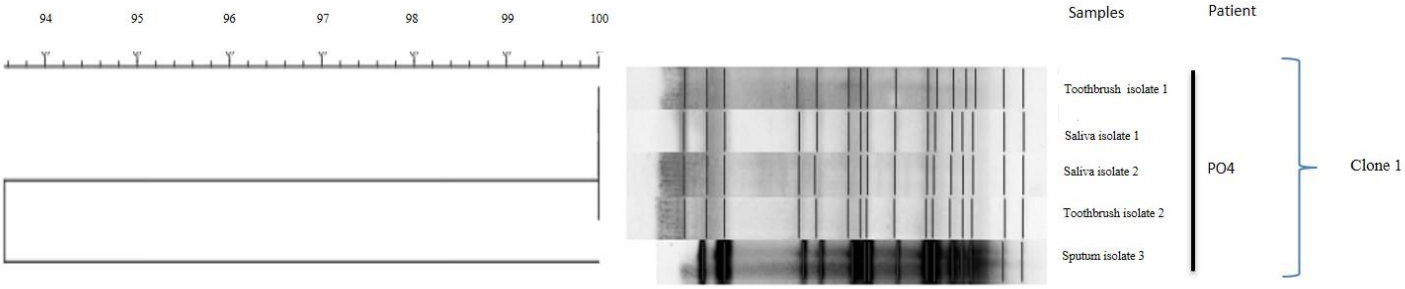


Figure 18. PFGE of salivary, toothbrush, and pulmonary *S.marcescens* isolates. Analysis of the genetic relationship with the SpeI restriction enzyme was performed with 1 patient. Only one clonal group was found (similarity ≥ 93%).

To *S. aureus* PFGE analysis restricted by SmaI and Cfr9I, identified 82 genotypes that grouped into 21 clones groups with ≥ 80% pattern similarity (Figure 19). The strains of pediatric patients PN12, PN7, PN24, and PO9 presented different clonal groups: clone 2, clone and clone 12; clone 11 and clone 12; clone 11 and clone 13; and clone 12 and sputum isolated 3 respectively. Clonal *S. aureus* strains were detected in four pairs of individuals (AN11 and AN9, PN7, PN12 and PO9, PN7 and PN24, PN1 and AN7)

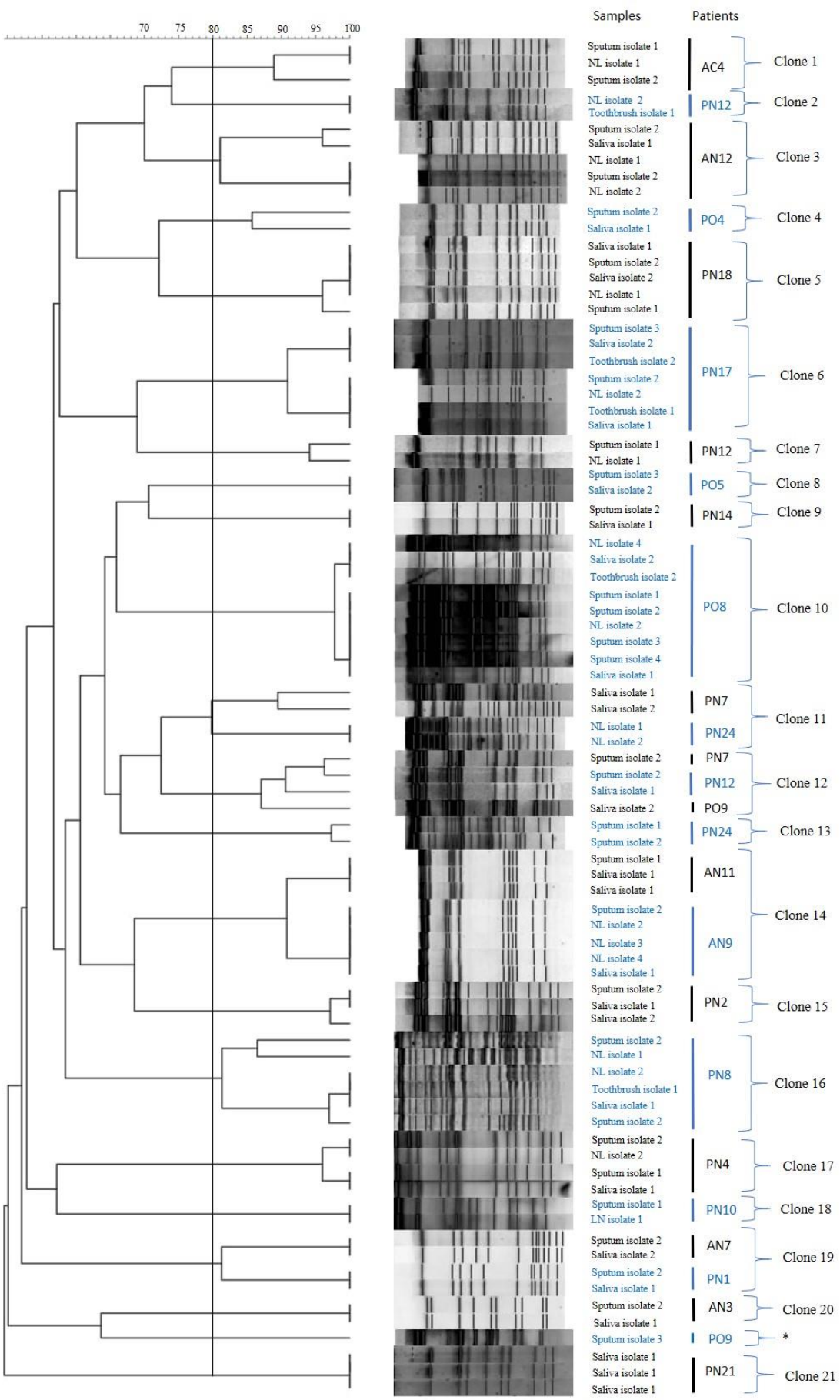
70 75 80 85 90 95 100
Samples
Patients
Sputum isolate 1
NL isolate 1
Sputum isolate 2
AC4
Clone 1
NL isolate 2
Toothbrush isolate 1
PN12
Clone 2
Sputum isolate 2
Saliva isolate 1
NL isolate 1
Sputum isolate 2
NL isolate 2
AN12
Clone 3
Sputum isolate 2
Saliva isolate 1
PO4
Clone 4
Saliva isolate 1
Sputum isolate 2
Saliva isolate 2
NL isolate 1
Sputum isolate 1
PN18
Clone 5
Sputum isolate 3
Saliva isolate 2
Toothbrush isolate 2
Sputum isolate 2
NL isolate 2
Toothbrush isolate 1
Saliva isolate 1
PN17
Clone 6
Sputum isolate 1
NL isolate 1
PN12
Clone 7
Sputum isolate 3
Saliva isolate 2
PO5
Clone 8
Sputum isolate 2
Saliva isolate 1
PN14
Clone 9
NL isolate 4
Saliva isolate 2
Toothbrush isolate 2
Sputum isolate 1
Sputum isolate 2
NL isolate 2
Sputum isolate 3
Sputum isolate 4
Saliva isolate 1
PO8
Clone 10
Saliva isolate 1
Saliva isolate 2
PN7
NL isolate 1
NL isolate 2
PN24
Clone 11
Sputum isolate 2
PN7
Sputum isolate 2
Saliva isolate 1
PN12
Clone 12
Saliva isolate 2
PO9
Sputum isolate 1
Sputum isolate 2
PN24
Clone 13
Sputum isolate 1
Saliva isolate 1
Saliva isolate 1
AN11
Sputum isolate 2
NL isolate 2
NL isolate 3
NL isolate 4
Saliva isolate 1
AN9
Clone 14
Sputum isolate 2
Saliva isolate 1
Saliva isolate 2
PN2
Clone 15
Sputum isolate 2
NL isolate 1
NL isolate 2
Toothbrush isolate 1
Saliva isolate 1
Sputum isolate 2
PN8
Clone 16
Sputum isolate 2
NL isolate 2
Sputum isolate 1
Saliva isolate 1
PN4
Clone 17
Sputum isolate 1
LN isolate 1
PN10
Clone 18
Sputum isolate 2
Saliva isolate 2
AN7
Clone 19
Sputum isolate 2
Saliva isolate 1
PN1
Sputum isolate 2
Saliva isolate 1
AN3
Clone 20
Sputum isolate 3
PO9
*
Saliva isolate 1
Saliva isolate 1
Saliva isolate 1
PN21
Clone 21

Figure 19. Dendrogram displaying PFGE genotypes of Nasal lavage (NL), sputum, saliva, and toothbrush *S. aureus* isolates. Analysis of the genetic relationship with the SmaI and Cftr9I restriction enzyme was performed with the 23 patients, were presented 21 different clonal groups. The vertical line at 80% genetic similarity was considered an arbitrary threshold for grouping the clones. * indicates a single genotype profile had not been observed before.

Of the confirmed *P. aeruginosa* isolates, 74 sequential *P. aeruginosa* isolates were characterized by PFGE restricted by SpeI. These included multiple *P. aeruginosa* isolates with different colonial morphologies (brown, lysis, mucoid, non-mucoid, green, and spread) from different sites of the same patient (Figure 21). Were detected eighteen different clone groups, with the seventeen patients, presenting ≥80% similarity between the strains (Figure 20). The strains of chronic adults patients AC9, AC4, and AC3 presented different clonal groups. Four groups of clones were observed in which different patients had the same clone of strains, from several isolates: clone 1 (with AC5 and PN8), clone 5 (with AC9 and AC4), clone 9 (with AC9 and AC3), and clone 16 (with AC3 and AC4). The contacts could show link among patients with the same PFGE profile.

Despite different morphologies of colonies of *P. aeruginosa* strains in a single patient, no difference in genotype profiles was noted.

