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Pseudomonas aeruginosa in cystic fibrosis patients: acquisition, early diagnosis and efficacy of eradication treatment

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Table of contents

List of abbreviations

AB: antibiotic(s)
ABC: ATP-binding cassette
ABPA: allergic bronchopulmonary aspergillosis
AFLP: amplified fragment length polymorphism analysis
AHL: acyl homoserin lactone
AP-PCR:arbitrarily primed polymerase chain reaction
ASL: airway surface liquid
ATP: adenosine triphosphate
A. xylosoxidans: *Achromobacter xylosoxidans*
BAL: broncho-alveolar lavage
BALF: broncho-alveolar lavage fluid
Bcc: *Burkholderia cepacia* complex
BID: bis in die (= twice a day)
BMI: body mass index
B. cepacia: *Burkholderia cepacia*
cAMP: cyclic adenosine monophosphate
CBAVD: congenital bilateral absence of vas deferens
CF: cystic fibrosis
CFF: cystic fibrosis foundation
CFTR: cystic fibrosis transmembrane conductance regulator
CI: confidence interval
CIE: counter immuno electrophoresis
Cl^-: chloride
c.q.: casu quo
DB: double blind
DIOS: distal intestinal obstruction syndrome
DNA: deoxyribonucleic acid
ELISA: enzym-linked immunosorbent assay
EnaC: epithelial sodium channel
fAFLP: fluorescent amplified fragment length polymorphism analysis
FEV_1: forced expiratory volume in one second
FVC: forced vital capacity
GT: genotype
HC: historic control
HCO_3^-: bicarbonate
HCW: health care worker
H. influenzae: *Haemophilus influenzae*
i.e.: id est
IL: interleukin
Inh: inhaled
ip: inpatient
IV: intravenous
LBR: Laboratory for Bacteriology Research
LES: Liverpool epidemic strain
LF: lung function
LPS: lipopolysaccharide
LS: laryngeal suction
m: month(s)
MCC: mucociliary clearance
MLST: multi locus sequence typing
MRSA: methicillin resistant *Staphylococcus aureus*
MSSA: methicillin senstitive *Staphylococcus aureus*

MU: million units
NACFC: North American Cystic Fibrosis Conference
NBS: newborn screening
Neg: negative
NF-κB: nuclear factor kappa B
NIH: National Institute of HealthNPA: nasopharyngeal aspirate
NPV: negative predictive value
NPD: nasal potential differences
NR: not reported
ns: not significant
NTM: non tuberculous mycobacteria
OP: oropharyngeal
op: outpatient
Pa: *Pseudomonas aeruginosa*
P. aeruginosa: *Pseudomonas aeruginosa*
PAMP: pathogen-associated molecular pattern
PC: placebo controlled
PCL: periciliary liquid layer
PCR: polymerase chain reaction
PFGE: pulsed field gel electrophoresis
PG: placebo group
Pos: positive
PPV: positive predictive value
QS: quorum sensing
RAPD: random amplification of polymorphic DNA analysis
RCT: randomized controlled trial
RNA: ribonucleic acid
ROS: reactive oxygen species
RSV: respiratory syncytial virus
S. aureus: *Staphylococcus aureus*
SD: standard deviation
S. maltophilia: *Stenotrophomonas maltophilia*
SNP: single nucleotide polymorphism
TB: triple blind
tDNA-PCR: tDNA intergenic length polymorphism analysis
TG: treatment group
TID: ter in die (= three times a day)
TIP: tobramycin inhalation powder
TLR: Toll-like receptor
tRNA: transfer RNA
TS: throat swab
UK: United Kingdom
y: year(s)

Chapter I: Cystic fibrosis

Introduction

Cystic fibrosis (CF) is the most common hereditary life shortening disease in the Caucasian population, affecting more than 100 000 people worldwide. The disease is caused by a defect in the CFTR protein, resulting in a defective transport of chloride across the cellular membrane. Hallmarks of the classic CF disease are progressive obstructive lung disease, pancreatic insufficiency and congenital absence of the vas deference in males. The prognosis for CF patients has improved substantially over the past 10-20 years, largely as a result of earlier diagnosis, more aggressive therapy, and provision of care in specialised centres. With a growing insight in the genetic defect and pathophysiology of the disease, major advances are made in the treatment of CF and in the recognition of atypical CF disease.

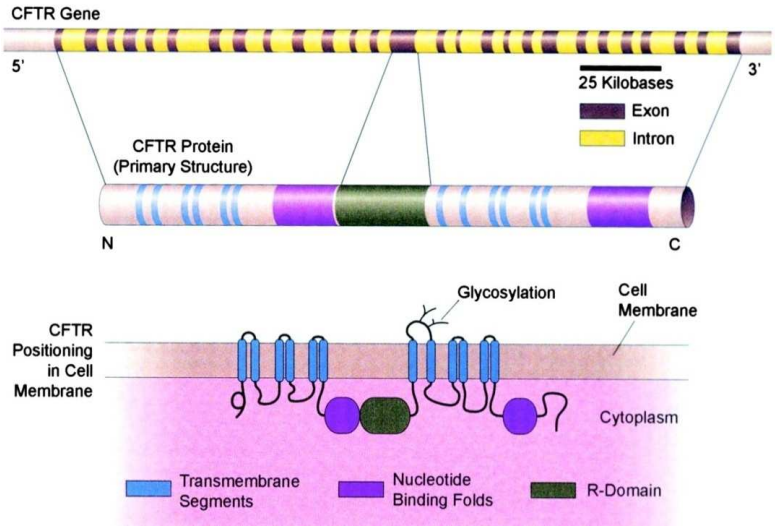
1. History

Although the first descriptions of young children dying from cystic fibrosis date back from the mid 16th century; the first clear description of cystic fibrosis as a clinical entity was made in 1938, by Dr Dorothy Andersen, pathologist at the New York Babies Hospital (1). She reported the clinical and pathological findings of 49 children, describing the characteristic neonatal meconium ileus, intestinal and respiratory complications and many other features of CF and particularly the characteristic pancreatic histology. Over the following years, CF was increasingly recognised as a hereditary multi-system disorder not only affecting the pancreas (displaying cystic fibrosis on pathological examination), but also other organs such as the respiratory tract and male reproductive system. Whereas in the early years CF babies did not survive early childhood, survival steadily increased thanks to the expanding knowledge on the disease, better therapies and better organised patient care, e.g. the advent of antibiotics and pancreatic enzyme replacement therapy in the fifties and the establishment of specialized CF centres for comprehensive care from the sixties on, with meticulous clinical and microbiological follow-up.

2. Genetics

The genetic defect causing CF was identified in 1989 by teams headed by Prof. Lap-Chee Tsui, Dr Collins and Prof. Riordan (2-4). They detected that CF results from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This large gene is located on the long arm of chromosome 7 and spans 250 kb. As shown in Figure 1, the gene is transcribed into a 6.5-kb messenger RNA that encodes a 1480 amino acid protein. The mature protein, properly folded, glycosylated and inserted in the cell membrane, is shown at the bottom of Figure 1.

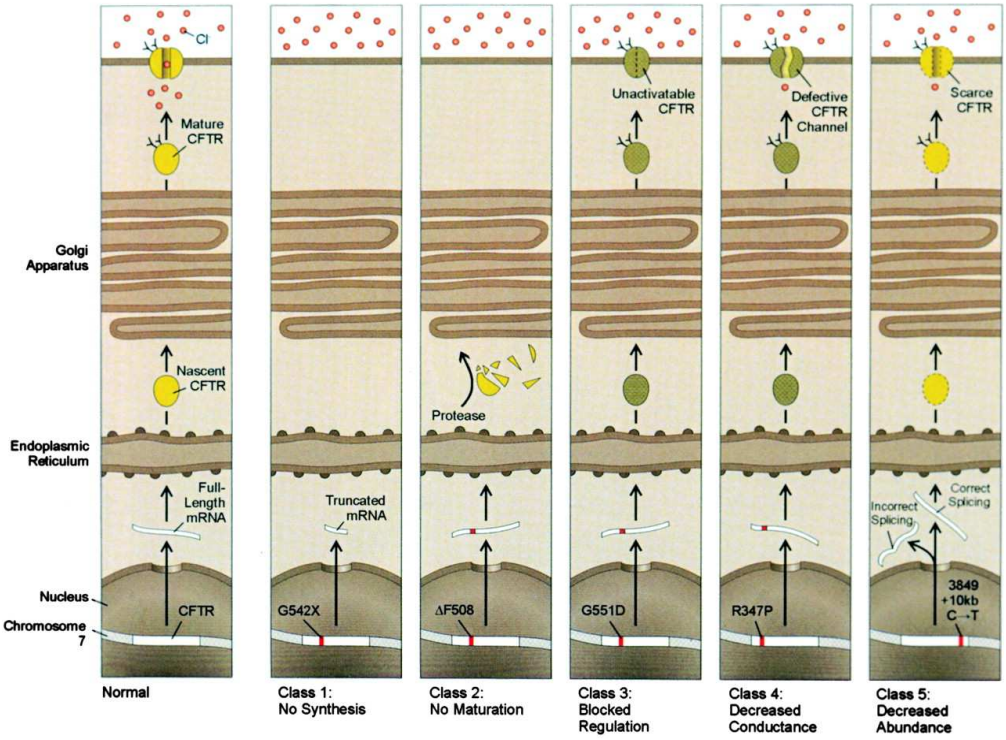
Figure 1. The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene and its encoded polypeptide.(5)



The CFTR protein is a member of the ATP-binding cassette (ABC) family of transporters. It contains two nucleotide-binding domains that bind and hydrolyze ATP, two dual sets of membrane-spanning segments that form the channel, and a central regulatory (R) domain. The CFTR protein mainly functions as a chloride (Cl^-) channel that regulates the ion and water balance across epithelia. CFTR is principally expressed in the apical membrane of epithelia and submucosal glands where it provides a pathway for Cl^- and bicarbonate (HCO_3) movement and controls the rate of fluid transport through its role as an anion channel on the one hand and through the regulation of other ion channels and transporters on the other hand (6). Thus, CFTR plays a fundamental role in transepithelial fluid and electrolyte transport. In respiratory airway, intestinal and pancreatic epithelia CFTR powers the secretion of Cl^- and HCO_3^-, while in sweat duct epithelia CFTR drives the reabsorption of salt. The mutations in the CF gene can disrupt CFTR function in different ways, ranging from complete loss of function to a reduced level of functional CFTR proteins. Mutations in the CFTR gene are currently classified into five groups according to their repercussion on CFTR protein synthesis and its chloride channel function (Figure 2).

Class I mutations produce premature transcription termination signals resulting in unstable, truncated non-functional CFTR. These mutations include the stop codon mutations.
Class II mutations, including F508del, result in an aberrantly folded CFTR protein that is degraded by the cell quality control system.
Class III mutations lead to defective regulation of the CFTR protein.
Class IV mutations give rise to a defective chloride conductance.
Class V mutations result in reduced amounts of functional protein.

Figure 2. Class I-V mutations of CFTR (5)



Since the identification of the gene, over 1500 CFTR mutations have been identified of which more than 1000 are known to cause CF while the remainder are involved in the milder CFTR related diseases or do not cause disease at all.
Mutation classes I to III usually lead to a classic CF phenotype with pancreatic insufficiency, although the severity of lung disease is highly variable. Class IV and V mutations are often associated with a milder expression of the disease and with pancreatic sufficiency. In general, genotype-phenotype correlation is good for pancreatic insufficiency, but is weak for respiratory disease. Modifier genes, lifestyle, treatment, environment and age may all play an additional role in determining the phenotype of clinical disease (7).

Cystic fibrosis is an autosomal recessively heritable disease. Two mutant CFTR genes are necessary to produce CF disease. A CF patient can either be homozygous (carrying an identical CFTR mutation on both CFTR alleles) or compound heterozygous (carrying two different CFTR mutations). The distribution of CFTR mutations differs between different ethnic populations. The most common mutation, F508del, reaches frequencies of 70% or more in northern European and North American populations, with lower frequencies in southern European populations. Although CFTR mutation frequency varies from population to population, worldwide no other single mutation accounts for more than approximately 5% of CFTR mutations (8).
Table 1 represents the relative frequencies of different mutations in the Belgian population (9).

Table 1: CFTR mutation frequency

F508del and other pairs

Mutation pair	n	%	cumulative %
F508del --- F508del	498	46.8	46.8
F508del ---OTHER	332	31.2	78.0
F508del ---NI	82	7.7	85.7
OTHER---OTHER	84	7.9	93.6
OTHER---NI	34	3.2	96.8
NI---NI	35	3.3	100.0
subtotal	**1065**		
missing	22		
total	**1087**		

NI = Not Identified
The mutation 'Poly-T tract variations' was treated as Not Identified

DNA ANALYSIS

Genotyped	n	%
Executed	1065	98.0
Unknown + Missing	22	2.0
total	**1087**	

Mutation	n	%
F508del*	1410	66.2
N1303K	58	2.7
G542X	56	2.6
1717-1G->A	32	1.5
3272-26A->G	27	1.3
R117H	26	1.2
S1251N	25	1.2
A455E	20	0.9
R553X	19	0.9
2789+5G->A	18	0.9
W1282X	16	0.8
R1162X	12	0.6
I507del	11	0.5
2183AA->G	11	0.5
L927P	10	0.5
3849+10kbC->T	10	0.5
394delTT	8	0.4
3659delC	8	0.4
E60X	7	0.3
3905insT	7	0.3
3199del6	6	0.3
W401X	5	0.2
G85E	5	0.2
D1152H	4	0.2
R334W	4	0.2

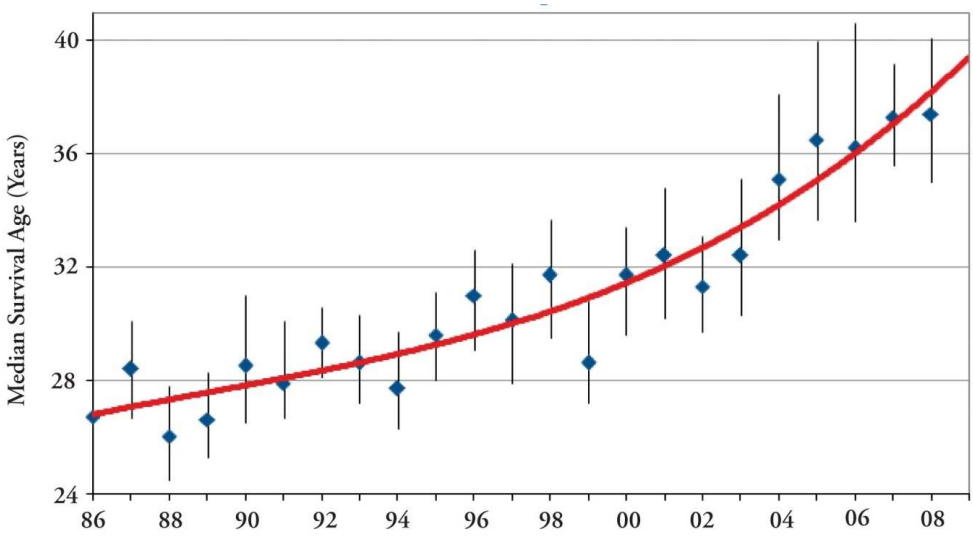
Mutation	n	%
L165S	4	0.2
Y913C	4	0.2
L227R	4	0.2
711+1G->T	4	0.2
3120+1G->A	4	0.2
poly-T tract variations	4	0.2
L997F	4	0.2
1078delT	3	0.1
Y1092X	3	0.1
CFTRdel2,3	3	0.1
711+5G->A	3	0.1
G970R	3	0.1
others	90	4.3
not identified	182	8.5
result unknown	44	.
subtotal	**2130**	
missing	44	
total	**2174**	

*The most common mutation is ***F508del*** with a prevalence of 66.2%.

3. Epidemiology

Cystic fibrosis affects more than 100 000 people worldwide. CF is more common in Caucasian populations. In Europe, prevalence varies from 1/1350 in Ireland to 1/25000 in Finland (10). The prevalence of CF in Belgium is 1/3700. Carrier frequency varies from 1/20 to 1/80. The prevalence of CF in North-America is approximately the same as in Europe (1/3500). CF is less common in Latin America, the Middle East and South Africa with prevalence rates ranging from 1/5000 to 1/20000 (6). Cystic fibrosis is uncommon in Africa and Asia, with a reported frequency of 1/350 000 in Japan (11). Due to better medical surveillance and better treatments CF no longer is a disease of childhood. Whereas in the early years of CF, babies did not survive early childhood, survival has increased considerably over the last decades (table 3). In Belgium, as in other countries, close to 50% of the CF patients are 18 years or older, reflecting the better survival, but probably also showing the result of increased diagnosis of atypical CF disease. At present, the median predicted survival of an individual with CF in the USA is 37.4 years (figure 3) (12). Data from the German Cystic Fibrosis registry in 2005 showed similar survival figures with a median cumulative survival of 37.4 years (13).

Figure 3 : Median predicted survival age 1986-2008 (12)



In 2008, 25651 patients (age range 0-82years) were followed in the US; of which 46,3% older than 18 years. Seventy percent of the CF patients are diagnosed before the age of 1 year, and 90% before their 8th birthday. Median age for diagnosis was 6 months; 42.8% of the newly diagnosed patients were detected through newborn screening (12).
In Belgium 1087 CF patients were followed in the CF Reference Centres in 2008 (Table 2) (9). The median age was 18.1 years (age range 0.1-67.4 years). The percentage of adults (>18 years) has risen steadily and represented 50.2% of the total number of patients. The median age at diagnosis was 6.9 months and at the age of 18 years 93% of the patients are diagnosed. In 2008, 20% percent of the newly diagnosed CF patients were detected by the neonatal screening test, which is locally organised in some Belgian provinces.

Table 2: General Data Belgisch Mucoviscidose Register Annual Data Report 2008 (14).

Number of CF patients: 1087
Number of CF patients with data records: 1072
Number of CF patients without observation: 15
New CF diagnoses: 25
Median patient age in years (range)*: 18.1 (0.1 - 67.4)
Median patient age males (range)*: 17.5 (0.1 - 57.5)
Median patient age females (range)*: 18.7 (0.2 - 67.4)
Males : 51.7%
Adults ≥ 18 years : 50.2%
Median age at diagnosis: 6.9m
Age range at diagnosis: 0.0 - 65.0y
Median age at diagnosis males : 7.0m
Age range at diagnosis males : 0.0 - 55.7y
Median age at diagnosis females: 6.8m
Age range at diagnosis females: 0.0 - 65.0y
Median age at diagnosis new cases in years (range): 1.3 (0.0 - 17.1)
Number of transplants performed in 2008: 14
Number of deaths reported occurring in 2008: 5
Median age at death in years (range): 25.5 (17.5 - 45.8)
Overall mean Wang-Hankinson FEV_1 % predicted (SD): 77.0 (25.0)
Mean FEV_1 % predicted males (SD): 78.9 (25.4)
Mean FEV_1 % predicted females (SD): 74.8 (24.3)
*Age of the patient at the last consultation

4. Clinical manifestations

The CFTR protein is expressed in many of the epithelial surfaces throughout the body, including sinuses, lungs, pancreas, liver and the reproductive system. Consequently, disease manifestations can be encountered in the different anatomical sites of the body. Signs and symptoms consistent with CF are listed in Table 3.

Table 3 **:** Phenotypic features consistent with a diagnosis of CF (15)

1) Chronic sinopulmonary disease manifested by
    - Persistent colonisation/infection with typical CF pathogens including *Staphylococcus aureus*, nontypeable *Haemophilius influenzae*, mucoid and nonmucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*.
    - Chronic cough and sputum production.
    - Persistent chest radiograph abnormalities (e.g. bronchiectasis, atelectasis, infiltrates, hyperinflation)
    - Airway obstruction manifested by wheezing and air trapping.
    - Nasal polyps; radiographic or computed tomographic abnormalities of the paranasal sinuses.
    - Digital clubbing.
2) Gastrointestinal and nutritional abnormalities including
    - Intestinal: meconium ileus, distal intestinal obstruction syndrome, rectal prolapse.
    - Pancreatic: pancreatic insufficiency, recurrent pancreatitis.
    - Hepatic: chronic hepatic disease manifested by clinical or histologic evidence of focal biliary or multilobular cirrhosis.
    - Nutritional: failure to thrive, hypoproteinemia and edema, complications secondary to fat-soluble vitamin deficiency.
3) Salt loss syndromes: acute salt depletion, chronic metabolic alkalosis.
4) Male urogenital abnormalities resulting in obstructive azoospermia (CBAVD).

Phenotypic features consistent with a diagnosis of CF

- **Pulmonary**

The most striking manifestations of the disease occur in the lungs. Symptoms of recurrent or chronic productive cough can start early on in life. Chronic pulmonary infections in CF patients progress to bronchiectasis, hypoxemia and hypercarbia. Respiratory insufficiency is responsible for at least 80% of CF-related deaths (12). CF patients often have digital clubbing as result of chronic inflammation. CF causes chronic pulmonary infections with only a few pathogens, not all of them are pathogenic for non-CF people. The most important infecting bacteria are: *P. aeruginosa*, *S. aureus*, *H. influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *B. cepacia* complex (16). The organism most frequently discovered in CF sputum is *P. aeruginosa.* As pulmonary infections with *P. aeruginosa* in CF patients are the main topic of this thesis, this subject will be elaborated in Chapters II and III.

- **Gastro-intestinal**

- Pancreas

Abnormal CFTR function in the pancreatic ducts causes a decreased volume of pancreatic secretions with reduced bicarbonate concentration (17). Autoactivation of retained digestive proenzymes leads to destruction and fibrosis of pancreatic tissue. Exocrine pancreatic insufficiency, leading to fat malabsorption and consequently steatorrhoea, fat-soluble-vitamin deficiency, and malnutrition is present in 85 to 90% of the CF patients. It can be present at birth or evolve over the first year of life. Typical signs of pancreatic insuffiency are greasy stools, flatulence, abdominal bloating, and poor weight gain. Exocrine pancreatic insufficiency can be overcome by the administration of pancreatic enzymes. Pancreatitis due to intermittent duct obstruction can occur in pancreatic-sufficient patients and may be the lead to diagnosis in older patients.

- Liver

Cholestasis secondary to common bile duct obstruction with inspissated bile may be the earliest manifestation of CF (18).
Patients with CF are at risk for focal biliary cirrhosis caused by obstruction of intrahepatic bile ducts, but clinically apparent cirrhosis occurs only in about 5% of patients (19). Liver cirrhosis may result in portal hypertension, with bleeding oesophageal varices and splenomegaly with signs of hypersplenism. In general, the clinical picture is one of very slowly progressive liver disease (20).

- Intestines

Meconium ileus is present in about 15% of newborns with cystic fibrosis. The bowel obstruction is secondary to the accumulation of inspissated intraluminal meconium. Distal intestinal obstructive syndrome (DIOS) is the equivalent of meconium ileus of the neonate. Patients present with signs of abdominal obstruction or more commonly subacutely with cramping abdominal pain.

Until recently many children with CF were of below normal weight and height and had delayed puberty. Those who reached adulthood were of relatively short stature. Factors which contribute to the poor nutritional status of many CF patients include an inadequate energy intake, the often severe and rarely completely controlled intestinal malabsorption and the increased energy demands resulting from chest infection. With present-day treatment, the majority of people with CF should have a normal rate of growth, nutritional status and body composition.

- **Upper airways**

Nasal polyps are found frequently in CF patients, the incidence raising with increasing age from 10% to 32%. Nasal obstruction is the most common symptom and the reason for removal of polyps but recurrence rate is high. The occurrence of nasal polyps can be a lead to diagnosis in older, more mildly affected CF patients.
The sinuses are infected in more than 90% of patients with CF but bacterial flora does not always correlate with the lower airways. It is however important to consider that sinuses may act as a long term reservoir for pulmonary infection (21).

- **Endocrine system**

The endocrine pancreatic function is initially spared from pancreatic fibrosis in CF patients. CF related diabetes mellitus (CFRDM) affects over 30% of adults with CF (12). These patients gradually develop carbohydrate intolerance and insulin insufficiency. Current guidelines advice to perform an annual oral glucose tolerance test from the age of 10 years on to detect CFRDM in an early stage (22).

- **Reproductive system**

- Male fertility

Ninety-five percent of the males with CF are infertile, but sexual function is not affected. Infertility results from structural abnormalities of the reproductive tract. The most common structural defect in CF males is the congenital absence of the vas deferens, giving rise to an obstructive azoospermia (23). This phenomenon can also be seen in men with only one CFTR mutation and no other manifestations of CF.

- Female fertility

Women with CF have anatomically normal reproductive tracts, but abnormalities of cervical mucus have been described. The formation of thick, tenacious cervical mucus may reduce sperm penetration. Women with severe respiratory disease and poor nutritional status are likely to have amenorrhoea and anovulatory cycles. Although there is some controversy about the effects of pregnancy in cystic fibrosis, the consensus is that a woman who has adequate nutritional and pulmonary reserve can successfully complete the term of pregnancy.

- **Osteoporosis**

Osteoporosis is more and more being recognised as a complication of CF. Osteoporosis in CF is of multifactorial origin and can be secondary to vitamin D deficiency, low body weight, decreased physical activity, delayed puberty, chronic inflammation and corticosteroid use. The 2008 CFF Patient Registry reports that about 20% of the CF patients from the age of 25 years on suffer from osteoporosis (12).

- **Variability of clinical spectrum and disease severity in CF patients**

Manifestations of cystic fibrosis can appear throughout life, with great variability within individuals. Median age at diagnosis according to the 2008 Annual Report of the Belgian Cystic Fibrosis Registry data is 6.9 months (14). The clinical symptoms leading to diagnosis are outlisted in table 4 (9).

Table 4: Clinical symptoms leading to diagnosis of CF (Belgisch Mucoviscidose Register Annual Data Report 2008) (14)

CF DIAGNOSIS SUGGESTED BY

	all data 1998 - 2008		new cases 2008	
	n	%	n	%
Acute or Recurrent Respiratory Problems	459	44.7	14	58.3
Failure to thrive	251	24.5	8	33.3
Chronic diarrhea/streatorrhea/malabsorption	232	22.6	5	20.8
Neonatal screening test	166	16.2	5	20.8
Meconium ileus	140	13.6	3	12.5
Family history	96	9.4	1	4.2
Nasal polyposis / chronic sinusitis	41	4.0	1	4.2
Rectal prolapse	32	3.1	2	8.3
Intestinal obstruction (other than meconium ileus)	30	2.9	0	0.0
Prenatal diagnosis	22	2.1	0	0.0
Dehydration / electrolyte imbalance	16	1.6	0	0.0
Neonatal jaundice	7	0.7	0	0.0
Infertility	7	0.7	0	0.0
Diagnosis other	73	7.1	0	0.0
*No diagnosis reasons given	61*		1	

*61 patients did not have information on any of the above reasons given.
From the 1087 patients, the overall percentages are based on 1026.
Patients with none of the above reasons given were excluded from the calculations.
There were 25 new diagnoses in 2008 and percentages based on 24 patients.
Reasons for diagnosis are not mutually exclusive.

5. Diagnosis of CF

The diagnosis of CF should be considered and diagnostic work-up should be done in any patient of any age presenting with phenotypic features consistent with CF (see table 4) or a family history of CF. Diagnosis of CF is mainly made on clinical grounds and should be confirmed by a positive sweat test. The sweat test, developed by Gibson and Cook in 1959 and using pilocarpine iontophoresis, remains the most gold standard in making the diagnosis of CF, provided it is done according to strict guidelines (24). Sweat test is positive if the concentration of chloride in sweat is greater than 60 mmol/L. A helpful method in assessing individuals who might have CF, but who do not meet classic diagnostic criteria (positive sweat test + 2 CFTR mutations), is measurement of nasal transepithelial potential difference (NPD) to assess chloride channel function (7, 25).
The number of children diagnosed through newborn screening for CF is growing. Newborn screening is done by the measurement of immunoreactive trypsinogen (IRT) in blood spots taken from newborn infants. A very high IRT concentration suggests pancreatic injury consistent with (but not specific for) cystic fibrosis. A positive screening result only indicates that a child is at increased risk for cystic fibrosis; a sweat test must still be done to confirm the diagnosis. Several studies have shown that newborn screening for cystic fibrosis leads to improved nutritional outcomes (26, 27). There is some evidence of improved survival in newborns diagnosed before the age of 1 month compared to infants diagnosed later (28) National programmes for neonatal screening exist in the USA, the UK, Russia, France , Austria and are organized locally in many other European countries (29). About 3 000 000 infants per year are screened (29).

6. Classic or typical CF; non-classic or atypical CF; CFTR related diseases.

With expanding knowledge on CFTR function and mutation analysis, milder CF disease and diseases associated with CFTR mutation are increasingly recognised (7).
Distinction should be made between these three entities:
- classic or typical CF:
Patients are diagnosed with classic or typical CF if they have one or more phenotypic characteristics consistent with CF and a sweat chloride concentration of >60 mmol/l. The vast majority of CF patients falls into this category. Usually one established mutation causing CF can be identified on each CFTR gene. Patients with classic CF can have exocrine pancreatic insufficiency or pancreatic sufficiency. The disease can have a severe course with rapid progression of symptoms or a milder course with very little deterioration over time.
- non-classic or atypical CF:
Patients with non-classic or atypical CF have a CF phenotype in at least one organ system and a normal (<30 mmol/l) or borderline (30–60 mmol/l) sweat chloride level. In these patients confirmation of the diagnosis of CF requires detection of one disease causing mutation on each CFTR gene or direct quantification of CFTR dysfunction by nasal potential difference measurement. Non-classic CF includes patients with multiorgan or single organ involvement. Most of these patients have exocrine pancreatic sufficiency and milder lung disease.
CFTR related disease:
Some patients with single organ involvement resulting from CFTR dysfunction may be more appropriately given an alternative diagnostic label as recommended in the World Health Organization (WHO) diagnostic list (Table 5) (30). The number of these people recognised as having milder problems possibly associated with CFTR dysfunction is growing. These problems include male infertility, recurrent pancreatitis, chronic sinusitis, and primary sclerosing cholangitis (31). Congenital bilateral absence of the vas deferens (CBAVD) in infertile males has been associated with a high incidence of CF mutations (32). Some infertile males have two mutations, the most common being F508del/R117H- (33). Eighty to

ninety percent of men with CBAVD carry at least one and 50-60% have two CFTR mutations, usually one is CF-causing and the other is a CFTR-related disorder associated mutation (7, 34).
Sharer and Cohn evaluated the possible role CFTR in chronic pancreatitis and detected that a significant proportion of people with pancreatitis were carriers of a CF mutation; the frequency of a single mutation was 11 times greater than the expected frequency in a control population (35, 36).

Table 5: WHO diagnostic list for single organ disease phenotypes associated with CFTR mutations

Isolated obstructive azoospermia
Chronic pancreatitis
Allergic bronchopulmonary aspergillosis
Disseminated bronchiectasis
Diffuse panbronchiolitis
Sclerosing cholangitis
Neonatal hypertrypsinogenaemia

7. Respiratory pathogens

i. Bacteria

The lungs of CF patients are colonized by bacterial pathogens, these are frequently acquired in an age-dependent sequence. The bacterial species most commonly associated with respiratory tract infection in CF include common human pathogens such as *Staphylococcus aureus* and *Haemophilus influenzae* as well as several opportunistic pathogens, of which *Pseudomonas aeruginosa* is the most important one. Other opportunists include *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* and species that are only occasionally associated with human infections apart from CF, such as the *Burkholderia cepacia* complex.
We can divide the infecting organisms in '**early infectors'** and **'late infectors'**.
Early infectors are *S. aureus*, *H. influenzae* and *P. aeruginosa*, while late infectors are *B. cepacia* complex, *S. maltophilia* and *A. xylosoxidans*. The other late infectors, the nontuberculous mycobacteria and fungi are discussed later on in this chapter.
The pattern of age-specific prevalence as well as overall prevalence of these pathogens in the CF population in the United States is demonstrated in Figure 4 from the Cystic Fibrosis Foundation Patient Registry 2008 data (12). The microbiology data of the Belgian CF Registry 2008 (9, 14) are represented in Figure 5. It must be noted that CF respiratory infection often is polymicrobial (37).
Other emerging bacteria which are not discussed below are *Ralstonia* and *Cupriavidus* species, *Pandoraea* species, *Inquilinus limosus* and anaerobic species (16).