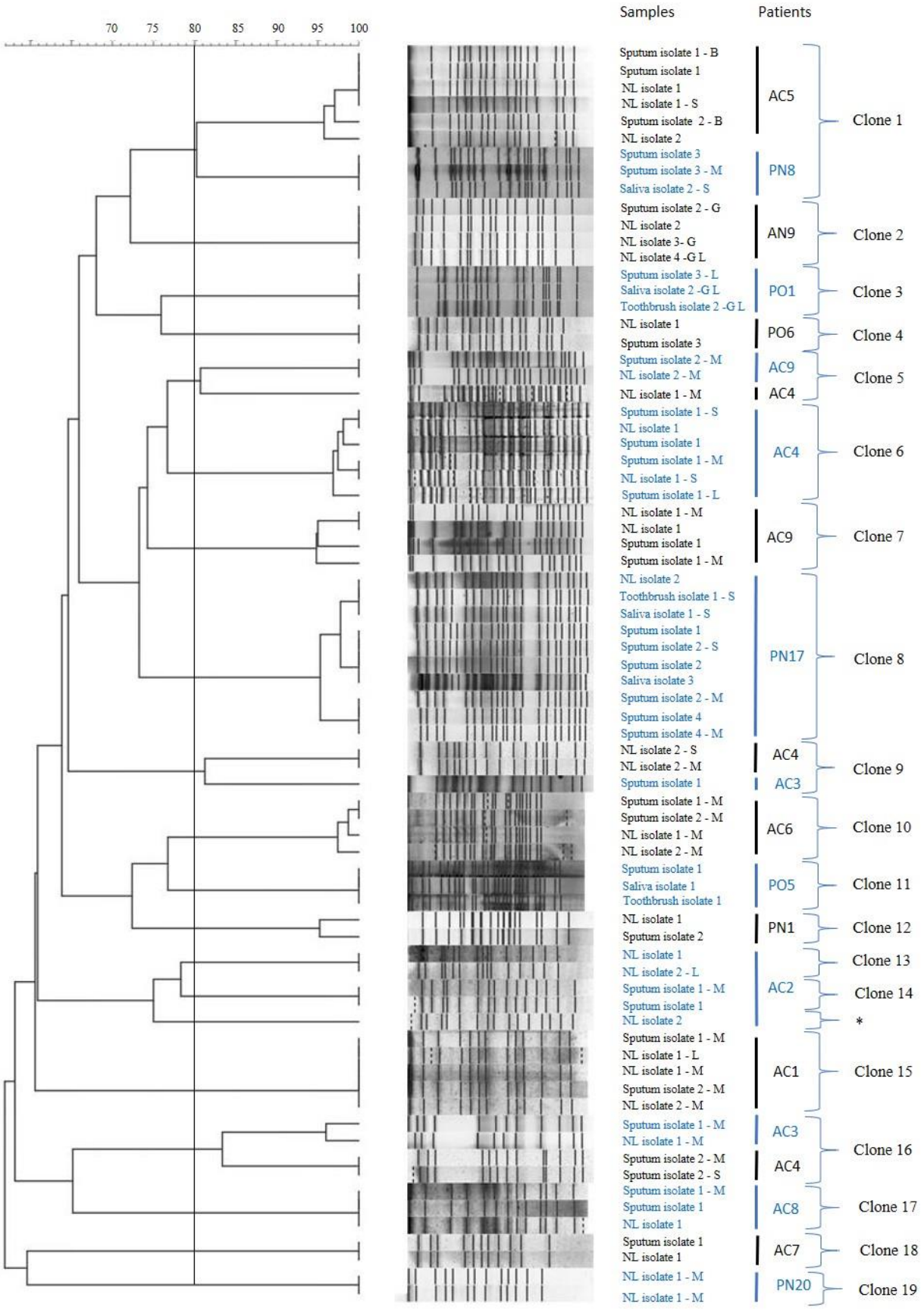
70
75
80
85
90
95
100
Samples
Patients
Sputum isolate 1 - B
Sputum isolate 1
NL isolate 1
NL isolate 1 - S
Sputum isolate 2 - B
NL isolate 2
AC5
Clone 1
Sputum isolate 3
Sputum isolate 3 - M
Saliva isolate 2 - S
PN8
Sputum isolate 2 - G
NL isolate 2
NL isolate 3- G
NL isolate 4 -G L
AN9
Clone 2
Sputum isolate 3 - L
Saliva isolate 2 -G L
Toothbrush isolate 2 -G L
PO1
Clone 3
NL isolate 1
Sputum isolate 3
PO6
Clone 4
Sputum isolate 2 - M
NL isolate 2 - M
AC9
Clone 5
NL isolate 1 - M
AC4
Sputum isolate 1 - S
NL isolate 1
Sputum isolate 1
Sputum isolate 1 - M
NL isolate 1 - S
Sputum isolate 1 - L
AC4
Clone 6
NL isolate 1 - M
NL isolate 1
Sputum isolate 1
Sputum isolate 1 - M
AC9
Clone 7
NL isolate 2
Toothbrush isolate 1 - S
Saliva isolate 1 - S
Sputum isolate 1
Sputum isolate 2 - S
Sputum isolate 2
Saliva isolate 3
Sputum isolate 2 - M
Sputum isolate 4
Sputum isolate 4 - M
PN17
Clone 8
NL isolate 2 - S
NL isolate 2 - M
AC4
Clone 9
Sputum isolate 1
AC3
Sputum isolate 1 - M
Sputum isolate 2 - M
NL isolate 1 - M
NL isolate 2 - M
AC6
Clone 10
Sputum isolate 1
Saliva isolate 1
Toothbrush isolate 1
PO5
Clone 11
NL isolate 1
Sputum isolate 2
PN1
Clone 12
NL isolate 1
Clone 13
NL isolate 2 - L
Sputum isolate 1 - M
Clone 14
Sputum isolate 1
NL isolate 2
AC2
*
Sputum isolate 1 - M
NL isolate 1 - L
NL isolate 1 - M
Sputum isolate 2 - M
NL isolate 2 - M
AC1
Clone 15
Sputum isolate 1 - M
NL isolate 1 - M
AC3
Clone 16
Sputum isolate 2 - M
Sputum isolate 2 - S
AC4
Sputum isolate 1 - M
Sputum isolate 1
NL isolate 1
AC8
Clone 17
Sputum isolate 1
NL isolate 1
AC7
Clone 18
NL isolate 1 - M
NL isolate 1 - M
PN20
Clone 19

Figure 20. Dendrogram displaying PFGE genotypes of Nasal lavage (NL), sputum, saliva, and toothbrush of *P. aeruginosa* isolates. Analysis of the genetic relationship with the SpeI restriction enzyme was performed with the 17 patients, were presented 19 different clonal groups. The numbers represented the visit of the collected samples. The letters in front of the numbers represented the morphology of the strains: M= mucoid, S= spread, L=lysis, G= green, and B= brown. The vertical line at 80% genetic similarity was considered an arbitrary threshold for grouping the clones. * indicates a single genotype profile had not been observed before.

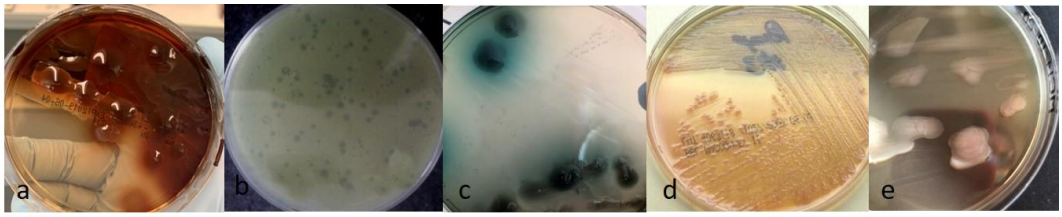


Figure 21. Phenotypes of *P. aeruginos*a CF isolates: a) brown and mucoid, b) lysis, c)green, d) non-mucoid, and e) spread. [184, 185]

## 6. DISCUSSION

The adaptation of some bacterial species, such as *P. aeruginosa*, to the lung environment in cystic fibrosis takes place through a high rate of mutation, expression of different pathogenicity factors, enhanced antibiotic resistance, changes in nutrient utilization, overproduction of the mucoid exopolysaccharide alginate, reduction in growth rate and loss of O-antigen, motility, type III secretion and quorum sensing and the huge stimulation of the host inflammatory response thus allowing the evolution from the acute to the chronic lung colonisation. In fact, one of the most important challenges in CF is avoiding, treating, and solving chronic lung infections which are the major cause of morbidity and mortality in this kind of patients. [93, 186-192].

A role for bacterial reservoirs located in other body districts connected to and feeding the lower airways has also been hypothesised [128, 131, 134, 137, 146, 147, 193, 194]. It seems clear that these reservoirs constitute a continuing risk of chronic lung recontamination [115]. Lack of eradication of potentially pathogenic bacteria in those sites could lead to a continuous source of infectious agents producing lung colonisation and finally chronic infection [115]. Once bacteria are established in the lung, eradication is rarely successful: although some improvement may be noticeable after using antibiotics by inhalation or intravenous, most patients will return to positive sputum within 3 months after treatment.[195, 196].

The nasal/paranasal sinuses have been proven to be a reservoir both in people with CF and Primary Ciliary Dyskinesia and have been proposed as an important source for chronic bronchopulmonary infection with opportunistic bacteria[117, 197]. A clinical study conducted by Mainz et al (2009) on over 180 patients compared the microbial flora in the UAW and LAW and assessed the genetic relatedness of *S. aureus* and *P. aeruginosa* strains collected in the two different environments [131].

In children with CF, the three main pathogens *H. influenzae, S. aureus,* and *P. aeruginosa* were recovered from the paranasal sinuses but not from the bronco lavage. There are several reasons to explain this difference in the infection/colonisation rate: it can be due to the anatomy of the sinus that, having small draining cavities, facilitates the retention of thick mucus while this mucus in the lung can be better eliminated by sneezing and coughing. Moreover, the heterogenicity of the CF lung can lead to the negativity of the BALF cultures when arriving from only one pulmonary lobe [198].

Morlacchi and colleagues (2018) demonstrated that a large number of patients (60%) had the same pathogens colonizing both LAW and UAW on the same visit after lung transplantation [199]. In addition, many studies have shown that *P. aeruginosa* and *S. aureus* clones can be found both in the upper and lower airways of these patients: Walter et al (1997) saw that *P. aeruginos*a isolated from transplanted lungs presented as clones that were identical to the genotypes isolated before lung transplantation while Hansen et al (2012) showed by evaluation of intraoperatively taken material that the paranasal sinuses are a potential niche for *P. aeruginosa* clones to adapt and diversify for subsequent chronic lung infections [131, 134, 135, 200]. The evolution of strains more adapted to the stressful lung environment could be easier due to the colonisation and adaptation to the sinuses before the infection of the lower airways. [93].