Figure 4: CF microbiology (USA CFF Patient Registry 2008) (12)

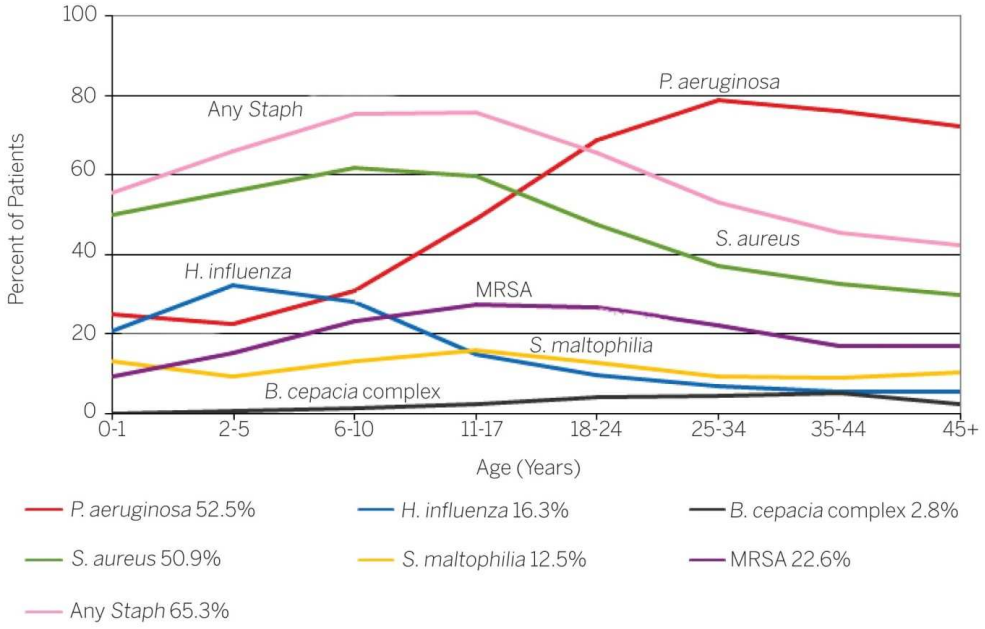
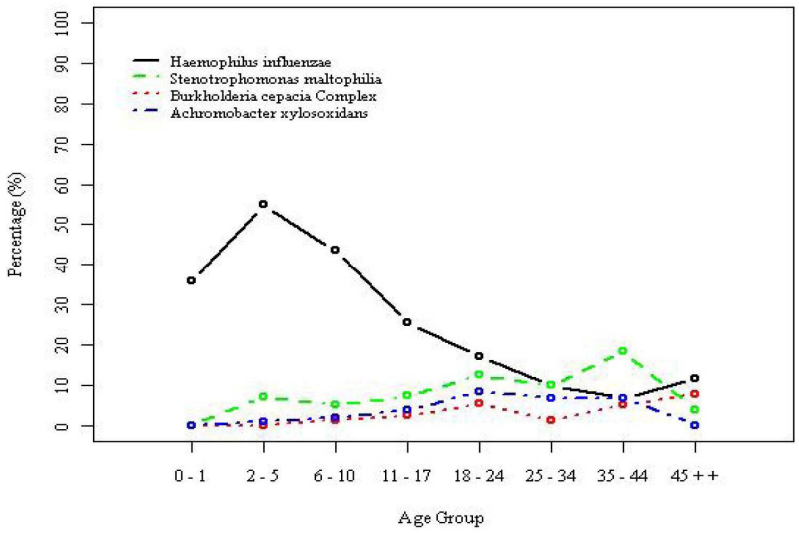
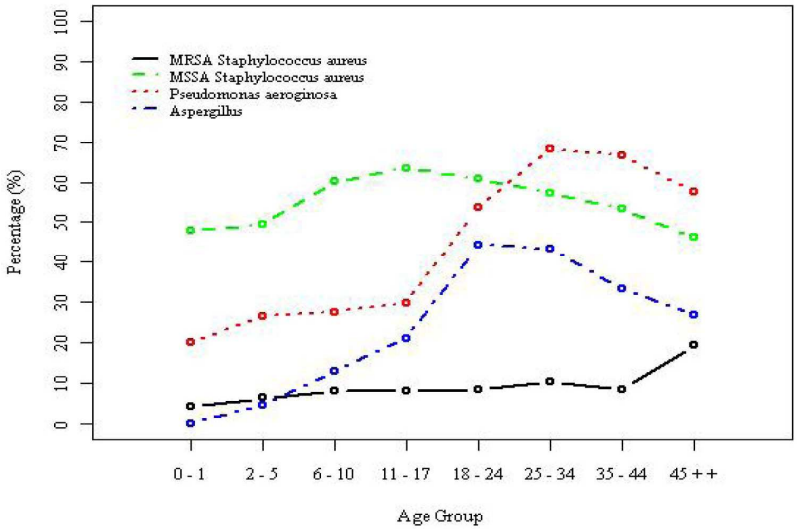


Figure 5: CF microbiology (Belgisch Mucoviscidose Register Annual Data Report 2008) (14)





Staphylococcus aureus

S. aureus is often the first and is among the most common bacterial pathogens recovered from the respiratory tract of persons with CF (38-40). In the pre-antibiotic area, *S. aureus* caused substantial morbidity and mortality in infants with CF (40). Continuous, anti-staphylococcal antibiotic prophylaxis is effective in reducing the incidence of infection with methicillin-sensitive *S. aureus* (MSSA) but an improvement in clinical outcome has not been shown (41). Moreover, there is some concern that antibiotic prophylaxis, especially with oral cephalosporins, might increase the incidence of *P. aeruginosa* (42, 43). Therefore, anti-staphylococcal antibiotic prophylaxis is not widely used. In recent years, the prevalence of methicillin resistant *S. aureus* (MRSA) in the CF population increased. Although initially questioned, there is now a growing evidence that MRSA might accelerate clinical deterioration and increase morbidity in CF patients (44, 45). Numerous protocols for eradication of MRSA in CF patients have been reported in literature, with an overall eradication rate of 80% (46-48).

Haemophilus influenzae

H. influenzae is also isolated from the respiratory tract early in the course of CF (39). The *H. influenzae* strains infecting CF patients are not capsulated and thus not prevented by childhood immunization against *H. influenzae* type b. The role of *H. influenzae* in progressive airway infection and inflammation in CF patients has not been clearly demonstrated. There are no trials to demonstrate benefit from eradication of *H. influenzae* from respiratory cultures in CF.

Pseudomonas aeruginosa

P. aeruginosa has been regarded in the previous decades as a 'late infector'. However, several recent studies have shown that infection occurs much earlier than previously believed (49, 50). Initially, *P. aeruginosa* grows as a non-mucoid strain that can be cleared by the host or eradicated with aggressive antibiotic treatment. Over time, *P. aeruginosa* colonies synthesize an alginate coat and form biofilms. These biofilms, once established, are difficult if not impossible to clear with standard antibiotic treatment. The epidemiology, clinical impact and treatment of *P. aeruginosa* in CF patients is reviewed extensively in Chapter II.

***Burkholderia cepacia* complex**

B. cepacia is a late infector in cystic fibrosis. The "*B. cepacia* complex" (Bcc) is not a single species but rather a group of closely related species, "genomovars". The vast majority of CF airway infections with *B. cepacia* complex are caused by genomovars II (*Burkholderia multivorans*), III (*Burkholderia cenocepacia*) and V (*Burkholderia vietnamiensis*) (51, 52). Genomovar III is higly virulent and transmissible and is feared because of its association with the *B. cepacia* syndrome, which can lead to death due to bacteremia and necrotizing pneumonia. Most patients infected with *B. cepacia* complex however have a more chronic course, associated with lung function decline and increased mortality (53). A combination of antibiotics is used to treat *B. cepacia* complex, as most organisms within the *B. cepacia* complex exhibit high levels of antibiotic resistance (54, 55).

Stenotrophomonas maltophilia

Isolation of *S. maltophilia* from sputa of patients with CF has increased markedly since the early 1980s (12, 56, 57). The precise reasons for this rise are unclear. The origin of *S. maltophilia* is unknown for most patients. Both the natural environment and other patients might be a possible source, although modes of transmission have not been identified exactly (58-60). Both transient and persistent infections can occur (16). The pathogenic role of *S. maltophilia* in the CF lung disease remains an area of uncertainty and debate (57, 60-65). *S. maltophilia* is resistant to most anti-pseudomonal antibiotics (66). In most studies only co-trimoxazole appears to have consistent activity, with more than 90% of the isolates being susceptible in vitro. UK guidelines (67) advice that only those patients chronically infected with *S. maltophilia* who exhibit evidence of clinical deterioration should receive antibiotic treatment targeted at this organism.

Achromobacter xylosoxidans

The prevalence of *A. xylosoxidans* in CF patients is rising (12, 68). *A. xylosoxidans*, as *S. maltophilia,* is encountered in CF patients with advanced lung disease. The source and routes of acquisition are unknown for most patients, although CF patients can share genetically identical strains (69, 70). Although infection with *A. xylosoxidans* is frequently transient, chronic infection can occur and is most often due to the persistence of the same strain (70-72).
Uncertainty still remains regarding its clinical significance in CF patients, although there is growing evidence that it causes an increase in morbidity. While Tan *et al*. (73) found no evidence of attributable clinical deterioration two years post-acquisition of *A. xylosoxidans*, De Baets *et al*. (74) observed that patients with chronic *A. xylosoxidans* infection had lower chest X-ray scores and pulmonary function parameters and required more courses of antibiotics than age- and *P. aeruginosa* colonization status matched CF patients. However, no accelerated decline in respiratory function was observed during the study period (1.5 +/- 0.9 years) . Hansen *et al*. (72) notified that *A. xylosoxidans* was associated with declining respiratory function if there was a rapid rise in specific precipitating antibodies in serum. *A.*

xylosoxidans is often multi-resistant and clinical data regarding optimal therapy are lacking. In vitro data suggest that the most active agents may be minocycline, meropenem or imipenem, piperacillin-tazobactam and chloramphenicol (75).
Possibly, the prevalence of this species may be higher, because *A. xylosoxidans* may be misidentified as an 'atypical *P. aeruginosa* isolate' in many laboratories (69, 74).

ii. Nontuberculous mycobacteria:

Nontuberculous mycobacteria (NTM) are increasingly reported from the respiratory secretions of patients with CF. The species most commonly isolated are *Mycobacterium avium* complex and *M. abscessus*. Studies conducted in the USA and France have demonstrated a prevalence ranging from 7% to 24% (76, 77). Different NTM types are found in different age groups and in different geographical regions (76, 78). The origin of NTM in CF is likely to be the environment, and there is little evidence for interpatient transmission of these species (76, 79). Jonsson and colleagues (80) observed that a shared strain of NTM was only found in one pair of siblings out of 14 CF patients with NTM infection.
The significance of the isolation of NTM from respiratory secretions remains unclear. NTM can represent transient contamination or colonization of the airway of CF patients but can also cause granulomatous disease (81). All CF patients should be screened annually for NTM. Patients with persistently positive sputum smears or cultures should be monitored closely for development of granulomatous disease (81). The American Thoracic Society criteria for diagnosis of disease have been revised in 2007 (82). Although not specifically designed for CF, they are helpful in guiding investigation.

iii. Fungi

Aspergillus *fumigatus* is the most common filamentous fungus involved in CF lung disease. The prevalence of *A. fumigatus* is low in young children with CF but increases steadily with age (83). Reported prevalence rates vary from 6% to nearly 60% (16). The origin of *A. fumigatus* is probably the environment, as it is an ubiquitous fungus, found in air, water, soil and rotting vegetation. The most frequently encountered fungal problem in CF is allergic bronchopulmonary aspergillosis (ABPA). Other clinical presentations include invasive pulmonary aspergillosis, aspergillus bronchitis, and aspergilloma.
ABPA is an immune-mediated bronchial disease causing bronchiectasis as a result of exposure to *A. fumigatus.* The cause of ABPA is not an invasive fungal infection but a type I and III sensitization against allergens from *A. fumigatus* in the environment, leading to a clinical syndrome with wheezing, pulmonary infiltrates and, if untreated, bronchiectasis and pulmonary fibrosis (84). Prevalence in CF is reported to be between 2 and 8% (85-87). Treatment of ABPA consists of systemic steroids in conjunction with an azole antifungal agent.
Invasive pulmonary aspergillosis is a rare but serious form of aspergillosis, mainly seen in immunosuppressed individuals. For persons with CF it is most likely to occur post transplantation.
Other fungal species, including *Exophiala dermatitidis* and *Scedosporium* species, are increasingly reported from CF respiratory samples (83). *Scedosporium apiospermum* has been associated with a symptom complex similar to that of ABPA (88). It is also capable of causing invasive disease with high mortality post lung-transplant (89, 90). The significance of *Exophiala dermatitidis* in CF remains uncertain (90).
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iv. Viruses

Viruses have a well-recognized role in exacerbations of respiratory disease in CF patients (91-99). The frequency of respiratory viral infections and distribution of viruses involved do not differ between CF patients and non-CF controls (93, 96, 100). However, viral respiratory

infections in CF patients are often more severe, of a longer duration (93, 96, 98) and can result in a decreased pulmonary function (91, 93, 98). Viral respiratory infections may predispose CF patients to bacterial infection, including infection with *P. aeruginosa* (92, 93, 96, 97, 101-104). Van Ewijck *et al*. (104) demonstrated that more than a third of non-CF individuals carry *P. aeruginosa* transitorily in the airways at times of acute viral respiratory infections during the winter months, but that they clear the organism spontaneously at recovery. In contrast, 30% of the CF people who become *P. aeruginosa* positive during colds often fail to clear the infection spontaneously when the viral infection settles. The same study group proved that RSV infection facilitates acute colonization with *P. aeruginosa* in mice (103).

8. Treatment

Appropriate and early initiated therapy is of paramount importance in CF patients. Treatment in CF is extensive and is started as soon as CF is diagnosed. Treatment includes pancreatic enzyme replacement therapy, high-fat and high-energy diet, chest physiotherapy and inhalation therapy. Treatment is burdensome: in a survey of adult patients with CF, subjects reported spending an average of 108 minutes (SD +/- 58 min) on daily CF treatments (105). In case of end stage pulmonary disease, lung transplantation can be performed. In patients with advanced lung disease, oxygen therapy and non-invasive ventilation is introduced awaiting lung transplantation. Future therapies and areas of intensive research include gene therapy, therapies to restore the airway surface liquid (ASL) and therapies to restore the CFTR protein defect.

- **Gastro-intestinal**

CF patients should maximize dietary intakes, taking a high-fat diet with appropriate pancreas enzyme replacement therapy (for pancreatic insufficient CF patients). All pancreatic insufficient patients should receive supplements of the fat-soluble vitamins. When nutritional failure occurs, patients can be fed by enteral tube.

- **Pulmonary**

Physiotherapy
Physiotherapy is usually introduced soon after CF is diagnosed. Forced expiratory manoeuvres and autogenic drainage assisted by positive end expiratory devices are probably the most effective part of chest physiotherapy (106).

Inhalation therapy
Effective and efficient inhalation therapy is an important component of CF care. Recent advances in inhalation therapy consist of alternative inhaled formulations (e.g. dry powder inhalations) and improved nebulizer devices, with shorter delivery time and improved lung deposition of medication (107, 108). Different drugs with different therapeutic goals can be inhaled, including antibiotics, hypertonic saline and dornase alfa. The main current therapeutic agents and future therapeutic perspectives are shortly described below. Reviews on this topic can be consulted elsewhere (6, 109, 110).

Inhaled tobramycin and colimycin are the most frequently used inhaled antibiotics in CF. Early studies with inhaled tobramycin, both low-dose (60-80 mg tid) (111, 112) and high-dose (600 mg tid) (113, 114) have shown an improved lung function and/or decreased hospitalisation rate in the treated patients. CF patients chronically colonized by *P. aeruginosa* on long-term nebulized tobramycin (300 mg bid, cycling 28 days on and 28 days off therapy) had a 10% increase in FEV_1 and 36% reduction of IV antibiotic use compared to the placebo group (115).

Nebulised colistin achieves low systemic and high local concentrations in the lung, supporting its use in patients with *P. aeruginosa* infection (116). It is frequently used as an alternative or in addition to inhaled tobramycin in patients chronically infected by *P. aeruginosa.* A randomized one-month trial comparing tobramycin solution for inhalation with inhaled colistin found no difference in reduction of *P. aeruginosa* in culture, but a significant improvement in lung function in the inhaled tobramycin group only (117). This result may be explained by previous exposure to colistin and treatment with a 1MU rather than a 2MU dose.

Nebulized human recombinant DNAse (dornase alfa, Pulmozyme®) is currently the most widely used mucolytic agent. It hydrolyzes the DNA present in sputum of cystic fibrosis patients, thereby reducing viscosity in the lungs and promoting the clearance of secretions. Daily inhalation improves lung function and reduces the frequency of infective exacerbations (118).
Inhaled Mesna and N-acetylcysteine are sometimes used in CF patients, without studies to support clinical evidence (119). However, the interest in the use of N-acetylcysteine as anti-oxidant drug, modulating inflammation in CF is growing (see future therapies p 26).
Inhaled hypertonic saline (HS) increases airway epithelium hydration, thereby improving mucociliary clearance (120-122). Elkins *et al.* (118, 123) conducted a double-blind trial in 146 CF patients, comparing inhalation with HS 7% bid with inhalation of normal saline 0.9% for 48 weeks. Although the rate of change (slope) in lung function during the 48 weeks of treatment did not differ significantly between both groups ($p = 0.79$), the absolute difference in lung function between the two groups was significant. Furthermore, the HS group also had a significantly higher percentage of patients without exacerbations. As HS can cause transient air flow obstruction (124, 125), patients are better pretreated with beta 2 agonists. A recent Cochrane review favours the use of HS (126).
Short and long-acting beta-2 agonists can be beneficial both in the short and long term in CF patients with obvious bronchodilator responsiveness or bronchial hyperrresponsiveness (127).
Inhaled corticosteroids are widely prescribed in CF. A recent Cochrane review concludes that evidence from trials is insufficient to establish whether ICS are beneficial in CF (128). CF patients with concurrent bronchial hyperresponsiveness may have some benefit of inhaled steroids (129)

Antimicrobial treatment
Antibiotics are the mainstay of treatment for pulmonary disease in CF and they are used for distinct situations. Antibiotics, either administered orally or intravenously, are used in the treatment of acute exacerbations, but also in attempts to eradicate early *P. aeruginosa* infection (see Chapter II). Once patients are chronically colonized with *P. aeruginosa*, chronic maintenance antibiotics are taken in order to slow the decline in pulmonary function and to reduce the frequency of pulmonary exacerbations. Both inhaled (eg colimicin, tobramycin – see inhalation medication) and oral antibiotics (azithromycin) are used for this purpose.
Maintenance treatment with azithromycin in CF patients chronically colonized with *P. aeruginosa* leads to a small but significant positive effect on lung function and less respiratory exacerbations (130-133). The exact mechanism of action of macrolides is unknown but is believed to be multiple. Both anti-infective and anti-inflammatory modes of action have been proposed to account for the efficacy of macrolides (134). Macrolides have an effect on formation of biofilms, as they inhibit the production of alginate and the expression of flagellin by *P. aeruginosa* and modulate quorum sensing (135-139). Antimicrobial effects are augmented by excellent biofilm penetration and intracellular accumulation in *P. aeruginosa* (140). It has been suggested that macrolides have an effect on reducing the sputum viscosity (141) and on *P. aeruginosa* adherence to respiratory epithelial cells (142). A recent study in *P. aeruginosa* free children and adolescents with CF does not favour the use of azithromycin: treatment with azithromycin for 24 weeks did not result in improved pulmonary function (143).

Anti-inflammatory agents

As airway inflammation starts early on in CF patients, it is logical to attempt to temper this ongoing inflammatory process.

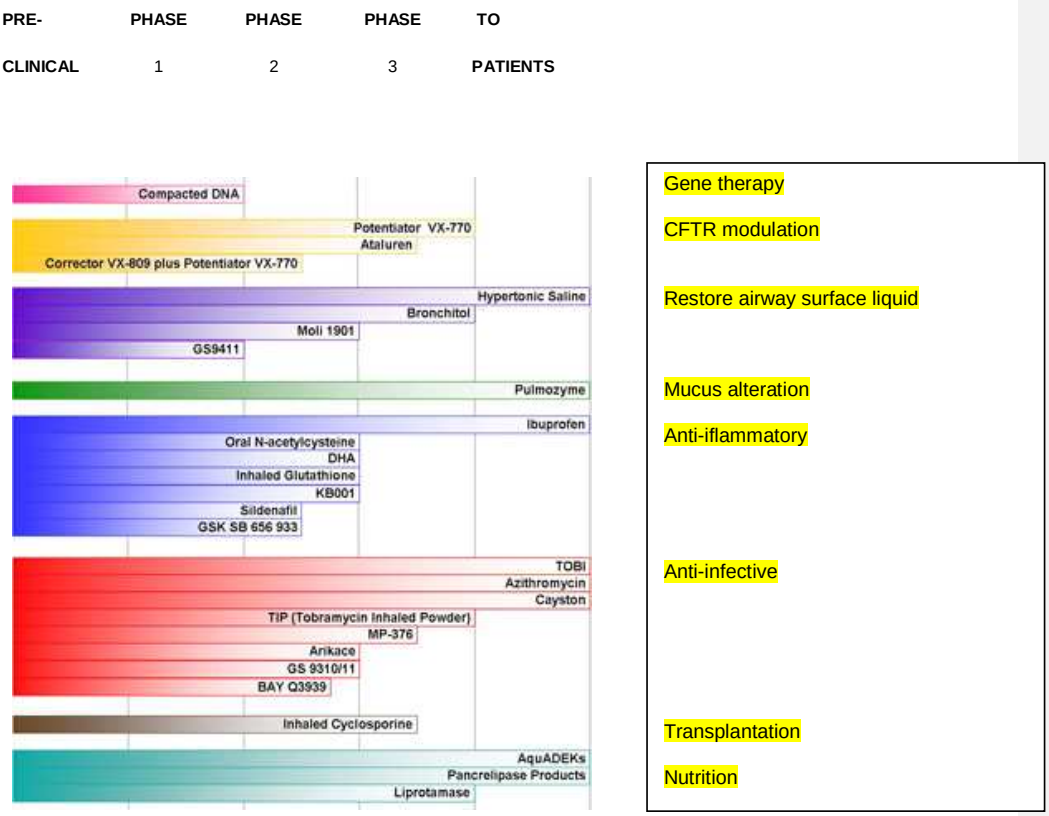
Oral corticosteroids have been shown to slow the progression of lung disease, but have unacceptable side effects for long-term use (144).

Konstan *et al*. (145) demonstrated that high-dose oral ibubrofen led to a significant reduction in the rate of decline in FEV_1 and fewer hospitalizations in CF patients with mild disease. Although ibuprofen has proven efficacy, it is not widely used because of its potential adverse effects, such as gastrointestinal bleedings and the need of monitoring peak plasma concentrations, which is not a standard laboratory technique (146).

Future therapies

The CF scientific field is expanding rapidly and several new possible treatment options are currently being examined. The therapeutic drug pipeline is represented below (http://www.cff.org/research/DrugDevelopmentPipeline).

Figure 7: CF therapies that are currently in development as of February 23, 2011.



These future therapies include:

New inhaled antibiotic treatments:
Aztreonam lysine for inhalation (Cayston®) has shown in a Phase III study in CF patients colonized with *P. aeruginosa* a significant improvement in respiratory symptoms and FEV_1 in the treatment group compared to the placebo group (147, 148). Cayston® received FDA approval in the US and EMA approval in Europe and became available to patients in the US in March 2010. In Belgium, we are awaiting reimbursement.
Tobramycin inhalation powder (TIP) was developed as an alternative to nebulized tobramycin (107). A Phase III trial has completed enrollment.

Other inhalation therapies:
Denufosol is a novel ion channel regulator designed to correct the ion transport defect and increase the overall mucociliary clearance in cystic fibrosis lung disease by increasing chloride secretion, inhibiting sodium absorption, and increasing ciliary beat frequency in the airway epithelium independently of cystic fibrosis transmembrane conductance regulator genotype. Patients who received denufosol demonstrated a significant increase in pulmonary function parameters compared to the placebo group (149). The results of a Phase 3, randomized, double-blind, placebo-controlled, 24-week trial in 352 CF patients with normal to mildly impaired lung function were published recently (150). A significant mean change in FEV_1 ($p = 0.047$) from baseline to week 24 in the patients inhaling denufosol 60mg 3 times a day compared with the placebo group was observed. A 48-week trial involving 466 CF patients however, showed that denufosol failed to meet its primary endpoint in generating a significant improvement in FEV_1. As a consequence Inspire Pharmaceuticals, the company producing denufosol decided to stop further investigations on this drug.
Inhaled dry powder mannitol (Bronchitol®) improves hydration and surface properties of sputum in CF patients (151), thereby improving mucociliary clearance. It has been shown to increase mucociliary clearance and FEV_1 (152). Phase III trials are ongoing.

Anti-inflammatory agents
The interest in the use of N-acetylcysteine, an anti-oxidant drug replenishing glutathione levels in neutrophils and modulating inflammation in CF is growing (153-155). Furthermore, N-acetylcysteine has shown in in vitro experiments in CF airway epithelial cells to increase chloride efflux, thereby improving hydration of mucus (156). A Phase 2 study on the efficacy and safety of high doses oral N-acetylcystein in CF patients failed to demonstrate an effect on clinical or inflammatory parameters after 12 weeks. However, extracellular glutathione in induced sputum tended to increase on high-dose N-acetylcysteine (154). A Phase 2b multi-center trial completed enrollment in 2010.

***Pseudomonas* vaccination**
Early studies of polyvalent *Pseudomonas* vaccines did not show a decrease in *P. aeruginosa* colonization in the vaccinated group and may have even predisposed some patients, once infected with *P. aeruginosa*, to a more severe pulmonary disease (157). A recent trial appeared to be more successful: Lang *et al.* (158) analyzed the 10-year follow-up data of 26 vaccinated CF patients, annually receiving a polysaccharide-exotoxin A conjugate *P. aeruginosa* vaccine and demonstrated that the time to infection with *P. aeruginosa* was longer and that the frequency of chronic infection with *P. aeruginosa* was significantly reduced in the vaccination group (32% of the vaccinated patients versus 72% of the control patients). In a prospective 2-year trial with a *P. aeruginosa* flagella vaccine (159), *P. aeruginosa* infections were documented in 37 of 189 vaccinated subjects versus 59 of 192 control subjects ($p = 0.02$). More recently published vaccine trials investigate alternative routes of administration of the vaccine in order to induce local immunity in the lung. For example, Bumann *et al.* (160) detected that only nasal and oral vaccinations, but not

systemic vaccination with a *P. aeruginosa* outer membrane porin vaccine resulted in a significant rise of IgA and IgG antibodies in the lower airways. Larger trials are needed to further validate the clinical benefit of these vaccines.

Gene therapy

Since the cloning of the CF gene in 1989, 25 Phase I/II clinical trials involving approximately 420 CF patients have been carried out, using a variety of viral and non-viral gene transfer agents. A gene therapy-based treatment however has not yet been developed (161). Barriers to effective gene transfer therapy are difficulties to deliver the vector to the cell, poor efficiency (162, 163), limited duration of gene expression and significant immune and inflammatory response to the virus-derived vector (162, 164, 165).

Mutation-specific therapies

Mutation-specific chloride channel correction pharmacotherapy is being developed at present and is a rapidly expanding area of research. These therapies are designed to correct or increase the function of the defective CFTR protein: "correctors" correct mutated CFTR function, "potentiators" improve channel gating. Some examples of these new therapeutical agents are described below:

In patients with one or more stop codon mutations, PTC 124 (Ataluren®) selectively induces read-through of premature stop codons. It has been studied in Duchenne muscular dystrophy and CF patients with a stop codon mutation (class I mutation) (166, 167). Its high oral bioavailability makes this product a promising medicine. In a Phase II study, oral administration of PTC124 reduced the epithelial electrophysiological abnormalities as assessed by transepithelial nasal potential difference (168, 169) and there was a trend toward improvements in pulmonary function (170). Phase III studies in CF completed enrollment in fall 2010.

In vitro studies have demonstrated that VX 770 is a CFTR potentiator that increases CFTR-mediated chloride transport (171). Accurso and colleagues recently published the results of a double-blind, placebo-controlled multicenter Phase 2 trial on the effect of orally administrated VX-770 in CF patients with at least one copy of the G551D mutation (172). They demonstrated improvements in both biological measures of CFTR function (nasal potential difference and sweat chloride) and pulmonary function measures (FEV_1). In February 2011, Vertex Pharmaceuticals, the company that developed VX 770, released promising results of a Phase 3 clinical trial of VX-770 in CF patients age 12 and older who carry at least one copy of the G551D mutation. In March, the company also released positive results from an ongoing Phase 3 clinical trial of VX-770 in children age 6 to 11 with the G551D mutation.

In the study of VX-770 in adults, patients who took the drug, compared to those on placebo, showed marked improvements in lung function (10 percent) and other key indicators of the disease, including sweat chloride levels, likelihood of pulmonary exacerbations and body weight. In the trial of VX-770 in young children, those who received the drug showed similarly significant improvements in lung function (12.5 percent) and in other key measures of the disease, including body weight and sweat chloride levels (www.vrtx.com).

The results of a phase II randomised placebo controlled clinical trial of 4 doses of the CFTR corrector VX-809 in CF patients homozygous for F506del CFTR mutation were presented at the NACFC 2010 by Clancy *et al*. The investigators noted a dose-dependent decrease in sweat chloride concentration but no effect on nasal potential differences nor on clinical parameters (FEV_1 and quality of life questionnaire) on day 28.

9. Pathogenesis of pulmonary disease and *P. aeruginosa* infection in cystic fibrosis

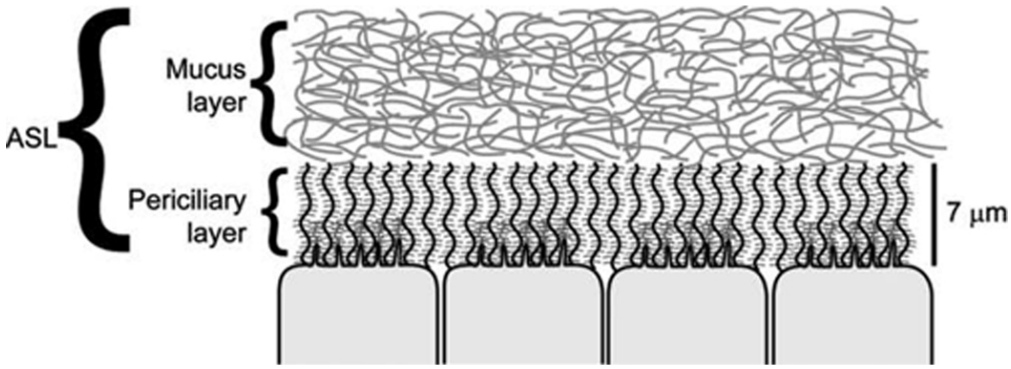
The mechanisms underlying CF lung disease are complex and not completely understood. There are several hypotheses as to how the CFTR defect causes the clinical picture of CF lung damage, of which the hallmarks are the intensive neutrophilic airway inflammation and the propensity to become infected with only a limited number of bacteria, non-pathogenic in the normal host. Some of these hypotheses will be outlined below, with the focus on pathogenesis and persistence of *P. aeruginosa* infection. In this chapter, the different theories are somewhat artificially classified into compositional, immunological, inflammatory and adherence hypotheses (figure 10). It is important to acknowledge that these hypotheses are not mutually exclusive. It is beyond the scope of this thesis to discuss all theories (with sometimes conflicting results), extensive reviews on this topic can be consulted elsewhere (173-180).