Also, the oral cavity, as well as objects introduced in the mouth, such as the toothbrush, could have a role as bacterial reservoirs [146, 194, 201]. The toothbrush is the main tool to keep the oral cavity and teeth clean to prevent dental problems such as caries, gingivitis, periodontitis, halitosis, and aggravations of systemic diseases related to the oral cavity [202]. However, when bacteria survive on the toothbrush's bristles, they may re-inoculate the oral cavity of the original host thus increasing the risk of infectious diseases [203, 204].

The toothbrush can be contaminated with microorganisms coming from both the oral cavity and the environment in which it is stored. The wet environment of the bathroom, as well as aerosols from toilet drains and contaminated hands, can contribute to the colonisation of the toothbrush. In addition, most families store the toothbrushes in common containers leading to the possibility of cross-infection [202]. Microorganisms can first infect the oral cavity and then spread to the rest of the body causing serious health problems (e.g. heart disease, gastrointestinal, respiratory and kidney disease, as well as heart attacks, arthritis, etc.) or exacerbate existing diseases [204, 205]. This can be particularly relevant in subjects at risk such as CF patients. The actual impact of the contaminated toothbrush on bronchial colonisation in these subjects is difficult to assess since bronchial colonisation can come from different sources and is highly dependent on the lung condition of the patients.

As highlighted in a recent literature review, it is still not clear whether typical CF

bacterial species are transient members of the oral flora in patients with occasional and chronic bronchopulmonary colonisation and more longitudinal studies are needed to learn about the prevalence of typical CF bacterial species and the genotypic concordance between specific strains present in reservoirs and causing lung colonisation [194]. Also, other latest studies evaluating the possible roles of oral/digestive pathways as possible bacterial reservoirs had been resulted incomplete because they are only focused on the bacteria most commonly found in the CF respiratory system (*P. aeruginosa* and *S. aureus*) [146, 206, 207]. Despite its potential impact on the management of CF, the association between UAW, LAW and oral cavity colonisation has never been systematically investigated.

For these reasons and differently from previous works, **our study was not limited to *Pseudomonas aeruginosa* and *Staphylococcus aureus* but also included other potentially pathogenic emerging bacterial species such as *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and *Serratia marcescens*,** that are frequently isolated from the sputum of CF subjects but still lack a clear clinical role [208, 209]. Besides, there is still a need to investigate a higher number of individuals with CF to assess whether the monitoring and eradication of the bacterial flora in the reservoirs would be a good practice to be introduced in the clinical field to avoid or delay chronic lung infection. **We have considered not only the classical sputum sample but also nasal lavage, saliva, and toothbrushes**, **have monitored patients longitudinally, and have genotyped the bacterial strains in different sites and times in the same patient within a large cohort of CF adults and children.** Moreover, non-invasive and simple methods for sampling the UAW that apply to daily clinical practice have been used.

W**e have been monitoring adults and paediatrics who were never or only occasionally colonised by *P. aeruginosa* since this is the population that might benefit more from monitoring bacterial reservoirs to avoid the insurgence of a chronic lung infection**. In addition, a small number of adults with *P. aeruginosa* chronic lung colonisation were monitored as a control: in these patients, as expected, we isolated the same *P. aeruginosa* clones from the two investigated sites. Genotypic and phenotypic variants may exist in the population of *P. aeruginosa* in the CF lung that contributes to different aspects of disease progression and lung anatomical

deterioration [210-212]. Heterogeneity manifests itself both in terms of genetic and physiological diversity and spatial distributions in the airways [213, 214]. In this study, chronically infected patients harboured a total of up to 6 different *P. aeruginosa* colony morphological types in the respiratory samples (brown, green, lysis, mucoid, non-mucoid, and spread) (Figure 21). Once *P. aeruginosa* has colonized the respiratory sites of CF patients, there is a trend to develop mucoidity, resulting in persistent infections with all chronically infected patients harbouring mucoid *P. aeruginosa* in their respiratory samples. This finding is in agreement with others studies where an increased association of mucoidity with chronicity of infection was observed [215-217] Chronic infections are commonly caused by a single *P. aeruginosa* clonal lineage. However, different lineages were found in the respiratory samples from our chronic patients or obtained longitudinally from the same patient ( E.g. one lineage of *P. aeruginosa* from AC3, belongs to clone 9, the other lineage, belongs to the clone 16); similar results were also noted in previous studies [211, 218].

In our study, several patients were enrolled at the beginning of the project and then followed up and monitored during subsequent visits. E.g. three enrolled patients (PN12, PO8, AN9) produced up to 5 longitudinal samples of sputum and nasal lavage while two enrolled patients (PO3, AN4) provided us with only 2 samples. These differences in the samples number was caused by some patients being unable to collect the samples due to absence on the day of the visit or due to hospitalization. Despite being a negative aspect in that decreases the number of all the theoretically possible samples, this does not represent a significant problem in our study. While the collection of sputum and nasal lavage initiated at time zero of the project, saliva and toothbrush sample collection started at a later moment (after one month using the toothbrush), thus for half of the patients (n=27) we recovered less oral than respiratory samples. Also, in few cases a sampling was missed (n=12) due to collection-related issues: patient PN10 missed the collection of nasal lavage at the second visit while in all the other cases the missing sample was saliva (n=9) or toothbrush (n=2). With an only exception, we included in the study two toothbrushes that were collected from patient AN15 after the end of the collection period. Despite these deviations in sampling, we managed to collect at least 2 longitudinal samples of sputum and nasal lavage from all patients and at least one sample of saliva and one toothbrush. This number of samples

was informative to allow the comparison between the species and strains isolated from the four different districts.

**About 80% of the 59 subjects monitored in this study resulted positive for at least one of the investigated bacterial species. 74% of the patients who resulted positive in the sputum also presented the same species in the nasal lavage and saliva.** The interpretation of the bacteria presence data in the different body sites is particularly challenging due to concerns about optimal sampling techniques and crossover between upper and lower airway bacterial communities [219]. Although we cannot exclude cross-contamination between sputum and saliva during the sampling, the repetitive isolation of the same bacterial strain in the different samples supports the persistence of bacteria also in the oral cavity. Thus, we stress the importance of monitoring saliva as a read-out of the mouth microflora. As regards toothbrushes, 26% of the samples were positive for the same investigated bacterial species found in sputum and, in some cases, also in the other two types of samples. These results are comparable with data from Genevois et al (2015) who detected *S. aureus* in 22% of analyzed toothbrushes. Although this percentage is lower than that of the sinuses and the oral cavity, it is significant when considering a source of bacteria potentially associated with LAW infection. **It, therefore, seems important to properly educate CF patients people with CF on how to maintain their toothbrush free of bacterial contamination.**

While *S. aureus* and *P. aeruginosa* were present in the samples from 69% and 48% of our patients, respectively, we detected *S. maltophilia* and *A. xylosoxidans* in the respiratory samples of 41% (n=24) and 22% (n=13) of all 59 patients enrolled, respectively. *S. marcescens* during the study was not isolated in the nasal/paranasal sinuses, but was associated to a percentage of positivity in the lungs of 8% (n=5). Interestingly, among other isolated species, we observed a high incidence of *Candida spp*. which was isolated from 17% of sputum samples, 43% of saliva samples and 17% of toothbrushes. In the nasal / paranasal sinuses no much yeast was isolated (2 patients), a similar result reported also by Mainz et al (2009), while the presence of Candida in saliva, and consequently in toothbrushes, could be expected in that this yeast is part of the normal oral microbiota, *Candida albicans* was found in 18.1% of the LAW cultures but in none of the UAW samples [131].

An interesting fact is the presence of *Aspergillus spp, Exophiala spp* and *Candida spp* that were isolated from the samples, our study was not aimed at investigating it, but we observed that in AC group presented concomitant colonisation with *P. aeruginosa* and filamentous fungus (data not shown) and there was noted a coexistence of *P. aeruginosa* and *Candida spp.* on the LAW, with more than half of patients. Analyzing all groups (AC, AN, PN/PNP and, PO), *Candida spp*. were isolated with high frequency in sputum, saliva, and toothbrushes. Chen et al (2014) showed that *C. albicans* produces ethanol, which in turn promotes both the colonisation and biofilm formation of *P. aeruginosa* [220]. In turn, however, *P. aeruginosa* forms a dense biofilm on *C. albicans* hyphae and kills the fungus [221]. These data highlighted the possibility that *Candida spp*. could have a pathogenic role in CF, however, currently there is not enough evidence to suggest that *Candida spp.* are responsible for a series of events including infectious exacerbations and no conclusions can be made and further studies should be performed, since *Candida spp* is a microorganism frequently colonized in the human mouth, and several other pathogenic fungi appear to be oral residents in some individuals.

To investigate the possibility of transmission between the UAW, LAW, and oral environment, we assessed the presence of the bacterial species in nasal lavage, sputum, saliva, and toothbrushes of the same patient and checked their clonal identity by PFGE analysis.

The molecular typing of the bacterial strains isolated from the different samples of each patient indicates that most of them belong to single clones circulating in the UAW, LAW, and oral cavity and, in some cases, in the toothbrush too.

Regarding the possibility of transmission between the investigated reservoirs and the lungs, **it is worth highlighting and analysing some interesting cases:**

Three children (PN1, PN22, PO6) initially showing colonisation of the UAW became positive for the same clone also in the LAW by the end of the study. PN1 presented *P. aeruginosa* only in the nasal lavage at the first visit while in the second visit the pathogen was detected in the sputum too. PN22 presented *S. aureus* only in the nasal lavage at the first two visits and also in the sputum at the third visit. At the first visit, PO6 presented P. *aeruginosa* only in the nasal lavage, in the third visit only in the sputum, and in the fourth visit only in the nasal lavage again; although *P.*

*aeruginosa* was undetected at the second visit, LAW colonisation with the same clone support the possible passage from UAW. Similarly, in two patients (PO4, PO9) the bacterial strains were present in the saliva and the same clones were later isolated also from the sputum. An adult patient (AN9) was colonised at the beginning of the study by *P. aeruginosa* in both LAW and UAW; after combined i.v. antibiotic therapy with ceftazidime and azithromycin, *P. aeruginosa* was successfully eradicated in the LAW. However, the infection persisted in the UAW and, at the end of the study, lung colonisation was detected again. This might indicate that the same microorganism when persisting in the nasal sinuses, can transfer to the lungs where it can cause a chronic infection. Particularly, the case of patient PO4 illustrated well the possibility of transmission of bacteria from the toothbrush to lower airways: at the first sampling, the toothbrush and saliva of this patient were positive for the presence of *S. marcescens* while the sputum sample was only positive for *S. aureus*. At the second sampling, sputum was also positive for the presence of the same *S. marcescens* clone previously isolated from saliva and toothbrush highly suggesting a passage of bacteria from the environment/mouth to the lung. These cases highlight the likelihood of transmission of potential pathogens from nasal/oral reservoirs to the lung environment and the importance of applying a specific-site CF bacteria eradication from these reservoirs to prevent lung infections.