Defense strategies in the lungs of healthy individuals

In healthy individuals, host bacteria entering the lungs are cleared rapidly, without the initiation of an inflammatory response, by an array of host defence strategies, both mechanical (i.e. mucociliary clearance) and immunological.

Mechanical: Mucociliary clearance (MCC) is mediated by the airway surface liquid (ASL) (figure 8). The ASL lies on top of the epithelial surface and consists of two layers: a mucus layer, generated by secreted mucins and a periciliary liquid layer (PCL), being a poly-anionic water layer. The mucus layer entraps inhaled particles and pathogens, this entrapped material is cleared through the coordinated beating of cilia, moving the mucus blanket over the periciliary liquid layer. In normal airway epithelia Cl^- secretion (mediated by CFTR- and Ca^{2+}-Cl^- -channels) and Na^+ absorption (mediated by the epithelial Na^+-channel (ENaC)) are coordinated to maintain ASL at a depth that allows cilia to beat efficiently and to move the mucus blanket over the periciliary liquid layer (181).
Immunological: The resident macrophages and antimicrobial peptides, such as lysozyme and lactoferrin, protect against invading pathogens.

Figure 8: the airway surface liquid (ASL)



Theories linking the altered CFTR function to the increased inflammation and early infection in CF lungs

- **Compositional theories** (figure 9)

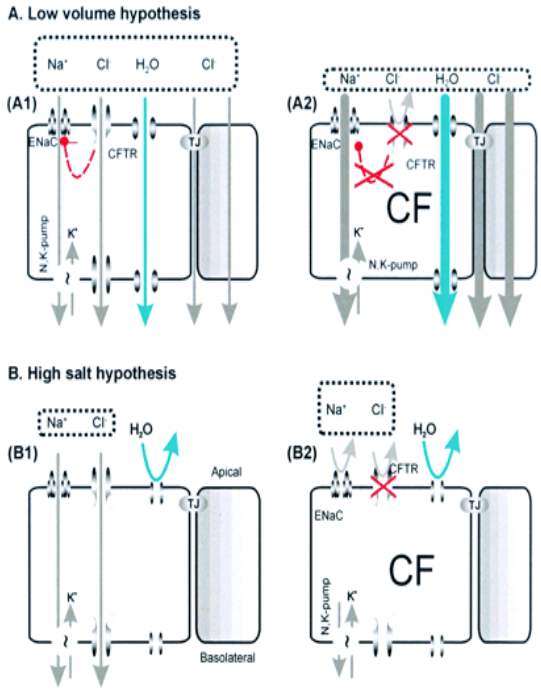
Low volume hypothesis

In the low volume hypothesis, defective CFTR results in increased sodium and water absorption, depleting the volume of ASL and of the mucus layer, within which cilia are unable to beat efficiently (182-185). Mucociliary clearance is impaired and the inhaled particles, bacteria and viruses become trapped in the viscous ASL and the mucus transport rates are reduced. Failure to clear inhaled environmental particles and irritants triggers release of pro-inflammatory chemokines, such as IL-8, from airway epithelia and/or macrophages patrolling airway surfaces. The cytokines become concentrated in the thickened mucus, recruit neutrophils into the airway lumina, and trigger airway inflammation even in the absence of bacteria.

High salt hypothesis

The high-salt hypothesis claims that in the absence of functional CFTR, excess sodium and chloride are retained in airway surface liquid (186, 187). The increased concentration of chloride in the periciliary layer disrupts the function of important innate antibacterial defense proteins, such as human β-defensin 1, lysozyme and lactoferrin, allowing bacteria, that would be cleared by normal airways, to persist in lungs (188). Although *in vitro* data have shown that the high salt content in CF airways disables these defense molecules (186), *in vivo* data demonstrated that ASL electrolyte levels are not different between CF and healthy airways (189), refuting this hypothesis.

Figure 9: low volume and high salt hypothesis (6)



(A) The low-volume hypothesis postulates that normal ASL (A1) has salt levels approximately equal to plasma. In CF (A2), the removal of CFTRs inhibition of epithelial sodium channels (EnaC) results in abnormally elevated isotonic fluid absorption, which depletes the ASL and leads to reduced mucociliary clearance. Key features of the low-volume model are the parallel pathway for Cl^- via shunt pathway(s) and inhibition of ENaC via CFTR.
(B) The high-salt hypothesis postulates that normal ASL has low levels of salt as a result of salt absorption in excess of water (B1). Even though the epithelium is water permeable, salt is retained in thin surface films by some combination of surface tension impermeant osmolytes. In CF (B2), salt is poorly absorbed resulting in excessively salty ASL that inactivates endogenous, salt-sensitive antimicrobial peptides. Key features of the high-salt model are: the lack of an appreciable shunt Cl^- conductance, central importance of CFTRs channel role, no specific role for inhibition of ENaC by CFTR, and a switch from isotonic volume absorption to hypertonic salt absorption as the surface layer thins and traps residual water.

- **Immunological theories:**

There are some indications that CFTR alterations lead to decreased local immunity in CF airways. These failures of local innate immunity might be risk factors for CF airway infections. There are several resports indicating that the oxidative killing is impaired in CF patients: In the bronchi, the highly viscous mucus generates a microaerobic milieu which may become anaerobic by a rapid oxygen consumption by pathogens such as *P. aeruginosa* (190). Consequently, the generation of reactive oxygen species (ROS) by neutrophils and other cells is abolished and bacterial killing impaired The microaerobic/anaerobic environmental conditions on the respiratory epithelium allow microbial pathogens, intrinsically resistant to non-oxidative killing or capable to change their phenotype to become rapidly resistant to non-oxidative killing, to persist in airways of individuals with CF. On top of that, Moskwa and colleagues (191) have demonstrated that a diminished transepithelial hypothiocyanite (SCN-) transport exists in CF airways, resulting in a defective killing of bacteria through the oxidative defense mechanism by means of ROS. Furthermore, according to the high-salt hypothesis, antibacterial peptides in the ASL are inactivated by the high salt concentration. In addition, there is accumulating evidence that expression of nitric oxide (NO) synthase is regulated by CFTR. Low levels of NO synthase are present in CF airways, resulting in low levels of nasal and exhaled NO (192). As NO has certain antibacterial and pro-inflammatory properties, the low levels could have,at least partly, a role in the predisposition to infection.

- **Inflammatory theories**:

Hallmark of CF airway disease is the intense neutrophilic inflammation, starting early on in life. Studies have attributed a central role of Toll-like receptors (TLRs), nuclear factor *κ*B (NF-κB) signalling pathway and neutrophil elastase in the development of CF lung disease. The adhesion of mucus to the epithelium surface brings epithelial cells into the first line of host defense against colonizing bacteria. Invading bacteria display pathogen-associated molecular patterns (PAMPs), which are recognized by TLRs on the airway epithelium and their activation results in a pro-inflammatory response, leading to activation of downstream signalling cascades such as NF-*κ*B and production of pro-inflammatory cytokines and chemokines. Each TLR has the ability to recognize and discriminate a specific PAMP present on invading pathogens. The most studied TLRs in CF are TLRs 2, 4 and 5. Heterodimerization of TLR2 with TLR1 or TLR6 results in the recognition of microbial components from the cell wall of Gram-positive bacteria, such as lipoproteins, peptidoglycan and lipoteichoic acid, whereas TLR4 is involved in the detection of lipopolysaccharide (LPS) and TLR5 with flagellin, which are both found in Gram-negative bacteria. LPS, which is found on *Ps. aeruginosa*, plays a major role in producing the chronic infection seen in the CF airways (178). Activation of any TLR results in a Th1 innate immune response, which is characterized by NF-*κ*B activation and the release of pro-inflammatory mediators.NF-*κ*B is one of the key regulating transcription factors of genes that are involved in inflammation and immunity. Activation of the NF-κB pathway in the CF airways leads to enhanced secretion of IL-8 and other chemokines by the airway epithelial cells (193-198). IL-8 is the principal

neutrophil chemoattractant in the CF lung (199), attracting neutrophils, which on their turn produce elastase. Extracellular neutrophil elastase has several roles: it cleaves CXCR-1, a chemokine receptor, from the neutrophil cell surface, thereby disarming neutrophils of their antibacterial capacity. The released CXCR-1 stimulates IL-8 production by the airway epithelial cells, thereby initiating a feedback circuit that amplifies neutrophil recruitment within the CF airways and amplifying inflammation (200). Elastase can also induce mucin overproduction by airway glands, potentiating obstruction (201). It can cleave scavenger receptors on macrophages and epithelial cells, inhibiting clearance of apoptotic neutrophils. Some studies also established that CF airways are relatively deficient in counter-regulatory molecules such as IL-10 and NO. Consequently, a lack of inhibition on the NF-κB activity results in prolonged and excessive production of inflammatory mediators (202-205). Despite the fact that the link between NF-κB signaling and inflammatory activation is established, the precise "activator mechanism" by which the deficient CFTR leads to an elevated NF-κB activation in the CF airway remains to be elucidated. Discussion continues whether inflammation is intrinsically inherent to CFTR defect or secondary to infection.

Studies favouring the theory of an intrinsically increased inflammation in CF:
Some studies have found increased numbers of inflammatory cells and IL-8 in BALF from CF patients with either mild disease symptoms or in the absence of demonstrable microorganisms (205-211). Many studies have found that CF airway epithelial cells in culture have constitutively active NF-κB and upregulated expression and secretion of IL-8 and other inflammatory mediators (207, 208, 211-215). This apparent inherent inflammatory state may be further stimulated by the presence of *P. aeruginosa* (211, 216), though not always (213).

Studies favouring the theory of inflammation resulting from infection:
Other authors hold the opinion that early infection in CF is the likely explanation for the enhanced inflammatory responses in CF lungs. In vivo measurements of inflammatory mediators in BAL fluids showed that increased inflammation in CF followed bacterial infections (38). Recent studies using terminal restriction fragment length polymorphism analysis (RFLP) of sputum from both adult (217) and pediatric (218) CF patients have shown many bacterial species that have not been previously identified in CF. It therefore seems possible that previous in vivo studies that observed inflammation in the apparent absence of infection may have suffered from low sensitive methods for bacterial detection.

- ***P. aeruginosa* adherence/ingestion theories**

These theories give some clue as to why CF lungs are especially vulnerable to infections caused by *P. aeruginosa*.

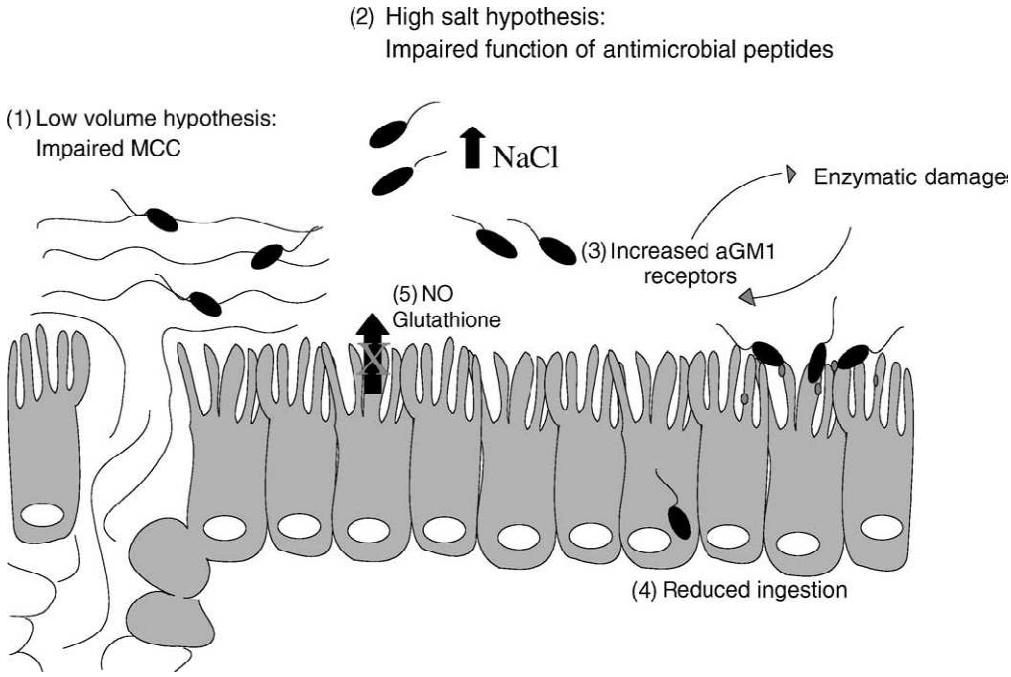
The adherence hypothesis
This theory alleges that the defective CFTR protein causes an increased availability of receptors for *P. aeruginosa. P. aeruginosa* possesses several different classes of adhesins (e.g. pili and flagellae) to bind to receptors on cell surfaces. One of these binding sites, the asyalated glycolipid GM1 (aGM1) is present in increased abundance on the surface of CF respiratory epithelial cells, allowing an increased binding of *P. aeruginosa* to CF cells (219, 220). Pseudomonads also produce neuraminidase, which cleaves sialic acid off glycolipids, thereby increasing the amount of aGM1 (221). In addition, adherence of *P. aeruginosa* to this receptor leads to a NF-κB-mediated increase in expression of the pro-inflammatory cytokine IL-8, with ensuing increase of the inflammatory cycle. Recently, Teichgraber *et al*. (222) detected an abnormal accumulation of ceramid (a sphingomyelin component of biological membranes) in the lungs of CFTR-deficient mice and in epithelial cells from CF patients. As a consequence, the rate of cell death increases in the respiratory epithelial cells, resulting in the formation of DNA deposits on the respiratory epithelium, facilitating bacterial adherence

of *P. aeruginosa*. Ceramide accumulation also provokes a pro-inflammatory status with an increased synthesis and release of cytokines and a subsequent recruitment of macrophages and neutrophils. This phenomenon precedes bacterial infection. How mutated CFTR mediates ceramide accumulation is still discussed.

The ingestion hypothesis

In this hypothesis, supported by Pier *et al*. (223), CF respiratory epithelial cells are less capable than normal cells to ingest *P. aeruginosa*. In contrast with the former hypothesis, Pier *et al*. hypothesize that the wild type CFTR is a receptor for *P. aeruginosa*. Binding of *P. aeruginosa* to the wild type CFTR airway epithelium results in phagocytosis and clearance by desquamation (223, 224). Defective CFTR consequently leads to reduced *P. aeruginosa* binding, resulting in reduced *P. aeruginosa* clearance from the CF airway.
Darling *et al*. (225), in contrast, proposes that internalized *P. aeruginosa* may play an important role in the pathogenesis of infection and that, by allowing greater internalization into epithelial cells, mutant CFTR results in an increased susceptibility of bronchial infection with this microbe.

Figure 10: Hypotheses on pathophsiology of CF airway disease



Chapter II: *PSEUDOMONAS AERUGINOSA*

Introduction:

CF patients can get infected by *P. aeruginosa* early on in life and the prevalence of *P. aeruginosa* in respiratory cultures increases with age, from 10–30% at ages 0–5 years to 80% at ages 25-34 years (US data – Chapter I figure 4)(12). In Belgium, prevalence rises from <10% in the children < 1year of age to nearly 70% at ages 25-34 years according to the 2008 data (Chapter I – figure 6) (14). *P. aeruginosa* infection in CF patients takes its course in distinct stages. After treatment of a first ever isolate of *P. aeruginosa*, patients may go through different episodes of colonization (intermittent colonization), preceding chronic colonization by months to years, eventually resulting in chronic infection (6, 49, 226). *P. aeruginosa* strains causing early infection usually have a nonmucoid phenotype, are present in low bacterial density and are relatively antibiotic susceptible. Over time, the distinct microenvironment in the CF airways allows selection of *P. aeruginosa* uniquely adapted for chronic, persistent infection. These organisms are mucoid, form biofilms, become increasingly antibiotic-resistant (227), are present at high density, and are virtually impossible to eradicate (228, 229). In this chapter, the origin of *P. aeruginosa* in CF patients, the evolution from initial to chronic infection and non-mucoid to mucoid *P. aeruginosa* in CF patients, the consequences of chronic colonization by this pathogen and finally the treatment of *P. aeruginosa* in CF patients will be discussed.

***P. aeruginosa*: natural habitat and survival:**

P. aeruginosa is an ubiquitous organism: it has been isolated from soil (230, 231), from plants and vegetables (231) and from water samples from creeks, rivers (232) and other water sources such as drinking water (233). Sewage frequently harbors *P. aeruginosa*. *P. aeruginosa* is not a marine organism, probably because the high salt concentrations inhibit its growth. *P. aeruginosa* is rarely isolated from healthy humans with only up tot 7% of healthy humans carrying *P. aeruginosa* in the throat, nasal mucosa or the skin (234), and 1.2% to 2.3% of healthy individuals harboring *P. aeruginosa* in the intestines (235-237). Animal experiments have shown that the addition of penicillin to drinking water leads to intestinal colonization by *P. aeruginosa* (238) and accordingly, the use of antibiotics in hospitalized patients increases considerably the incidence of *P. aeruginosa* -positive stools (239). Carriage rates of *P. aeruginosa* in stool as high as 24% have been reported in hospitalized patients (234, 240).
P. aeruginosa is tolerant to temperatures as high as 45 to 50 ℃ an d sensitive to desiccation and light and hence short-lived in aerosols and on dry surfaces (241). However, *P. aeruginosa* strains may survive considerably longer in the presence of protein and other organic material, e.g. in sputum. Mucoid *P. aeruginosa* strains survive longer than non-mucoid strains and strains suspended in sputum of CF patients can survive on dry surfaces for as long as 8 days (242, 243).

Origin of *P. aeruginosa* in CF patients

Numerous studies have attempted to identify the initial source of *P. aeruginosa* in CF patients. Both transmission from the natural environment, home and hospital environment and from other CF patients has been investigated. This chapter gives an overview on the current knowledge on possible sources and transmission of *P. aeruginosa* to CF patients.

- **Patient-to-patient transmission:**

Transmission of *P. aeruginosa* from one CF patient to another CF patient has been well studied. The so-called "Danish experience" is the first documented observation of patient-to-patient transmission and described the effect of preventive measures to limit the spread of *P. aeruginosa* among CF patients. In the 1980s, after the observation that one epidemiological *P. aeruginosa* strain predominated in CF patients in the Danish CF centres (244, 245), studies were undertaken to identify routes of cross-infection. The CF ward was found to be massively contaminated by *P. aeruginosa* of the same type as those cultured from the patients (243) and it was shown that patients could spread *P. aeruginosa* when coughing. Furthermore, the observation that CF patients treated outside the centre had a lower prevalence of chronic *P. aeruginosa* infection and contracted the infection later (246) confirmed the suspicion of patient-to-patient transmission of *P. aeruginosa*. Hoiby and Pedersen (244) calculated that the risk of cross infection increased with "contact density", i.e. the number of days a noninfected CF patient spent at the CF centre. Based on these results, preventive measures were introduced: *P. aeruginosa* infected patients were segregated from non-infected patients in separate wards and on separate days in the outpatient clinic, and improved hygienic measures were undertaken in the clinic (243). After the introduction of cohort isolation in the CF centre, an epidemic spread of multi-resistant *P. aeruginosa* strain was observed in the CF ward for patients with chronic *P. aeruginosa* infection (246). Further development of this epidemic was immediately stopped by establishing a third cohort for the patients harbouring the multi-resistant strain. As a result of the segregation measures and the improved hygiene the yearly incidence of new chronic *P. aeruginosa* infection dropped from 17% in 1976-1980 to 3% in 1986-1987 (247). Later on, the existence of patient-to-patient transmission was confirmed by genotyping of *P. aeruginosa* (248, 249).

Different conclusions on the degree of transmissibility of *P. aeruginosa* have been drawn from two molecular epidemiological studies from two large centres without segregation policies in Australia (250) and Canada (251). The Australian cross-sectional study found a widespread clone of *P. aeruginosa* in 55% of 118 infected patients in a paediatric CF clinic. In contrast, the Canadian longitudinal study, run over two decades, showed a low risk of patient-to-patient spread among 174 patients, except for patients with prolonged and close contacts, such as siblings. A non-exhaustive overview of the studies supporting the positions of both groups can be consulted in Table 1.

Table 1: studies on patient-to-patient transmission of *P. aeruginosa*.

Author year (ref)	Number of patients/sputum samples/isolates	Genotyping method	% of identical isolates	Remarks
Studies indicating the existence of highly transmissible P. aeruginosa strains				
Cheng 1996 (252) Liverpool, UK	92/92/NR	PFGE	60	Liverpool strain
Jones 2001(253) Manchester, UK	154/154/NR	PFGE	14	
Edenborough 2004 (254) Sheffield, UK	43/407/NR	PFGE	16/23	Liverpool strain/Sheffield strain
Scott 2004 (255) UK	NR/NR/ 849	PFGE	11/10/1,7	Liverpool (15 centres), Midlands (9 centres), other strain (8 centres)
Armstrong 2002 (250) Victoria, Australia	118/118/NR	PFGE/fAFLP	55	Melbourne strain, also in 5 distant centres (256)
O'Carroll 2004 (257) Brisbane, Australia	100/100/NR	PFGE	59	Largest cluster: 39 patients; 8 patients Melbourne strain
Studies indicating the existence of a limited patient-to-patient transmission risk of P. aeruginosa				
Speert 2002 (251) Vancouver, Canada	174/NR/NR	RAPD/PFGE	12/10	157 genotypes of which 123 unique ones 48% and 42% of shared Pa strains transiently present
Tubbs 2001 (258) North Staffordshire, UK	72/NR/NR	PFGE	NR	10 clusters (4 sibling pairs). Largest cluster: 5 patients
Da Silva Filho 2001 (259) Sao Paulo, Brazil	38/65/86	RAPD	0	
Van daele 2006 (260) 7 CF centres , Belgium	213/213/910	RAPD/fAFLP	31	163 genotypes. 16 clusters (66 patients) Largest cluster: 12 patients

Abbreviations : see list of abbreviations p. 4-5

Reports on highly transmissible strains of *P. aeruginosa* mainly originate from the UK and Australia (250, 252-257).
Furthermore, data suggest that cross-infection with these highly transmissible strains has occurred between CF centres both within England and within Australia (255, 256).
Some highly transmissible *P. aeruginosa* strains are more virulent: The "Melbourne" epidemic *P. aeruginosa* strain caused 5 deaths in young CF children (250) and CF patients who were infected with a transmissible strain had an increased number of exacerbations and days of intravenous antibiotics and hospital days than those with sporadic *P. aeruginosa* (261). In a retrospective study, 12 patients infected with the Liverpool epidemic strain had a greater loss of lung function and deterioration in BMI than those not chronically infected (262). In an 8-year prospective study of the clinical impact, Jones *et al*. (263) concluded that infection by transmissible *P. aeruginosa* does not increase mortality but is associated with an increased healthcare utilization and antibiotic use. This increased virulence does not apply to all epidemic strains of *P. aeruginosa*: De Vrankrijker *et al*. (264) did not detect unfavorable clinical outcome (pulmonary function, nutritional status, hospitalization days) among 40 CF patients, infected by the highly prevalent Dutch
P. aeruginosa clone ST 406.
In contrast with the reports on highly transmissible strains, other groups (including ours) observed a limited risk of patient-to-patient spread of *P. aeruginosa* even when patients were not segregated according to *P. aeruginosa* colonization status. Patient-to-patient spread in these studies was limited to prolonged close contact, such as between siblings and close friends (251, 258-260).

Segregation of patients has been shown in several CF centres to prevent cross-infection with *P. aeruginosa* (247, 265). In most studies however, attempts of limiting *P. aeruginosa* infection has not only been made by segregating *P. aeruginosa* infected patients from not infected patients, but also by implementation of hygienic precautions. Effects of segregation itself on patient-to-patient spread of *P. aeruginosa* is difficult to assess. Nevertheless, some observations support the effect of segregation policy on the acquisition of *P. aeruginosa.* After the observation that in 4 years time (1983-1986) the prevalence of *P. aeruginosa* infection had risen from 37% (33 patients) to 60% (104 patients) and that 12 out of the 40 patients newly colonized by *P. aeruginosa* had acquired *P. aeruginosa* at CF recreation camps, clinics or rehabilitation centres, hygienic and segregation precautions were introduced at the CF clinic in Hannover Germany. This led to only a single episode of nosocomial transmission of *P. aeruginosa* during the subsequent 2 years (266).
Following the identification of the Melbourne epidemic *P. aeruginosa* strain, cohort segregation according to *P. aeruginosa* culture status and genotype was introduced. To determine if these strategies had interrupted cross-infection within the clinic, patients from the initial study were followed prospectively (265). Three years after the introduction of cohort segregation, the epidemic strain prevalence had decreased from 21% to 14% ($p = 0.03$). Farrell and coworkers (267) in Wisconsin, USA reported later acquisition of *P. aeruginosa* among newly diagnosed CF patients who were segregated from *P. aeruginosa* colonized patients compared with CF patients at another outpatient clinic where no segregation measures were taken. McKay and colleagues (268) in Sydney, Australia, introduced segregation for children under five from older patients and from *P. aeruginosa* positive peers. They compared data on *P. aeruginosa* acquisition before (1999-2002) and after introduction of segregation (2004-2007). There was a significant decrease in the acquisition of mucoid but not in acquisition of non-mucoid *P. aeruginosa* after segregation, indicating that mucoid *P. aeruginosa* were likely to be transmitted from other patients, nonmucoid *P. aeruginosa* however must come from other (environmental?) sources.

Interpretation of the different studies on patient-to-patient transmission of *P. aeruginosa* are somehow hampered by several factors. In the first place, no agreement or even discussion exists on the definition of a "higly transmissible" strain: from what percentage of shared

P. aeruginosa in a CF community on is a strain called “highly transmissible”? This results in different conclusions of authors on sometimes comparable results: e.g. in the study of Speert *et al*. (251), the largest clone is present in 12% of the patients, nearly the same percentage (14%) is seen in the study of Jones *et al*. (253). Authors however come to different conclusions and different degrees of alertness:
Speert *et al*. (251): “It appears that prolonged close contact, such as occurs between siblings, is necessary for patient-to-patient spread. Considering these observations, we do not recommend segregation of patients with CF on the basis of their colonization status with P. *aeruginosa*.”
Jones *et al* (253): Cross-infection by a multiresistant *P aeruginosa* strain has therefore occurred in patients attending our cystic fibrosis centre. We recommend microbiological surveillance in other cystic fibrosis centres.
Another example of different interpretation of results holds true for Fluge *et al* (269). Although 45% of the 60 Norwegian CF patients studied over a 5 year period (1994-1998) had a common *P aeruginosa* genotype, authors conclude: “Our results indicate that cross-infection with *P aeruginosa* between CF patients has occurred”; but also: “The advisory board of the Norwegian cystic fibrosis society has decided that segregation should not be advocated, except in cases of infection with multiresistant strains. Hygienic measures have been introduced and CF patients and health care personnel are now aware of the possibility of cross-infection. We believe that our new guidelines will be sufficient in preventing cross-infection with *P aeruginosa*”.

Conclusion: Several studies have clearly demonstrated the existence of patient-to-patient spread of *P. aeruginosa* between CF patients. Conflicting data however exist on the extent of this spread: does it only result from longstanding and close contact between CF patients or is *P. aeruginosa* highly transmissible? Previous studies have supported the position of both groups, indicating the difficulty of making general statements about this highly diverse and adaptable pathogen. It seems reasonable that both opinions are correct and that different strains of *P. aeruginosa* circulate: some of low and other of high transmissibility. As a result of these studies on patient-to-patient transmission of *P. aeruginosa*, segregation measures according to *P. aeruginosa* status have been advised in the European consensus guidelines, but not (yet) in the US guidelines.

- **Environment-to-patient transmission**

There are some indications that the environment might be the initial source of *P. aeruginosa* in CF patients. First of all, *P. aeruginosa* is widely present in the natural environment in soil, plants and water. Initial isolates from infants and young children with CF usually display an “environmental phenotype” (49): they are nonmucoid, fast growing and relatively susceptible to antibiotics. Romling and colleagues (270) demonstrated that the most prevalent clone in CF patients was also frequently isolated in aquatic environment and in various spatially and temporally separated habitats. In addition, the environmental origin of *P. aeruginosa* in CF patients is suspected by the observation that young CF children can be protected from the acquisition of mucoid *P. aeruginosa* by patient segregation and that the acquisition of non-mucoid *P. aeruginosa* is likely to be from environmental sources, as the rate of acquisition of non-mucoid *P. aeruginosa* did not decrease after segregation (268).
The contamination with *P. aeruginosa* especially in health care settings and to a lesser degree in non-health care settings and possible transmission from these environments to the CF patients has been studied.

Studies on the presence of *P. aeruginosa* in CF health-care settings
P. aeruginosa has been recovered from numerous environmental sources in both in- and outpatient CF healthcare settings, e.g. sinks, soap, baths, toys, tables, brushes, cloths, and

air (243) tap-water, sinks, wash-basins and creams (271), pulmonary function machines (251), hands of health care workers and CF patients (242, 243, 251).
A lot of studies extend on the contamination of CF clinics (both in- and outpatient) with *P. aeruginosa*, some of these also try to link environmental *P. aeruginosa* genotypes with *P. aeruginosa* genotypes encountered in the CF patients. These studies, outlined in Table 2, demonstrate that especially the sinks in CF wards can be contaminated by *P. aeruginosa* (242, 243, 271-274). Sometimes, environmental *P. aeruginosa* strains were identical to the CF patient *P. aeruginosa* strains, transmission however was difficult to prove (243, 272-274).
An interesting observation on possible environment-to-patient transmission was made by Doring *et al*. (242). The authors performed a 4 week prospective study of *P. aeruginosa* strain transmission in a pediatric CF ward in Tubingen, Germany. Samples were taken weekly from sink drains, toilets, baththubs and showers in the CF ward and from hands of the hospital personnel. Cultures form sputum, throat, nose and anus were taken weekly from 14 CF patients admitted to the CF ward during that time period . *P. aeruginosa* strains were compared by means of PFGE. One out of the five CF patients not colonized by *P. aeruginosa* entering the ward became positive for an environmental *P. aeruginosa* strain one week after entering the ward and remained positive for the following 3 weeks during his stay on the ward. Strain transmission from the environment to this patient was highly probable in this case. The authors suggested that transmission occurred through the personnel, as the environmental strain was detected in a distant washbasin one week before it was isolated from the patient, although this could not be proven.
Conclusion: The different studies on the prevalence of *P. aeruginosa* in CF health care settings demonstrate that especially sinks are heavily contaminated. Genotypes of environmental *P. aeruginosa* sometimes matched patient strains and in one patient environment-to-patient transmission was strongly suspected.

Table 2: studies on the prevalence of *P. aeruginosa* in healthcare settings.

Reference	Geographical location	Sampling site	Sample	Number	% positive for *P. aeruginosa*	Number of isolates identical to patient isolates
Zimakoff 1983	Copenhagen, Denmark	CF IP & OP wards	Dry surfaces	258	2,7	
(243)		CF IP & OP wards	Sinks/baths	43	30	8
		non-CF IP ward	Dry surfaces	72	0	
		non-CF IP ward	Sinks/baths	27	11	0
Speert 1987	Vancouver, Canada	CF IP ward	Dry surfaces	385	1.3	
(272)			Bathroom/Patient room sinks	126	11	5
			Hands personnel	43	0	
Bosshammer 1995	Hannover, Germany	CF IP & OP wards	Bathroom	328	8,8	NR
(271)			Sink drains (35)	280	63 of sink drains	
			Water taps (35)	280	31 of water taps	
			Dry surfaces	NR	0	
			Hands of personnel	NR	0	
Doring 1996	Tubingen, Germany	CF IP ward	Hands of personnel	79	14	1
(242)			Sink drains	24	87,5	
Festini 2007	Florence, Italy	CF OP ward	Dry surfaces in 5 rooms	230	NR	
(273)			Sinks + sink drains (5)	230	44,3 (sinks) - 21-71 (sink drains)	
			All samples	460	22.8	19/21 investigated strains
Panagea 2005	Liverpool, UK	CF IP & OP ward	Bathroom	22	73	1 "Liverpool" strain
(274)			Dry surfaces	22	13,6	All 3 " Liverpool" strains, all from respiratory equipment
			Personnel	12	0	

Abbreviations : see list of abbreviations p 4-5.