At the Verona Cystic Fibrosis Centre, patients are visited on specific days to avoid cross-infection by *P. aeruginosa*. Patients with chronic *P. aeruginosa* infection are seen on different days than those who are free or occasionally infected by *P. aeruginosa*. However, cross-infection may occur between patients who have close social contact or present the same day to the medical visit despite all precautionary and hygiene measures taken. The cross-infection was unremarkable throughout the study period, eight of 23 patients were colonised with isolates of 4 clones of *S. aureus,* between these groups we didn´t notice cross-infection between an AC with AN/PN/PO; likewise, genotyping confirmed the presence of 4 *P. aeruginosa* clonal groups including 5 of 17 patients, where only the “Clone 1” presented different status of *P. aeruginosa* lung colonisation (AC5 and PN8). The others clone groups from different bacterial strains included isolates belonging to one patient per clone (independent of the number of samples and phenotype of the bacterial species). This means that the

diversity among the strains in an individual patient was very low and shows that during the period of observation the incidence of infection by another clone strain was rare.

To prevent the toothbrush from becoming a receptacle of potentially pathogenic microorganisms, it is recommended to change it frequently, at least every 3 months, or even more often for the most vulnerable subjects [222]. An alternative might be its decontamination. Although there are little data in the literature regarding toothbrush sanitation, one study indicates that soaking the toothbrush in 3% hydrogen peroxide or Listerine mouthwash greatly reduces (i.e. 85%) the bacterial load while 20 min immersion in a standard disinfectant every 3 days did not eliminate the microorganisms present on the toothbrush [223, 224]. Recent studies demonstrated that chlorhexidine had greater efficacy against the microorganism colonisation/biofilms on the toothbrush's bristles and exhibited inhibition of microbial growth [225]. Toothbrush sanitizer devices are available. Therefore, we encourage the development of oral hygiene protocols to be shared with CF patients as part of their daily routine in order to expand their hygiene education and decrease the chance of lung infection.

While further evidence regarding the role of oral reservoir might be needed, our study confirms the need for eradication of bacterial CF species from the UAW in addition to standard LAW eradication and to dedicate special attention to the oral and environmental reservoirs.

The limitation of this study was due to the heterogeneity of the number of samples from each patient, as some patients on the day of the visit did not bring their toothbrushes or refused, because they were not feeling well, to carry out the collection of some samples.

However, this did not interfere with the results and objectives of the study in that a sufficient number of samples were obtained from each patient.

Future studies are being programmed to evaluate the success of the protocol change at the Verona Cystic Fibrosis Centre, in the context of preventing chronic pulmonary infection, as well as the extension for analysis of other microorganisms not involved in this project.

A prevention video was prepared to teach cystic fibrosis patients the correct hygiene of toothbrushes, available on youtube: "Toothbrushes as vehicles for

environmental bacteria”  https://youtu.be/9gVJWK4HTk0 .

## 7. CONCLUSION

This study strongly supports the role of nasal/paranasal sinuses and oral cavity as "reservoir" in CF subjects with positive sputum in that 74% of them presented the same species in the UAW, nasal lavage, and saliva while 26% have the same species on the toothbrush. The clonal identity between the strains present in the same patient in different sites was confirmed in almost all cases supporting the hypothesis of bacterial transmission among the sites, and confirm, as elucidated in some cases in this study, that oral cavity and UAW are reservoirs of potentially pathogenic microorganisms that lead to pulmonary colonisation.

The lack of eradication of bacteria from these sites might undermine the antibiotic therapy applied to treat the lung infection and allowing the persistence of the bacteria within the patient in a continuous cycle of lung eradication and re-infection. Thus, it might be useful to introduce in the clinical routine of CF Centres the monitoring of bacterial reservoirs such as nasal sinuses and oral cavity and the training of CF patients to properly clean and store toothbrushes to prevent the establishment of chronic infections due to infectious agents colonising and persisting in these sites.

An important result of this project was the understanding established with the clinicians for considering the importance of eradicating bacterial reservoirs: a new protocol has been proposed and accepted by the Cystic Fibrosis Centre of Verona for monitoring the analyzed bacterial reservoirs and to eradicate the microorganisms present to prevent chronic lung infection.

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# 9. BIBLIOGRAPHY


1. Molina S.A., H.W.R., *Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease*. Chapter 12 - Cystic Fibrosis: An Overview of the Past, Present, and the Future., ed. S.V.k.K. M. 2017.
2. Tsui, L.C. and M. Buchwald, *Biochemical and molecular genetics of cystic fibrosis.* Adv Hum Genet, 1991. **20**: p. 153-266, 311-2.
3. *Condition, gene, or chromosome summary: National Library of Medicine (US).* 2012 [cited 2020 may]; Available from: https://ghr.nlm.nih.gov/condition/cystic-fibrosis.
4. Davis, P.B., *Cystic fibrosis since 1938.* Am J Respir Crit Care Med, 2006. **173**(5): p. 475-82.
5. Veit, G., et al., *From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations.* Mol Biol Cell, 2016. **27**(3): p. 424-33.
6. *Cystic Fibrosis Foundation. Patient registry annual data report 2018*. 2019 [cited 2020 April 1]; Available from: https://www.cff.org/Research/Researcher-Resources/Patient-Registry/2018-Patient-Registry-Annual-Data-Report.pdf.
7. Claustres, M., et al., *Spectrum of CFTR mutations in cystic fibrosis and in congenital absence of the vas deferens in France.* Hum Mutat, 2000. **16**(2): p. 143-56.
8. Giordani, B., Amato, A., Majo, F., et al, *Italian Cystic Fibrosis Registry (ICFR). Report 2015-2016*. 2019: Rivista dell'Associazione italiana di epidemiologia. p. 1-36.
9. *Cystic Fibrosis Foundation. Patient Registry Annual Report 2010*. 2012: Bethesda: Cystic Fibrosis Foundation.
10. Quinton, P.M., *Physiological basis of cystic fibrosis: a historical perspective.* Physiol Rev, 1999. **79**(1 Suppl): p. S3-S22.
11. Di Sant'Agnese, P., et al., *Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas.* AMA Am J Dis Child, 1953. **86**(5): p. 618-9; discussion, 619.
12. Gibson, L.E. and R.E. Cooke, *A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis.* Pediatrics, 1959. **23**(3): p. 545-9.
13. Kerem, B., et al., *Identification of the cystic fibrosis gene: genetic analysis.* Science, 1989. **245**(4922): p. 1073-80.
14. Berkhout, M.C., et al., *CT-abnormalities, bacteriology and symptoms of sinonasal disease in children with Cystic Fibrosis.* J Cyst Fibros, 2016. **15**(6): p. 816-824.
15. Frizzell, R.A. and J.W. Hanrahan, *Physiology of Epithelial Chloride and Fluid Secretion.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(6).
16. Rowe, S.M., S. Miller, and E.J. Sorscher, *Cystic fibrosis.* N Engl J Med, 2005. **352**(19): p. 1992-2001.
17. Riordan, J.R., *Assembly of functional CFTR chloride channels.* Annu Rev Physiol, 2005. **67**: p. 701-18.
18. Moran, O. and O. Zegarra-Moran, *On the measurement of the functional properties of the CFTR.* J Cyst Fibros, 2008. **7**(6): p. 483-94.
19. Morral, N., et al., *The origin of the major cystic fibrosis mutation (delta F508) in European populations.* Nat Genet, 1994. **7**(2): p. 169-75.
20. Saint-Criq, V. and M.A. Gray, *Role of CFTR in epithelial physiology.* Cell Mol Life Sci, 2017. **74**(1): p. 93-115.