***P. aeruginosa* in home environment of CF and non-CF patients**

In contrast with the extensive studies on patient-to-patient transmission and on the presence of *P. aeruginosa* in the hospital environment, there are only a few reports on the prevalence of *P. aeruginosa* in the home environment of both CF and non-CF patients. These studies provide conflicting results. None of them studied the possibility of environment-to-patient transmission, neither did they compare genotypes of *P. aeruginosa* isolates recovered from either patients or from their home environment. The lack of knowledge about the exact role of the environment in the acquisition of *P. aeruginosa* by the CF patient is of concern to parents and physicians, and sometimes leads to questionable preventive measures, such as not drinking tap water unless it has been boiled or not visiting swimming pools (275, 276) and is also reflected in national guidelines with different policies regarding the preventive hygienic measures to be taken in daily life.

Studies on prevalence of *P. aeruginosa* in both CF and non-CF houses

Mortensen and colleagues (277) investigated 14 houses of CF patients colonized with *B. cepacia complex* and 13 non-CF houses in the same geographical area. The *P. aeruginosa* colonization status of the CF patients was not reported. All houses harboured *P. aeruginosa.* Unfortunately, results were not reported separately for CF and non-CF houses.
P. aeruginosa was recovered from 26 out of 407 (6.4%) cultures taken in the homes. The main source of *P. aeruginosa* were drains: this pathogen was cultured from 10/106 (9.4%) samples of the drains of the kitchen sink and bathroom, shower heads and refrigerator drain pans. Other sources of *P. aeruginosa* were "personal items" (5/130 (3.8%) samples of clothes washer, soap, toothpaste, toothbrush) and vegetables (10/61 (16.4%) of the cultured vegetables).
Regnath *et al*. (278) looked closely into the prevalence of *P. aeruginosa* in 102 households of 118 CF patients. Cultures were taken of different aqueous sites in the homes and *P. aeruginosa* was detected in 71.4% of the CF houses. *P. aeruginosa* was mainly encountered in drains of bathroom and kitchen (40% of the shower drains, 35% of the bathroom and kitchen washbasin drains), but also in 26.5% of the drainpipes of the toilet. Only 1.3% of the sponges, and 6.3% of the dish brushes harboured *P. aeruginos.*
P. aeruginosa colonization status was known for 88 studied patients, of whom 28 were colonized. In this study, the frequency and intensity of cleaning procedures did not have an impact on the detection rate of *P. aeruginosa*.
In a Swiss study, Barben and colleagues (279) documented the presence of *P. aeruginosa* in bathroom water of patients with cystic fibrosis. The prevalence of *P. aeruginosa* in 204 tap water specimens was low, with only two positive specimens of standing water, sampled during the summer.
Studies on prevalence of *P. aeruginosa* in non-CF houses:
Whitby *et al*. (280) examined 33 houses of hospital staff members and of people not working in hospitals. Of a total of 171 samples, only 9 *P. aeruginosa* posive cultures (5.3%) from 4 homes, two of which belonging to people working with *P. aeruginosa*, were detected. The vast majority of the positive samples (7 out of the 141 samples taken) was from the sink, bathroom and washbasin drains, and just one out of the 21 samples of taps. The remaining *P. aeruginosa* was detected in a soap dish (1/15), no *P. aeruginosa* was cultured from 21 floor-cloths, dish-cloths, mops and sponges.
Ojima (281) scrutinized 86 Japanese houses for the presence of different bacteria. In each house, cultures of 100 different sites were taken by direct agar contact plate method. *P. aeruginosa* was mainly found in sinks, dish washing tubs and drains in the kitchen (respectively 12.9, 19.6 and 27.1% of the cultures). Eighteen percent of the bathroom floors was contaminated with *P. aeruginosa*, and 6% of the wash pans and wash stools, nearly without contamination of the bathtubs. This was explained by the authors being the result of a different cultural-based use of the bathroom in Japan, although this different use was not

specified. Ojima detected *P. aeruginosa* in 16% of the dish-washing sponges in the kitchen an 22% of the cleaning sponges in the bathroom.

Conclusion: Studies on prevalence of *P. aeruginosa* in both CF and non CF houses indicate that drains in bathroom and kitchen are important reservoirs of *P. aeruginosa*. No comparison of contamination in CF versus non-CF houses has been made nor was possible transmission of *P. aeruginosa* from the environment to the CF patient studied. This uncertainty on possible acquisition concerns CF patients and parents.

Other potential environmental sources of *P. aeruginosa* mentioned in literature.
Numerous other potential environmental sources of *P. aeruginosa* for CF patients have been discussed in literature. Acquisition through one of these sources however has never been proven.
Dental units: In the early 1980s, a German study (282) identified *P. aeruginosa* in 74% of the water in dental chair units of private dental practices. Jensen and colleagues (283) reported contamination with *P. aeruginosa* in 18 out of 327 (5.5%) water samples from nine (11%) dental sessions. In one case, a genotypically identical *P. aeruginosa* strain was found both in water from the dental session and in the CF patient's sputum. In a survey in a dental hospital, 43% of the suction hoses were infected with *P. aeruginosa* and corroded at their attachment to the main dental chair unit. The problem was eliminated by replacement of the hose connectors (284).
Whirlpools and hot tubs frequently harbour *P. aeruginosa* (285). In a study from Northern Ireland, 4/13 (30.8%) of hydrotherapy pools and 37/51 (72.5%) of jaccuzis/spas contained *P. aeruginosa* (286).
Swimming pools, if correctly managed and disinfected by chlorine, are free of or contain very little *P. aeruginosa*. Standards however vary. In the study of Barben *et al*. (279) , none of the 72 specimens from 28 outdoor pools in 2002 and three specimens from 3 different paddling pools out of 46 pools (7%) in 2003 revealed *P. aeruginosa*. Both samples were from non-public hydrotherapy pools. Moore *et al.* (286) detected *P. aeruginosa* in 26/68 (38.2%) of the swimming pools. Plastic animals used in swimming pools may be contaminated with *P. aeruginosa* and have been linked to an outbreak of folliculitis (287).
P. aeruginosa is isolated from a number of plants and soil in the natural environment: it was detected in 24% of the soil samples but only in 0.13% of the vegetable samples from various agricultural areas of California (231). Ornamental potted plants can also harbour *P. aeruginosa* (288).
Contamination of prepared vegetable salads with *P. aeruginosa* at home and in the hospital has also been described as a possible source of infection. Kominos *et al*. (289) detected *P. aeruginosa* from diverse fresh vegetables in the hospital kitchen. *P. aeruginosa* was also present on the hands of the kitchen personnel and on cutting boards and knives, suggesting acquisition of the organism through contact with the contaminated vegetables. Pyocine types of *P. aeruginosa* isolated from the clinical specimens were frequently identical to those recovered from vegetables. *P. aeruginosa* was recovered from 44% of the fresh vegetable salads from the hospital kitchen (290). Ingestion of *P. aeruginosa*-contaminated food may lead to colonization of the oropharynx or colonization of the gastrointestinal tract . One study in CF patients however concluded that gastrointestinal colonization occurs as a result of respiratory infection and that the gut is not a significant reservoir of *P. aeruginosa* prior to pulmonary colonization as they detected a low percentage (<10%) of positive stool cultures for *P. aeruginosa* in CF patients not chronically colonized by this pathogen in contrast with positive stools in 8 of the 10 chronically colonized patients (291).
The role of home nebulizers in transmitting potential pathogens has been studied. A significant proportion (25 to 55%) of the home nebulizers of CF patients are contaminated with *P. aeruginosa* (292-294)**.** When adequately disinfected and cleaned, nebulizers do not contain *P. aeruginosa*, as proved by Hutchinson *et al*. (295) and Reychler *et al*. (296). The latter assessed the in vitro effectiveness of 5 methods of disinfecting nebulizer equipment.

Following disinfecting procedures were studied: hypochlorite solution (0.02% active chlorine), acetic acid 3.5%, Hexanios 0.5%, washing-up detergent 0.5% and a dishwasher, tested with and without drying. One hundred sixty mouthpieces and 160 masks of nebulizers were artificially and massively contaminated with 16 strains of germs, including *P. aeruginosa*. Acetic acid without drying was not effective to get completely rid of *P. aeruginosa*, and was not effective against *S. aureus* and *B. cenocepacia*.

Routes of transmission: contact, droplet or airborne?

Most patient-to-patient transmission of *P. aeruginosa* is thought to result from direct contact with infected secretions (e.g., during kissing), indirect contact with infected secretions (e.g., sharing a toothbrush, drinking from the same glass as another CF patient, or shaking hands with someone whose hands are contaminated with secretions), or via droplets (i.e., inspiration of large infectious particles that are spread by coughing, sneezing or singing and that can be inspired within a 1-meter distance from of an infected patient). Droplet transmission has been demonstrated by isolating *P. aeruginosa* from agar plates placed 0.5 to 1 meter (242, 243), and even, though to a smaller degree, beyond the distance of one meter from a coughing CF patient (297).
More recent studies also describe the possibility of airborne transmission (i.e. inspiration of smaller infectious particles that remain suspended in shared air supplies that are transported over long distances via air currents). *P. aeruginosa* has been isolated out of the air surrounding *P. aeruginosa* colonized CF patients by using an air sampler (243, 253, 298) (299) especially after coughing, performing lung function tests and physiotherapy. Real airborne transmission however is difficult to prove and debated (300).
While the routes of transmission are not fully understood, infected respiratory secretions may contaminate the health care environment, which then serves as a potential reservoir for *P. aeruginosa.* Contaminated hands of health care workers may further facilitate transmission. As *P. aeruginosa* is mainly found in sink drains, hand washing in contaminated sinks, generating aerosol of *P. Aeruginosa,* might be important in transmission of *P. aeruginosa*.

General conclusion on environment-to-patient transmission in CF patients:

The contamination with *P. aeruginosa* of CF health care settings and of the natural environment, including CF and non CF houses has been studied. Sometimes, *P. aeruginosa* strains matched patient's strains, but it is unclear whether patients were the initial source of environmental contamination or whether the patient strains originated from the contaminated source. No studies conclusively demonstrate that the environment is the major source for early infection. The uncertainty on possible acquisition of *P. aeruginosa* from the environment concerns both patient and physicians, and sometimes leads to questionable preventive measures.

***P. aeruginosa* in CF patients: from initial infection to chronic colonization**

The first *P. aeruginosa* colonization episode can occur very early in the life of CF patients. After a first ever colonization episode by *P. aeruginosa*, patients may go through different episodes of colonization (intermittent colonization), preceding chronic colonization by months to years, eventually resulting in chronic infection (6, 49, 226). *P. aeruginosa* strains causing early infection usually have a nonmucoid phenotype, whereas *P. aeruginosa* strains in chronically colonized patients are mucoid and form biofilms. In this chapter the pathogenetic events associated with evolution from nonmucoid to mucoid *P. aeruginosa,* the course of *P. aeruginosa* infection in CF patients (from nonmucoid to mucoid *P. aeruginosa* , from initial colonization to chronic infection) and the different and confusing definitions of *P. aeruginosa* colonization and infection in CF patients are discussed.

Pathogenic events hypothesized to lead to chronic *P. aeruginosa* colonization.

Once initial infection has occurred, *P. aeruginosa* in CF lungs sometimes survives despite both innate and acquired host defence strategies and repeated courses of both systemic and inhaled antibiotics. To achieve this, *P. aeruginosa* has developed a huge armamentarium of immuno-evasive strategies including exoproducts, antibiotic resistance proteins and phenotypic changes that render them virtually unrecognisable from the initially colonizing *P. aeruginosa*. *P. aeruginosa* found in the CF lung are hypermutable (301). They possess the ability to react promptly to their environment not only by switching genes on or off but also by an increased frequency of mutations.

This hypermutability plays an important role in the change to the *P. aeruginosa* phenotype seen in chronically infected CF patients. This phenotype is characterized by slow growth, auxotrophy, alginate overproduction (mucoidity), antibiotic resistance and loss of motility (302-307).

Exoproducts

Pseudomonads produce a wide array of exoproducts, of which both elastase and alkaline protease protect against immune destruction by immunoglobulins, complement and cytokines. Exotoxin A inhibits phagocytosis and suppresses the cell-mediated immune response. The siderophores produced by *P. aeruginosa*, such as pyocyanin, break down intercellular tight junctions, slow ciliary beat frequency and thereby impair mucociliary clearance (176).

Antibiotic resistance proteins

P. aeruginosa disposes of a wide variety of intrinsic antibiotic resistance mechanism such as the production of beta-lactamases, efflux pumps and a low permeability of the outer membrane. Moreover, *P. aeruginosa* has a remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including beta-lactams, aminoglycosides and fluoroquinolones. Practically all known mechanisms of antimicrobial resistance can be observed: derepression of chromosomal AmpC cephalosporinase, production of plasmid or integron mediated beta-lactamases from different molecular classes (carbenicillinases, extended spectrum beta-lactamases , oxacillinases and carbapenem hydrolysing enzymes), diminished outer membrane permeability (through loss of OprD proteins), overexpression of active efflux systems with wide substrate profiles, synthesis of aminoglycoside modifying enzymes and structural alterations of topoisomerases II and IV determining quinolone resistance. These mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes (308).

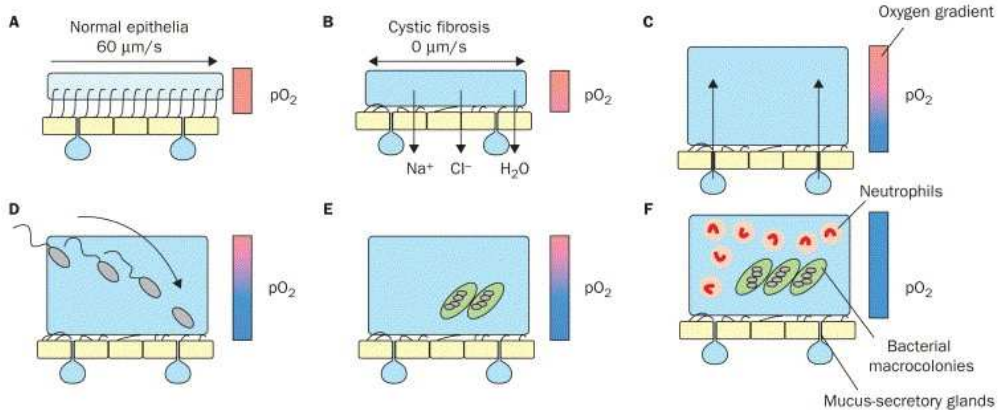
Phenotypic changes

One of the mutation events in *P. aeruginosa* in chronically infected CF patients triggers conversion to a mucoid phenotype, which is almost pathognomonic for CF (309). In response to environmental triggers, such as nutritional stress and hypoxia found within a CF mucus plug, mutants are selected that overproduce mucoid exopolysaccharide (alginate), as represented in Figure 1 (190). This surrounds them, protecting them from adverse external

challenges, such as mucociliary clearance, the host immune response (non-oxidative killing by phagocytes) and antibiotics and is highly pro-inflammatory.