21. Gibson, R.L., J.L. Burns, and B.W. Ramsey, *Pathophysiology and management of pulmonary infections in cystic fibrosis.* Am J Respir Crit Care Med, 2003. **168**(8): p. 918-51.
22. Barth, A.L. and T.L. Pitt, *Microbial Pathogens Associated With Cystic Fibrosis: Special Focus on Pseudomonas aeruginosa.* Braz J Infect Dis, 1998. **2**(2): p. 43-61.
23. Buchanan, P.J., et al., *Role of CFTR, Pseudomonas aeruginosa and Toll-like receptors in cystic fibrosis lung inflammation.* Biochem Soc Trans, 2009. **37**(Pt 4): p. 863-7.
24. MacDonald, K.D., K.R. McKenzie, and P.L. Zeitlin, *Cystic fibrosis transmembrane regulator protein mutations: 'class' opportunity for novel drug innovation.* Paediatr Drugs, 2007. **9**(1): p. 1-10.
25. Welsh, M.J. and A.E. Smith, *Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis.* Cell, 1993. **73**(7): p. 1251-4.
26. Zielenski, J. and L.C. Tsui, *Cystic fibrosis: genotypic and phenotypic variations.* Annu Rev Genet, 1995. **29**: p. 777-807.
27. Marson, F.A.L., C.S. Bertuzzo, and J.D. Ribeiro, *Classification of CFTR mutation classes.* Lancet Respir Med, 2016. **4**(8): p. e37-e38.
28. De Boeck, K. and M.D. Amaral, *Progress in therapies for cystic fibrosis.* Lancet Respir Med, 2016. **4**(8): p. 662-674.
29. Marson, F.A.L., C.S. Bertuzzo, and J.D. Ribeiro, *Personalized or Precision Medicine? The Example of Cystic Fibrosis.* Front Pharmacol, 2017. **8**: p. 390.
30. Marson, F.A.L., *Disease-modifying genetic factors in cystic fibrosis.* Curr Opin Pulm Med, 2018. **24**(3): p. 296-308.
31. Pereira, S.V.N., et al., *Novel, rare and common pathogenic variants in the CFTR gene screened by high-throughput sequencing technology and predicted by in silico tools.* Scientific Reports, 2019. **9**.
32. Protasevich, I., et al., *Thermal unfolding studies show the disease causing F508del mutation in CFTR thermodynamically destabilizes nucleotide-binding domain 1.* Protein Science, 2010. **19**(10): p. 1917-1931.
33. Derichs, N., *Targeting a genetic defect: cystic fibrosis transmembrane conductance regulator modulators in cystic fibrosis.* Eur Respir Rev, 2013. **22**(127): p. 58-65.
34. Rogan, M.P., D.A. Stoltz, and D.B. Hornick, *Cystic Fibrosis Transmembrane Conductance Regulator Intracellular Processing, Trafficking, and Opportunities for Mutation-Specific Treatment.* Chest, 2011. **139**(6): p. 1480-1490.
35. Shanthikumar, S. and J. Massie, *A Review of Treatments That Improve Cystic Fibrosis Transmembrane Conductance Regulator Function.* Clinical Medicine Insights-Therapeutics, 2017. **9**.
36. Athanazio, R.A., et al., *Brazilian guidelines for the diagnosis and treatment of cystic fibrosis.* Jornal Brasileiro De Pneumologia, 2017. **43**(3): p. 219-245.
37. Farrell, P.M., et al., *Diagnosis of Cystic Fibrosis: Consensus Guidelines from the Cystic Fibrosis Foundation.* Journal of Pediatrics, 2017. **181**: p. S4-S15.
38. Rosa, F.R., et al., *Cystic fibrosis: a clinical and nutritional approach.* Revista De Nutricao-Brazilian Journal of Nutrition, 2008. **21**(6): p. 725-737.
39. Castellani, C., et al., *ECFS best practice guidelines: the 2018 revision.* J Cyst Fibros, 2018. **17**(2): p. 153-178.
40. Wagener, J.S., et al., *Pulmonary function outcomes for assessing cystic fibrosis care.* Journal of Cystic Fibrosis, 2015. **14**(3): p. 376-383.
41. Burity, E.F., et al., *Bronchodilator response cut-off points and FEV 0.75 reference values for spirometry in preschoolers %J Jornal Brasileiro de Pneumologia.* 2016. **42**: p. 326-332.

42. Stallings, V.A., et al., *Evidence-based practice recommendations for nutrition-related management of children and adults with cystic fibrosis and pancreatic insufficiency: Results of a systematic review.* Journal of the American Dietetic Association, 2008. **108**(5): p. 832-839.
43. Bech, B., et al., *Long-term outcome of lung transplantation for cystic fibrosis--Danish results.* Eur J Cardiothorac Surg, 2004. **26**(6): p. 1180-6.
44. Caverly, L.J. and J.J. LiPuma, *Cystic fibrosis respiratory microbiota: unraveling complexity to inform clinical practice.* Expert Rev Respir Med, 2018. **12**(10): p. 857-865.
45. Lyczak, J.B., C.L. Cannon, and G.B. Pier, *Lung infections associated with cystic fibrosis.* Clinical Microbiology Reviews, 2002. **15**(2): p. 194-+.
46. Gibson, R.L., J.L. Burns, and B.W. Ramsey, *Pathophysiology and management of pulmonary infections in cystic fibrosis.* American Journal of Respiratory and Critical Care Medicine, 2003. **168**(8): p. 918-951.
47. Dooring, G. and E. Gulbins, *Cystic fibrosis and innate immunity: how chloride channel mutations provoke lung disease.* Cellular Microbiology, 2009. **11**(2): p. 208-216.
48. RichmanEisenstat, J., *Cytokine soup: Making sense of inflammation in cystic fibrosis.* Pediatric Pulmonology, 1996. **21**(1): p. 3-5.
49. Guan, X., et al., *Dysregulated Chemokine Signaling in Cystic Fibrosis Lung Disease: A Potential Therapeutic Target.* Curr Drug Targets, 2016. **17**(13): p. 1535-44.
50. Schmitt-Grohe, S., et al., *Lipopolysaccharide binding protein, cytokine production in whole blood, and lipoproteins in cystic fibrosis.* Pediatr Res, 2005. **58**(5): p. 903-7.
51. Ng, H.P., et al., *Neutrophil-mediated phagocytic host defense defect in myeloid Cftr-inactivated mice.* PLoS One, 2014. **9**(9): p. e106813.
52. Ramsey, K.A., et al., *Early respiratory infection is associated with reduced spirometry in children with cystic fibrosis.* Am J Respir Crit Care Med, 2014. **190**(10): p. 1111-6.
53. Marteyn, B.S., et al., *Harnessing Neutrophil Survival Mechanisms during Chronic Infection by Pseudomonas aeruginosa: Novel Therapeutic Targets to Dampen Inflammation in Cystic Fibrosis.* Front Cell Infect Microbiol, 2017. **7**: p. 243.
54. Charman, S.C., R.; Cosgriff, R.; Lee, A.; and Carr, S., *UK Cystic Fibrosis Registry Annual Data Report 2017*. 2018, Cystic Fibrosis Trust.
55. Rowbotham, N.J., et al., *Infection prevention and control in cystic fibrosis: a systematic review of interventions.* Expert Rev Respir Med, 2019. **13**(5): p. 425-434.
56. Chotirmall, S.H., et al., *Sputum Candida albicans Presages FEV1 Decline and Hospital-Treated Exacerbations in Cystic Fibrosis.* Chest, 2010. **138**(5): p. 1186-1195.
57. Gileles-Hillel, A., et al., *Association of chronic Candida albicans respiratory infection with a more severe lung disease in patients with cystic fibrosis.* Pediatric Pulmonology, 2015. **50**(11): p. 1082-1089.
58. Crull, M.R., et al., *Change in Pseudomonas aeruginosa prevalence in cystic fibrosis adults over time.* BMC Pulm Med, 2016. **16**(1): p. 176.
59. Oplustil, C.P.e.a., *Procedimentos Básicos em Microbiologia Clínica*. Thirth ed. 2010: Sarvier. 544.
60. Koneman, E., and Elmer, W., *Diagnóstico Microbiológico - Texto e Atlas Colorido*. 2001, Guanabara Koogan.
61. Trabulsi, F., Alterthum, L.R., *Microbiologia*. fourth ed. 2004: Atheneu. 718.
62. Zemanick, E.T. and L.R. Hoffman, *Cystic Fibrosis: Microbiology and Host Response.* Pediatr Clin North Am, 2016. **63**(4): p. 617-36.
63. Lipuma, J.J., *The changing microbial epidemiology in cystic fibrosis.* Clin Microbiol Rev, 2010. **23**(2): p. 299-323.

64. Juliet, L.C.F., V.A., *Stenotrophomonas Maltophilia.* Rev Chilena Infectol, 2006. **3**: p. 247-8.
65. Stewart, J.D., *The effect of Stenotrophomonas maltophilia on Gram-negative cystic fibrosis pathogens*, in *Biology*. 2019, University of Louisville.
66. Pompilio, A., et al., *Adhesion to and biofilm formation on IB3-1 bronchial cells by Stenotrophomonas maltophilia isolates from cystic fibrosis patients.* BMC Microbiol, 2010. **10**: p. 102.
67. Hansen, C.R., *Stenotrophomonas maltophilia: to be or not to be a cystic fibrosis pathogen.* Curr Opin Pulm Med, 2012. **18**(6): p. 628-31.
68. Yabuuchi, E., et al., *Emendation of genus Achromobacter and Achromobacter xylosoxidans (Yabuuchi and Yano) and proposal of Achromobacter ruhlandii (Packer and Vishniac) comb. nov., Achromobacter piechaudii (Kiredjian et al.) comb. nov., and Achromobacter xylosoxidans subsp. denitrificans (Ruger and Tan) comb. nov.* Microbiol Immunol, 1998. **42**(6): p. 429-38.
69. Bennett, J.E.B., M.J.; Dolin, R., *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. Eighth ed. 2015: Elsevier. 4320.
70. Papalia, M., et al., *Diversity of Achromobacter species recovered from patients with cystic fibrosis, in Argentina.* Rev Argent Microbiol, 2020. **52**(1): p. 13-18.
71. Ronne Hansen, C., et al., *Chronic infection with Achromobacter xylosoxidans in cystic fibrosis patients; a retrospective case control study.* J Cyst Fibros, 2006. **5**(4): p. 245-51.
72. Filipic, B., et al., *Uncovering Differences in Virulence Markers Associated with Achromobacter Species of CF and Non-CF Origin.* Front Cell Infect Microbiol, 2017. **7**: p. 224.
73. Mantovani, R.P., C.E. Levy, and T. Yano, *A heat-stable cytotoxic factor produced by Achromobacter xylosoxidans isolated from Brazilian patients with CF is associated with in vitro increased proinflammatory cytokines.* J Cyst Fibros, 2012. **11**(4): p. 305-11.
74. Kim, J.Y., et al., *Disruption of tight junctions during traversal of the respiratory epithelium by Burkholderia cenocepacia.* Infect Immun, 2005. **73**(11): p. 7107-12.
75. Baron. E.J.; Pfaller, M.A.T., F.C.; Yolken, R.H. , *Manual of Clinical Microbiology*. seventh ed. 1999: American Society Microbiology. 1773.
76. Mahlen, S.D., *Serratia infections: from military experiments to current practice.* Clin Microbiol Rev, 2011. **24**(4): p. 755-91.
77. Crawford, S. and R. Daum, *Bacterial Pneumonia, Lung Abscess, and Empyema*. 2008. p. 501-553.
78. Yu, V.L., *Serratia marcescens: historical perspective and clinical review.* N Engl J Med, 1979. **300**(16): p. 887-93.
79. Henjyoji, E.Y., et al., *Bacteremia due to Serratia marcescens.* J Trauma, 1971. **11**(5): p. 417-21.
80. Fortun, J., et al., *Treatment of long-term intravascular catheter-related bacteraemia with antibiotic-lock therapy.* J Antimicrob Chemother, 2006. **58**(4): p. 816-21.
81. Lata, C., et al., *Epidemiology and Clinical Outcomes of Serratia marcescens Infections in Adults With Cystic Fibrosis*. Vol. 3. 2016.
82. Colombo, A.L., et al., *Brazilian guidelines for the management of candidiasis - a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical.* Braz J Infect Dis, 2013. **17**(3): p. 283-312.
83. Calderone, R.A. and W.A. Fonzi, *Virulence factors of Candida albicans.* Trends Microbiol, 2001. **9**(7): p. 327-35.

84. Brown, A.J. and N.A. Gow, *Regulatory networks controlling Candida albicans morphogenesis.* Trends Microbiol, 1999. **7**(8): p. 333-8.
85. Chandra, J., et al., *Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance.* J Bacteriol, 2001. **183**(18): p. 5385-94.
86. Chotirmall, S.H., et al., *Sputum Candida albicans presages FEV(1) decline and hospital-treated exacerbations in cystic fibrosis.* Chest, 2010. **138**(5): p. 1186-95.
87. Gileles-Hillel, A., et al., *Association of chronic Candida albicans respiratory infection with a more severe lung disease in patients with cystic fibrosis.* Pediatr Pulmonol, 2015. **50**(11): p. 1082-9.
88. Kean, R., et al., *Candida albicans Mycofilms Support Staphylococcus aureus Colonisation and Enhances Miconazole Resistance in Dual-Species Interactions.* Frontiers in Microbiology, 2017. **8**.
89. Cook, G.M., et al., *Energetics of pathogenic bacteria and opportunities for drug development.* Adv Microb Physiol, 2014. **65**: p. 1-62.
90. Tang, Y.-W., et al., *Molecular Medical Microbiology 2nd edition*. 2014.
91. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.* J Infect Dis, 2008. **197**(8): p. 1079-81.
92. Nichols, D., J. Chmiel, and M. Berger, *Chronic inflammation in the cystic fibrosis lung: Alterations in inter- and intracellular signaling.* Clinical Reviews in Allergy & Immunology, 2008. **34**(2): p. 146-162.
93. Folkesson, A., et al., *Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective.* Nat Rev Microbiol, 2012. **10**(12): p. 841-51.
94. Long, S.S., C.G. Prober, and M. Fischer, *Principles and Practice of Pediatric Infectious Diseases*. 2017. 1-1662.
95. Moradali, M.F., S. Ghods, and B.H.A. Rehm, *Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence.* Frontiers in Cellular and Infection Microbiology, 2017. **7**.
96. Lister, P.D., D.J. Wolter, and N.D. Hanson, *Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms.* Clin Microbiol Rev, 2009. **22**(4): p. 582-610.
97. Costerton, J.W., R.T. Irvin, and K.J. Cheng, *The bacterial glycocalyx in nature and disease.* Annu Rev Microbiol, 1981. **35**: p. 299-324.
98. Govan, J.R. and V. Deretic, *Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia.* Microbiol Rev, 1996. **60**(3): p. 539-74.
99. Martin, D.W., et al., *Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients.* Proc Natl Acad Sci U S A, 1993. **90**(18): p. 8377-81.
100. Boucher, J.C., et al., *Mucoid Pseudomonas aeruginosa in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection.* Infect Immun, 1997. **65**(9): p. 3838-46.
101. Wood, L.F. and D.E. Ohman, *Identification of genes in the sigma(2)(2) regulon of Pseudomonas aeruginosa required for cell envelope homeostasis in either the planktonic or the sessile mode of growth.* mBio, 2012. **3**(3).
102. Ramsey, D.M. and D.J. Wozniak, *Understanding the control of Pseudomonas aeruginosa alginate synthesis and the prospects for management of chronic infections in cystic fibrosis.* Mol Microbiol, 2005. **56**(2): p. 309-22.

103. Deretic, V., et al., *Conversion of Pseudomonas aeruginosa to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors.* J Bacteriol, 1994. **176**(10): p. 2773-80.
104. Chitnis, C.E. and D.E. Ohman, *Genetic analysis of the alginate biosynthetic gene cluster of Pseudomonas aeruginosa shows evidence of an operonic structure.* Mol Microbiol, 1993. **8**(3): p. 583-93.
105. DeVries, C.A. and D.E. Ohman, *Mucoid-to-nonmucoid conversion in alginate-producing Pseudomonas aeruginosa often results from spontaneous mutations in algT, encoding a putative alternate sigma factor, and shows evidence for autoregulation.* J Bacteriol, 1994. **176**(21): p. 6677-87.
106. Wood, L.F. and D.E. Ohman, *Use of cell wall stress to characterize sigma 22 (AlgT/U) activation by regulated proteolysis and its regulon in Pseudomonas aeruginosa.* Mol Microbiol, 2009. **72**(1): p. 183-201.
107. Wood, L.F., A.J. Leech, and D.E. Ohman, *Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in Pseudomonas aeruginosa: Roles of sigma (AlgT) and the AlgW and Prc proteases.* Mol Microbiol, 2006. **62**(2): p. 412-26.
108. Hauser, A.R., *The type III secretion system of Pseudomonas aeruginosa: infection by injection.* Nat Rev Microbiol, 2009. **7**(9): p. 654-65.
109. Sato, H., J.B. Feix, and D.W. Frank, *Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU.* Biochemistry, 2006. **45**(34): p. 10368-75.
110. Veesenmeyer, J.L., et al., *Pseudomonas aeruginosa virulence and therapy: evolving translational strategies.* Crit Care Med, 2009. **37**(5): p. 1777-86.
111. Amiel, E., et al., *Pseudomonas aeruginosa evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression.* Infect Immun, 2010. **78**(7): p. 2937-45.
112. Leid, J., *Bacterial Biofilms Resist Key Host Defenses.* Microbe, 2009. **4**: p. 66-70.
113. Olsen, I., *Biofilm-specific antibiotic tolerance and resistance.* Eur J Clin Microbiol Infect Dis, 2015. **34**(5): p. 877-86.
114. McDaniel, C., W. Panmanee, and D. Hassett, *An Overview of Infections in Cystic Fibrosis Airways and the Role of Environmental Conditions on Pseudomonas aeruginosa Biofilm Formation and Viability*. 2015.
115. Aanaes, K., et al., *Sinus biofilms in patients with cystic fibrosis: is adjusted eradication therapy needed?* Eur Arch Otorhinolaryngol, 2015. **272**(9): p. 2291-7.
116. Damron, F.H. and J.B. Goldberg, *Proteolytic regulation of alginate overproduction in Pseudomonas aeruginosa.* Mol Microbiol, 2012. **84**(4): p. 595-607.
117. Gysin, C., G.A. Alothman, and B.C. Papsin, *Sinonasal disease in cystic fibrosis: clinical characteristics, diagnosis, and management.* Pediatr Pulmonol, 2000. **30**(6): p. 481-9.
118. Moller, M.E., et al., *Sinus bacteriology in patients with cystic fibrosis or primary ciliary dyskinesia: A systematic review.* Am J Rhinol Allergy, 2017. **31**(5): p. 293-298.
119. Parsons, D.S., *Sinusitis and cystic fibrosis*, R.P. Lusk, Editor. 1992, Raven Press, New York Pediatric sinusitis. p. 65.
120. Eggesbo, H.B., et al., *CT characterization of inflammatory paranasal sinus disease in cystic fibrosis.* Acta Radiol, 2002. **43**(1): p. 21-8.
121. Mainz, J.G. and A. Koitschev, *Management of chronic rhinosinusitis in CF.* J Cyst Fibros, 2009. **8 Suppl 1**: p. S10-4.
122. Mak, G.K. and N.R. Henig, *Sinus disease in cystic fibrosis.* Clin Rev Allergy Immunol, 2001. **21**(1): p. 51-63.

123. Robertson, J.M., E.M. Friedman, and B.K. Rubin, *Nasal and sinus disease in cystic fibrosis.* Paediatr Respir Rev, 2008. **9**(3): p. 213-9.
124. Ramsey, B. and M.A. Richardson, *Impact of sinusitis in cystic fibrosis.* J Allergy Clin Immunol, 1992. **90**(3 Pt 2): p. 547-52.
125. Coste, A., et al., *Endoscopic and CT-scan evaluation of rhinosinusitis in cystic fibrosis.* Rhinology, 1995. **33**(3): p. 152-6.
126. Koitschev, A., et al., *[Routine otorhinolaryngological examination in patients with cystic fibrosis].* HNO, 2006. **54**(5): p. 361-4, 366, 368.
127. Fokkens, W., et al., *EP3OS 2007: European position paper on rhinosinusitis and nasal polyps 2007. A summary for otorhinolaryngologists.* Rhinology, 2007. **45**(2): p. 97-101.
128. Aanaes, K., *Bacterial sinusitis can be a focus for initial lung colonisation and chronic lung infection in patients with cystic fibrosis.* Journal of Cystic Fibrosis, 2013. **12**: p. S1-S20.
129. Berkhout, M.C., et al., *CT-abnormalities, bacteriology and symptoms of sinonasal disease in children with Cystic Fibrosis.* Journal of Cystic Fibrosis, 2016. **15**(6): p. 816-824.
130. Sakano, E., et al., *Nasal and paranasal sinus endoscopy, computed tomography and microbiology of upper airways and the correlations with genotype and severity of cystic fibrosis.* International Journal of Pediatric Otorhinolaryngology, 2007. **71**(1): p. 41-50.
131. Mainz, J.G., et al., *Concordant genotype of upper and lower airways P aeruginosa and S aureus isolates in cystic fibrosis.* Thorax, 2009. **64**(6): p. 535-40.
132. Goerke, C., et al., *Molecular epidemiology of community-acquired Staphylococcus aureus in families with and without cystic fibrosis patients.* J Infect Dis, 2000. **181**(3): p. 984-9.
133. Matsui, H., et al., *Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease.* Cell, 1998. **95**(7): p. 1005-15.
134. Hansen, S.K., et al., *Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection.* ISME J, 2012. **6**(1): p. 31-45.
135. Walter, S., et al., *Epidemiology of chronic Pseudomonas aeruginosa infections in the airways of lung transplant recipients with cystic fibrosis.* Thorax, 1997. **52**(4): p. 318-21.
136. Fischer, N., et al., *Non-Invasive Assessment of Upper and Lower Airway Infection and Inflammation in CF Patients.* Pediatric Pulmonology, 2014. **49**(11): p. 1065-1075.
137. Muhlebach, M.S., et al., *Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis?* Pediatric Pulmonology, 2006. **41**(5): p. 445-451.
138. Scannapieco, F.A., et al., *A randomized trial of chlorhexidine gluconate on oral bacterial pathogens in mechanically ventilated patients.* Critical Care, 2009. **13**(4).
139. Caldas, R.R. and S. Boisrame, *Upper aero-digestive contamination by Pseudomonas aeruginosa and implications in Cystic Fibrosis.* Journal of Cystic Fibrosis, 2015. **14**(1): p. 6-15.
140. Preza, D., et al., *Bacterial profiles of root caries in elderly patients.* J Clin Microbiol, 2008. **46**(6): p. 2015-21.
141. Amaral, S.M., Q. Cortes Ade, and F.R. Pires, *Nosocomial pneumonia: importance of the oral environment.* J Bras Pneumol, 2009. **35**(11): p. 1116-24.
142. Bensel, T., et al., *Periodontal pockets as potential sources of cystic fibrosis lung infection.* Journal of Cystic Fibrosis - J CYST FIBROS, 2010. **9**.

143. Heo, S.M., et al., *Genetic relationships between respiratory pathogens isolated from dental plaque and bronchoalveolar lavage fluid from patients in the intensive care unit undergoing mechanical ventilation.* Clin Infect Dis, 2008. **47**(12): p. 1562-70.
144. Bunetel, L., et al., *In vitro evaluation of the retention of three species of pathogenic microorganisms by three different types of toothbrush.* Oral Microbiol Immunol, 2000. **15**(5): p. 313-6.
145. Frazelle, M.R. and C.L. Munro, *Toothbrush contamination: a review of the literature.* Nurs Res Pract, 2012. **2012**: p. 420630.
146. Genevois, A., et al., *Bacterial colonisation status of cystic fibrosis children's toothbrushes: A pilot study.* Arch Pediatr, 2015. **22**(12): p. 1240-6.
147. Mehta, A., P.S. Sequeira, and G. Bhat, *Bacterial contamination and decontamination of toothbrushes after use.* N Y State Dent J, 2007. **73**(3): p. 20-2.
148. Karibasappa, G.N., L. Nagesh, and B.K. Sujatha, *Assessment of microbial contamination of toothbrush head: an in vitro study.* Indian J Dent Res, 2011. **22**(1): p. 2-5.
149. Glass, R.T., *The infected toothbrush, the infected denture, and transmission of disease: a review.* Compendium, 1992. **13**(7): p. 592, 594, 596-8.
150. Lipuma, J., et al., *Burkholderia, Stenotrophomonas, Ralstonia, Cupriavidus, Pandoraea, Brevundimonas, Comamonas, Delftia, and Acidovorax.* Manual of Clinical Microbiology, 2007: p. 692-713.
151. Cherkaoui, A., et al., *Comparison of Two Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry Methods with Conventional Phenotypic Identification for Routine Identification of Bacteria to the Species Level.* Journal of clinical microbiology, 2010. **48**: p. 1169-75.
152. Veen, S., E. Claas, and E. Kuijper, *High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories.* Journal of clinical microbiology, 2010. **48**: p. 900-7.
153. O'Hara, C.M., *Evaluation of the Phoenix 100 ID/AST system and NID panel for identification of Enterobacteriaceae, Vibrionaceae, and commonly isolated nonenteric gram-negative bacilli.* J Clin Microbiol, 2006. **44**(3): p. 928-33.
154. Hortin, G.L., *The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome.* Clin Chem, 2006. **52**(7): p. 1223-37.
155. Seng, P., et al., *MALDI-TOF-mass spectrometry applications in clinical microbiology.* Future Microbiol, 2010. **5**(11): p. 1733-54.
156. Krasny, L., R. Hynek, and I. Hochel, *Identification of bacteria using mass spectrometry techniques.* International Journal of Mass Spectrometry, 2013. **353**: p. 67-79.
157. Risch, M., et al., *Comparison of MALDI TOF with conventional identification of clinically relevant bacteria.* Swiss medical weekly, 2010. **140**: p. w13095.
158. Croxatto, A., G. Prod'hom, and G. Greub, *Applications of MALDI-TOF MS mass spectrometry in clinical diagnostic microbiology.* FEMS microbiology reviews, 2011. **36**: p. 380-407.
159. Cañas, B., et al., *Mass spectrometry technologies for proteomics.* Brief Funct Genomic Proteomic, 2006. **4**(4): p. 295-320.
160. Lo, C., et al., *Value of matrix assisted laser desorption ionizationtime of flight (MALDI-TOF) mass spectrometry in clinical microbiology and infectious diseases in Africa and tropical areas.* African Journal of Microbiology Research, 2017. **11**: p. 1360-1370.
161. Murray, P.R., *Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: usefulness for taxonomy and epidemiology.* Clin Microbiol Infect, 2010. **16**(11): p. 1626-30.

162. Emonet, S., et al., *Application and Use of Various Mass Spectrometry Methods in Clinical Microbiology.* Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 2010. **16**: p. 1604-13.
163. *Principles and Techniques of Biochemistry and Molecular Biology*. 7 ed. 2010, Cambridge: Cambridge University Press.
164. Carbonnelle, E., et al., *MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory.* Clinical biochemistry, 2011. **44**: p. 104-9.
165. Gorton, R., et al., *Evaluation of a Short, On-Plate Formic Acid Extraction Method for Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-Based Identification of Clinically Relevant Yeast Isolates.* Journal of clinical microbiology, 2014. **52**.
166. Dubois, D., et al., *Performances of the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry System for Rapid Identification of Bacteria in Routine Clinical Microbiology.* Journal of clinical microbiology, 2012. **50**: p. 2568-76.
167. Souza, H., et al., *MALDI-TOF: A Useful Tool for Laboratory Identification of Uncommon Glucose Non-Fermenting Gram-Negative Bacteria Related to Cystic Fibrosis.* Journal of medical microbiology, 2014. **63**.
168. Mironova, L.V., M.V. Afanasev, and S.V. Balakhonov, *[Application of Pulsed Field Gel Electrophoresis for Molecular Typing of Causative Agents of Especially Dangerous Infections].* Mol Gen Mikrobiol Virusol, 2015. **33**(3): p. 28-32.
169. Schwartz, D.C. and C.R. Cantor, *Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis.* Cell, 1984. **37**(1): p. 67-75.
170. Tenover, F.C., et al., *Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing.* J Clin Microbiol, 1995. **33**(9): p. 2233-9.
171. Benjamin, F., et al., *The Use of Pulsed Field Gel Electrophoresis in Listeria monocytogenes Sub-Typing - Harmonization at the European Union Level*. 2012.
172. Roselino, A.M., *Biologia molecular aplicada às dermatoses tropicais %J Anais Brasileiros de Dermatologia.* 2008. **83**: p. 187-203.
173. Chu, G., D. Vollrath, and R.W. Davis, *Separation of large DNA molecules by contour-clamped homogeneous electric fields.* Science, 1986. **234**(4783): p. 1582-5.
174. Goering, R.V., *Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease.* Infect Genet Evol, 2010. **10**(7): p. 866-75.
175. Doring, G., et al., *Antibiotic therapy against Pseudomonas aeruginosa in cystic fibrosis: a European consensus.* Eur Respir J, 2000. **16**(4): p. 749-67.
176. Courtney, J.M., et al., *Clinical outcome of Burkholderia cepacia complex infection in cystic fibrosis adults.* J Cyst Fibros, 2004. **3**(2): p. 93-8.
177. Vandamme, P., et al., *Burkholderia anthina sp nov and Burkholderia pyrrocinia, two additional Burkholderia cepacia complex bacteria, may confound results of new molecular diagnostic tools.* Fems Immunology and Medical Microbiology, 2002. **33**(2): p. 143-149.
178. Beiersdorf, N., et al., *Soluble inflammation markers in nasal lavage from CF patients and healthy controls.* J Cyst Fibros, 2013. **12**(3): p. 249-57.
179. Liu, H.H., et al., *Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry.* Applied and Environmental Microbiology, 2007. **73**(6): p. 1899-1907.

180. Iriart, X., et al., *Routine Identification of Medical Fungi by the New Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight System with a New Time-Effective Strategy.* Journal of Clinical Microbiology, 2012. **50**(6): p. 2107-2110.
181. Ribot, E., et al., *Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of Escherichia coli O157:H7, Salmonella , and Shigella for PulseNet.* Foodborne pathogens and disease, 2006. **3**: p. 59-67.
182. *The Pulsed-Field Gel Electrophoresis Process*. 2013 [cited 2020; Available from: https://www.cdc.gov/pulsenet/pathogens/pfge.html.
183. Mehta, G., et al., *Cystic fibrosis across Europe: EuroCareCF analysis of demographic data from 35 countries.* J Cyst Fibros, 2010. **9 Suppl 2**: p. S5-S21.
184. Azizian, R., et al., *Sewage as a rich source of phage study against Pseudomonas aeruginosa PAO.* Biologicals, 2015. **43**(4): p. 238-241.
185. Harper, D., et al., *Bacteriophages and Biofilms.* Antibiotics, 2014. **3**: p. 270-284.
186. Lam, J., et al., *Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis.* Infection and immunity, 1980. **28**(2): p. 546-556.
187. Yang, L., et al., *In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections.* J Bacteriol, 2008. **190**(8): p. 2767-76.
188. Hancock, R.E., et al., *Pseudomonas aeruginosa isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains.* Infect Immun, 1983. **42**(1): p. 170-7.
189. Mahenthiralingam, E., M.E. Campbell, and D.P. Speert, *Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis.* Infect Immun, 1994. **62**(2): p. 596-605.
190. Dacheux, D., I. Attree, and B. Toussaint, *Expression of ExsA in trans confers type III secretion system-dependent cytotoxicity on noncytotoxic Pseudomonas aeruginosa cystic fibrosis isolates.* Infect Immun, 2001. **69**(1): p. 538-42.
191. D'Argenio, D.A., et al., *Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients.* Mol Microbiol, 2007. **64**(2): p. 512-33.
192. Hoffman, L.R., et al., *Nutrient availability as a mechanism for selection of antibiotic tolerant Pseudomonas aeruginosa within the CF airway.* PLoS Pathog, 2010. **6**(1): p. e1000712.
193. Mainz, J.G., et al., *Cystic fibrosis upper airways primary colonisation with Pseudomonas aeruginosa: eradicated by sinonasal antibiotic inhalation.* Am J Respir Crit Care Med, 2011. **184**(9): p. 1089-90.
194. Rivas Caldas, R. and S. Boisrame, *Upper aero-digestive contamination by Pseudomonas aeruginosa and implications in Cystic Fibrosis.* J Cyst Fibros, 2015. **14**(1): p. 6-15.
195. Guggenbichler, J.P., et al., *Spaced administration of antibiotic combinations to eliminate pseudomonas from sputum in cystic fibrosis.* Lancet, 1988. **2**(8613): p. 749-50.
196. Høiby, N., *The management of pseudomonas chest infections-the way forward.* Cystic fibrosis horizons. Proceedings of the 9th International Cystic Fibrosis Congress, 1984: p. 87-95.
197. Tinkelman, D.G. and H.J. Silk, *Clinical and bacteriologic features of chronic sinusitis in children.* Am J Dis Child, 1989. **143**(8): p. 938-41.
198. Muhlebach, M.S., et al., *Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis?* Pediatr Pulmonol, 2006. **41**(5): p. 445-51.

199. Morlacchi, L.C., et al., *The burden of sinus disease in cystic fibrosis lung transplant recipients.* Transpl Infect Dis, 2018. **20**(5): p. e12924.
200. Mainz, J.G., et al., *Sinonasal persistence of Pseudomonas aeruginosa after lung transplantation.* J Cyst Fibros, 2012. **11**(2): p. 158-61.
201. Linnane, B., et al., *Medical devices for cystic fibrosis care may be portable reservoirs of potential pathogens.* J Hosp Infect, 2017. **96**(4): p. 397-398.
202. Susheela, P. *STUDIES ON THE MICRO-BIOLOGICAL CONTAMINATION OF TOOTHBRUSHES AND IMPORTANCE OF DECONTAMINATION USING DISINFECTANTS*. 2016.
203. Assed Bezerra Da Silva, L., et al., *Mutans streptococci remained viable on toothbrush bristles, in vivo, for 44 h.* Int J Paediatr Dent, 2014. **24**(5): p. 367-72.
204. Domingos, P., et al., *Efficiency of cares over toothbrush storage to prevent contamination.* Journal of Research in Dentistry, 2013. **1**: p. 298.
205. Peker, S., et al., *Related factors of dental caries and molar incisor hypomineralisation in a group of children with cystic fibrosis.* Eur Arch Paediatr Dent, 2014. **15**(4): p. 275-80.
206. Komiyama, K., et al., *Pseudomonas aeruginosa in the oral cavity and sputum of patients with cystic fibrosis.* Oral Surg Oral Med Oral Pathol, 1985. **59**(6): p. 590-4.
207. Rivas Caldas, R., et al., *Pseudomonas aeruginosa and Periodontal Pathogens in the Oral Cavity and Lungs of Cystic Fibrosis Patients: a Case-Control Study.* 2015. **53**(6): p. 1898-1907.
208. Green, H. and A.M. Jones, *Emerging Gram-negative bacteria: pathogenic or innocent bystanders.* Curr Opin Pulm Med, 2018. **24**(6): p. 592-598.
209. Recio, R., et al., *Effect of respiratory Achromobacter spp. infection on pulmonary function in patients with cystic fibrosis.* J Med Microbiol, 2018. **67**(7): p. 952-956.
210. Tai, A.S., et al., *Genotypic Diversity within a Single Pseudomonas aeruginosa Strain Commonly Shared by Australian Patients with Cystic Fibrosis.* PLoS One, 2015. **10**(12): p. e0144022.
211. Workentine, M.L., et al., *Phenotypic heterogeneity of Pseudomonas aeruginosa populations in a cystic fibrosis patient.* PLoS One, 2013. **8**(4): p. e60225.
212. Feliziani, S., et al., *Coexistence and within-host evolution of diversified lineages of hypermutable Pseudomonas aeruginosa in long-term cystic fibrosis infections.* PLoS Genet, 2014. **10**(10): p. e1004651.
213. Mahenthiralingam, E., et al., *Random amplified polymorphic DNA typing of Pseudomonas aeruginosa isolates recovered from patients with cystic fibrosis.* Journal of Clinical Microbiology, 1996. **34**(5): p. 1129-1135.
214. Bhagirath, A.Y., et al., *Cystic fibrosis lung environment and Pseudomonas aeruginosa infection.* BMC Pulm Med, 2016. **16**(1): p. 174.
215. Agarwal, G., et al., *Characterization of Pseudomonas aeruginosa isolated from chronically infected children with cystic fibrosis in India.* Bmc Microbiology, 2005. **5**.
216. Pedersen, S.S., *Lung Infection with Alginate-Producing, Mucoid Pseudomonas-Aeruginosa in Cystic-Fibrosis.* Apmis, 1992. **100**: p. 5-79.
217. Thomassen, M.J., et al., *Multiple of isolates of Pseudomonas aeruginosa with differing antimicrobial susceptibility patterns from patients with cystic fibrosis.* J Infect Dis, 1979. **140**(6): p. 873-80.
218. Lozano, C., et al., *Great phenotypic and genetic variation among successive chronic Pseudomonas aeruginosa from a cystic fibrosis patient.* PLoS One, 2018. **13**(9): p. e0204167.

219. Zemanick, E.T., et al., *Assessment of airway microbiota and inflammation in cystic fibrosis using multiple sampling methods.* Ann Am Thorac Soc, 2015. **12**(2): p. 221-9.
220. Chen, A.I., et al., *Candida albicans ethanol stimulates Pseudomonas aeruginosa WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines.* PLoS Pathog, 2014. **10**(10): p. e1004480.
221. Hogan, D.A. and R. Kolter, *Pseudomonas-Candida interactions: an ecological role for virulence factors.* Science, 2002. **296**(5576): p. 2229-32.
222. Abraham, N.J., U.K. Cirincione, and R.T. Glass, *Dentists' and dental hygienists' attitudes toward toothbrush replacement and maintenance.* Clin Prev Dent, 1990. **12**(5): p. 28-33.
223. Beneduce, C., et al., *Germicidal activity of antimicrobials and VIOlight® Personal Travel Toothbrush Sanitizer: An in vitro study.* Journal of dentistry, 2009. **38**: p. 621-5.
224. Konidala, U., et al., *Efficacy of various disinfectants on microbially contaminated toothbrushes due to brushing.* Contemporary clinical dentistry, 2011. **2**: p. 302-7.
225. Nelson-Filho, P., et al., *Evaluation of the contamination and disinfection methods of toothbrushes used by 24- to 48-month-old children.* Journal of dentistry for children (Chicago, Ill.), 2005. **73**: p. 152-8.

## 10. PUBBLICATIONS

- Passarelli Mantovani, R., Sandri, A., Boaretti, M., Burlacchini, G., Li Vigni, V., Scarazzai, M., Melotti, P., Signoretto, C., & Lleo, M. M. (2020). **Longitudinal monitoring of sinonasal and oral bacterial reservoirs to prevent chronic lung infection in people with cystic fibrosis**. *ERJ open research*, *6*(3), 00115-2020. https://doi.org/10.1183/23120541.00115-2020.

**Abstract**

**Background**

Paranasal sinuses act as bacterial reservoirs and contribute to transmitting bacteria to the lower airway of patients with cystic fibrosis (CF). Also, passage of bacteria from the oral cavity to the lungs may occur.

**Methods**

We evaluated the presence of Pseudomonas aeruginosa, Staphylococcus aureus, Stenotrophomonas maltophilia, Achromobacter xylosoxidans and Serratia marcescens in sputum and nasal lavage of 59 patients with CF, and also collected saliva and used toothbrushes from 38 of them. We assessed the clonal identity of the strains isolated from the different samples by pulsed-field gel electrophoresis.

**Results**

About 80% of the patients were positive for at least one of the bacterial species examined in nasal lavage and sputum. Among the subjects with positive sputum, 74% presented the same species in the nasal lavage and saliva, and 26% on their toothbrush. S. aureus was the most abundant species in all samples. Clonal identity (≥80% similarity) of the strains isolated among the different samples from each patient was confirmed in almost all cases. Longitudinal observation helped to identify five patients who were colonised in the lower airways after an initial period of nasal or oral colonisation.

**Conclusion**

Nasal and oral sites act as bacterial reservoirs, favouring the transmission of potentially pathogenic microorganisms to the lower airway. The lack of eradication from these sites might undermine the antibiotic therapy applied to treat the lung infection,

allowing the persistence of the bacteria within the patient if colonisation of these sites is not assessed, and no specific therapy is performed.

- Passarelli Mantovani R, Sandri A, Boaretti M, Grilli A, Volpi S, Melotti P, Burlacchini G, Lleò MM, Signoretto C. **Toothbrushes may convey bacteria to the cystic fibrosis lower airways.** J Oral Microbiol. 2019 Aug 7;11(1):1647036. doi: 10.1080/20002297.2019.1647036. PMID: 31489126; PMCID: PMC6713191.

**Abstract**

Recent findings indicate that the oral cavity acts as a bacterial reservoir and might contribute to the transmission of bacteria to the lower airways. Control of a potentially pathogenic microbiota might contribute to prevent the establishment of chronic infection in cystic fibrosis. We evaluated the presence of CF microorganisms in saliva and toothbrushes of CF patients and verify their possible transmission to lower airways.

**Methods**

We assessed the presence of *P. aeruginosa, S. aureus, S. maltophilia, A. xylosoxidans, S. marcescens,* and yeasts in saliva, toothbrushes and sputum of 38 CF patients and assessed the clonal identity of the strains occurring contemporary in multiple sites by PFGE.

**Results**

At least one of the investigated species was isolated from 60 saliva samples and 23 toothbrushes. *S. aureus* was the most abundant species, followed by Candida spp. 31 patients contemporary had the same species in sputum and saliva/toothbrush: in most cases, clonal identity of the strains among the different sites was confirmed.

**Conclusion**

Toothbrushes may be sources of oral contamination and might act as reservoirs favoring transmission of potentially pathogenic microorganisms from the environment to the oral cavity and eventually to the LAW. Oral hygiene and toothbrush care are important strategies to prevent CF lung infections.

## 11. BEST PRESENTATION AT CONGRESS AWARD

Third place for the best oral presentation award: "**Investigação do papel dos reservatórios bacterianos para infecção pulmonar crônica em pacientes com fibrose cística**" in the VII Brazilian Multidisciplinary Cystic Fibrosis Congress. 2019

## 12. SCIENTIFIC CONTRIBUITION FROM THE PhD THESIS

Italian Cystic Fibrosis Research Foundation (project FFC#22/2016)