Figure 1: Pathogenic events hypothesized to lead to chronic *P. aeruginosa* colonization

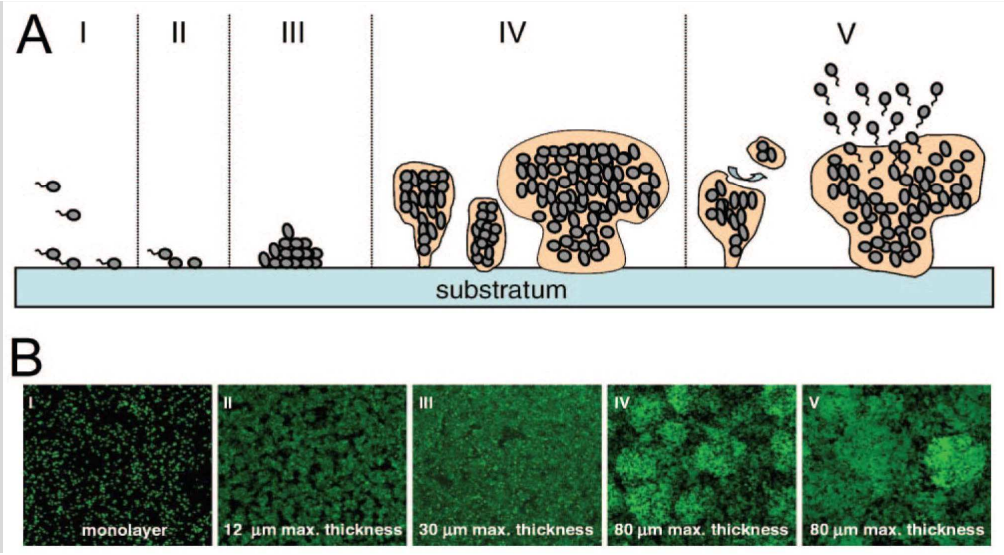
(190)



(*A*) In normal airway epithelia, the presence of a low-viscosity periciliary layer (PCL) of normal volume promotes efficient mucociliary clearance. A normal rate of epithelial cell oxygen consumption (QO_2) results in no gradient in the partial pressure of oxygen (pO_2) within the airway surface liquid (ASL). In the CF airway, (*B*) isotonic volume depletion of the PCL (denoted by *downward arrows* and bent cilia) results in reduced mucociliary transport (*bidirectional horizontal arrow*) and (*C*) persistent mucus hypersecretion (denoted by *upward arrows* from secretory gland/goblet cell units) with time increases the height of the luminal mucus layer/plugs. Elevated CF epithelial QO_2 generates steep hypoxic gradients (*dark colour* in pO_2 *bar*) in the thickened mucus layer. (*D*) *P. aeruginosa* bacteria deposited on mucus surfaces penetrate actively or passively (due to mucus turbulence) into hypoxic zones of the mucus masses. *P. aeruginosa* adapt within the hypoxic environment with increased alginate expression and the formation of microcolonies with potential evolution into biofilms. (*E*) Increased *P. aeruginosa* microcolony density and the presence of neutrophils render the mucus layer more hypoxic. *P. aeruginosa* microcolonies within the hypoxic mucus plugs resist host lung defenses, including neutrophils, and result in chronic airway infection (190).

Another highly successful survival strategy involves the formation of biofilms. Singh *et al*. provided the first evidence that *P. aeruginosa* exists in biofilms within the CF lung (310). Initiation of biofilm formation is dependent on a process of quorum sensing (QS). QS allows bacteria to determine, by means of freely diffusible molecules such as acyl homoserin lactones (AHLs) the presence of similar bacteria in the vicinity. Once these molecules reach a high concentration within the organism, genes controlling the formation of biofilm are expressed. In this state, microcolonies of bacteria are surrounded by a dense matrix, which protects against phagocytosis and prevents penetration by antibiotic agents. This phenotypic change is likely to play a major role in the persistence of *P. aeruginosa* infection in CF patients. The steps involved in biofilm formation are represented in Figure 2.

Figure 2: Steps thought to be involved in biofilm formation (177).



The top row (A) diagrams what is believed to be occurring in the bottom row (B), which shows confocal microscopy of fluorescently labelled organisms. Single (planktonic) bacterial organisms are inhaled (I) and settle onto a surface such as the respiratory mucosa (II), where they multiply into microcolonies (III). These evolve into mushroom-like structures and a biofilm matrix (pink)is produced, which surrounds the by now large colonies of less metabolically active organisms (IV). On occasion, the biofilm matrix breaks down into parts and a shower of organisms leave the biofilm (V), which might cause respiratory exacerbation.

Natural history of acquisition of *P. aeruginosa* and the evolution from non mucoid to mucoid *P. aeruginosa* infection in CF patients.

Interesting data regarding the natural history of acquistion of *P. aeruginosa* and evolution from non mucoid to mucoid *P. aeruginosa* infection in CF patients come from follow-up of CF patients detected through neonatal screening projects: Li and co-workers (311) evaluated prospectively 56 CF patients, diagnosed through the Wisconsin CF Neonatal Screening Project. Patients were followed every 6 weeks during the first year of life and every 3 months thereafter from birth up to age 16 years. Cultures of respiratory secretions (sputum or oropharyngeal swabs) were taken every 6 months and in between if clinically indicated. Patients did not routinely receive anti–*P. aeruginosa* antibiotics after detection of *P. aeruginosa*, but could be started on at the discretion of treating physicians. A total of 1921 cultures were collected with a mean interval of 4.04 months between cultures. Nearly one third (29%) of the patients acquired nonmucoid *P. aeruginosa* already in the first 6 months of life. Nonmucoid *P. aeruginosa* was acquired at a median age of 1 year (95% CI 0.6- 1.5 years), mucoid *P. aeruginosa* was detected at a median age of 13 years (95% CI, 10-14.9 y). All patients acquired *P. aeruginosa* reaching 13 years. The age-specific prevalence of nonmucoid *P. aeruginosa* increased from birth to age 4 years (86%); of mucoid *P. aeruginosa* from age 4 years (4%) to 16 years, when 92% of the 13 patients reaching that age developed mucoid *P. aeruginosa*. All patients who developed mucoid *P. aeruginosa* had acquired nonmucoid *P. aeruginosa* first. In contrast with the short transition time from no *P. aeruginosa* to nonmucoid *P. aeruginosa*, the transition time from nonmucoid to mucoid *P. aeruginosa* was relatively long (median 6.10 years (95% CI 4.47- 8.21y)).

Data on the early first acquisition of *P. aeruginosa* in CF patients were confirmed by other investigators. Burns *et al*. (49) investigated *P. aeruginosa* from oropharynx (OP) and bronchoalveolar lavage fluid (BALF) in a cohort of 40 CF patients during the first 3 years of life. Bronchoscopy with BAL was performed annually. OP cultures were taken at 3-month intervals, until the third bronchoscopy. In 72.5% of patients, *P. aeruginosa* could be demonstrated in BALF and/or OP cultures during their first 3 years of life.
Whereas in the data of Li *et al*. (311), all patients acquired nonmucoid *P. aeruginosa* before conversion to mucoid *P. aeruginosa*, other studies on acquisition of *P. aeruginosa* in birth cohorts detected by newborn screening (312, 313) report mucoid *P. aeruginosa* at detection in a substantial proportion of the patients. In the study of Nixon *et al*. (312), more than half of the patients (13 out of 24) had mucoid *P. aeruginosa* at detection. *P. aeruginosa* was mucoid at detection in 6 out of the 33 CF patients in the data of Douglas *et al*. (313). It is however worth noting that in both studies detection of *P. aeruginosa* was done only by yearly culture of BALF, and that no OP or sputum cultures were performed in between. The authors explain the observation of high prevalence of the mucoid phenotype by the possible delay in detection of *P. aeruginosa*, due to taking samples only annually. However, the possibility of acquisition of mucoid *P. aeruginosa* right from the start through patient-to-patient spread cannot be excluded.

Studies on the natural evolution from first isolation to chronic colonization by *P. aeruginosa*
Without antibiotic treatment, the natural history of *P. aeruginosa* infection in CF is often towards a rapid development of chronic colonization by *P. aeruginosa*. Johansen *et al*. (314) reviewed the Danish data (1965-1990) from intermittent and chronic *P. aeruginosa* infection. During the study period, 239 patients had intermittent episodes of *P. aeruginosa* colonization and 182 patients became chronically infected, all having been intermittently colonized previously. The median time interval before patients became chronically infected was 12 months (range 0-69 months). In 33 of 182 patients with chronic infection the initial episode of *P. aeruginosa* colonization developed immediately into chronic infection; the remaining 149 patients first became *P. aeruginosa* -free for a varying period of time before ultimately developing chronic infection. The mean age of patients acquiring chronic infection with *P. aeruginosa* was 9.7 years. Kerem *et al*. (315) reported that 199 out of 309 (69%) CF patients had more than 50% *P. aeruginosa* positive cultures over the two years following initial colonization; indicating chronic colonization.

Definitions of (chronic) colonization and infection by *P. aeruginosa*
Unfortunately, comparison of the different studies on chronic colonization by *P. aeruginosa* is hampered by the lack of agreement in the definitions of chronic colonization/infection. Currently, there is no universally accepted definition for chronic *P. aeruginosa* colonization/infection. An overview of the different criteria is presented in Table 3. Most European CF centers use the “European consensus criteria” agreed by Döring *et al*. (316) or the “Leeds criteria” defined by Lee *et al.* (317). In this thesis, “colonization” and “infection” are used respecting the definitions used by the different authors.

Table 3: overview of the different definitions of (chronic) *P. aeruginosa* colonization/infection.

European consensus criteria (316)

- Lung colonization by *P. aeruginosa*: "Presence of *P. aeruginosa* in the bronchial tree without direct (inflammation, fever, *etc*.) or indirect (specific antibody response) signs of infection and tissue damage"
- Chronic lung colonization by *P. aeruginosa*: "Presence of *P. aeruginosa* in the bronchial tree for at least 6 months, based on at least three positive cultures with at least one month intervals between them without direct (inflammation, fever, etc.) or indirect (specific antibody response) signs of infection and tissue damage".
- Lung infection by *P. aeruginosa*: "Presence of *P. aeruginosa* in the bronchial tree with direct (inflammation, fever, *etc*.) or indirect (specific antibody response) signs of infection and tissue damage. Infection can also be diagnosed on the basis of a positive antibody response in at least two examinations for patients who do not expectorate and present with negative bacterial cultures"
- Chronic lung infection by *P. aeruginosa*: "Presence of *P. aeruginosa* in the bronchial tree for at least 6 months, based on at least three positive cultures with at least one month intervals between them with direct (inflammation, fever, *etc*.) or indirect (specific antibody response) signs of infection and tissue damage. Chronic infection can also be diagnosed on the basis of a positive antibody response in at least two examinations for patients who do not expectorate and present with negative bacterial cultures."

The Leeds criteria (317) classify CF patients as:

- Never infected by *P. aeruginosa*: *P. aeruginosa* has never been cultured from sputum or cough swab.
- Free of *P. aeruginosa* infection: no growth of *P. aeruginosa* during the previous 12 months, having previously been *P. aeruginosa* culture positive.
- Intermittently infected by *P. aeruginosa*: when ≤ 50% of months, when samples had been taken, *P. aeruginosa* cultures are positive.
- Chronically infected by *P. aeruginosa*: when > 50% of months when samples had been taken, *P. aeruginosa* cultures are positive.

The Cystic Fibrosis Trust in the UK uses in the consensus guideline "Suggestions for prevention and infection control" (318) the definition proposed by Brett *et al*. (319) defining chronic infection with *P. aeruginosa* as "the regular culture of *P. aeruginosa* from the sputum or respiratory secretions, on two or more occasions, extending over 6 months or a shorter period if accompanied by a sustained rise of anti-*Pseudomonas* antibodies", although they also mention the Leeds criteria in this consensus guideline.

In Denmark, following definitions are used, known as the "Copenhagen criteria" (247):

- Chronic *P. aeruginosa* infection: "Persistent presence of *P. aeruginosa* for at least 6 consecutive months, or less when combined with the presence of two or more *P. aeruginosa* precipitins".
- Intermittent *P. aeruginosa* colonization: "Culture of *P. aeruginosa* at least once and the presence of normal levels of precipitating antibodies against *P. aeruginosa*"'.

According to Ballmann *et al*. (320) and Kerem *et al*. (315) a patient is considered chronically infected by *P. aeruginosa* when having more than 50% of cough swabs or sputum samples positive in a 12-month period. Fitzsimmons *et al*. (321) classified a patient as chronically infected if the last sputum sample of each year grows *P. aeruginosa*.

Each definition holds its advantages and drawbacks.
The definitions proposed by Brett (319), Ballman (320), Kerem (315) and Fitzsimmons (321) are vague and do not offer any advantage in practical organisation of patient care.
The European consensus criteria (316) are clinically relevant, allowing an easy tool for categorizing CF patients according to *P. aeruginosa* colonization status. By also implementing the results of *P. aeruginosa* antibodies, they overcome the problem of difficult–to-classify patients, e.g. those with intermittently positive *P. aeruginosa* cultures. The difference between infection and chronic infection however is not clear as there is an overlap in definition, both mentioning "Infection can also be diagnosed on the basis of a positive antibody response in at least two examinations for patients who do not expectorate and present with negative bacterial cultures". Furthermore, *P. aeruginosa* serology tests are not widely available.
This also holds true for the "Copenhagen" definition of intermittent colonization and chronic infection (247), although it offers a precise classification tool but is very laborious, needing monthly respiratory cultures.
The Leeds criteria (317) exclusively rely on culture methods, in contrast with the Danish and European consensus criteria, and give a detailed classification of CF patients according to *P. aeruginosa* status. The major drawback of the these criteria is that it is very laborious to set up segregation for the different cohorts. The Leeds criteria are the best documented and show good agreement with clinical parameters of the CF patients. In the initial report of Lee *et al*. (317) the' chronic' group had significantly worse chest X-ray scores, and lower FEV_1 values than the 'free' or 'never' categories (p<0.004). The 'intermittent' group had significantly higher chest X-ray scores than the 'chronic' group (p<0.0001), and a significantly lower % predicted FEV_1 value than the 'free' or 'never' groups (p<0.0003). 'Chronic' patients were associated with a positive, and 'never' patients with a negative *P. aeruginosa* antibody result (p<0.001). These criteria proved also to be relevant for predicting future infection status: 88% of the 'chronic' patients remained in this category five years later. Patients defined as 'intermittent' were most likely to remain in this category when reassessed 5 years later, but with 15% of the 'intermittent" reverting to 'free' and 15% progressing to 'chronic'.
The value of the Leeds criteria for *P. aeruginosa* infection was later on confirmed by Proesmans *et al.* (322). They evaluated the use of this definition in 193 CF patients, using clinical, immunological and lung function parameters and confirmed the agreement between *P. aeruginosa* status, the clinical status and level of *P. aeruginosa* antibodies. Disease markers, such as FVC,FEV_1 and weight for height were significantly worse in the chronic group. *P. aeruginosa* antibodies differed between the groups and were very high and statistically significant different from all other groups in the 'chronic' group and very low in the 'never infected' group en control group.

Conclusion: Studies demonstrate that the first *P. aeruginosa* infection occurs early in life. The time span from first isolation of *P. aeruginosa* to chronic colonization is variable and dependent on several factors, such as patient segregation and early treatment regimens, but also on definitions of chronic colonization used. The role of segregation and early treatment in the delay of chronic *P. aeruginosa* colonization is discussed in Chapter II.

Clinical impact of *P. aeruginosa* infection in CF patients

Plenty of reports extend on the deleterious effect of *P. aeruginosa* in CF patients. The presence of *P. aeruginosa* in respiratory cultures is associated with lower survival rates (312, 323-326), lower pulmonary function tests (312, 315, 325-328), more respiratory symptoms (39), worse chest X-ray scores (39, 210, 325, 326, 328) and worse clinical parameters (210, 312). Acquisition of mucoid *P. aeruginosa* was associated with an accelerated rate of decline in pulmonary function (326). Emerson *et al.* (329) analyzed prognostic indicators of 8-year mortality and morbidity in patients ages 1–5 years from the 1990 US Cystic Fibrosis Foundation Patient Registry. The 8-year risk of death was 2.6 times higher in patients who had respiratory cultures positive for *P. aeruginosa* in 1990 than in children without *P. aeruginosa*. Children from whom *P. aeruginosa* was isolated in 1990 had higher rates of continued infection in 1998, more frequent hospitalizations for acute respiratory exacerbations, lower percent predicted FEV_1 values, and lower weight percentiles.

Conclusion *: P. aeruginosa* infection is clearly associated with poorer clinical outcome in CF patients.

Eradication therapy for *P. aeruginosa* colonization episodes in CF patients not chronically colonized by *P. aeruginosa*

P. aeruginosa strains causing early infection usually are antibiotic sensitive and of low bacterial density in the airways (49, 226). Aggressive treatment of these early infecting strains often successfully eradicates *P. aeruginosa*. Chronic *P. aeruginosa* infection is clearly associated with poorer clinical outcomes among CF patients. As a result, the treatment strategy has shifted from suppressive therapy in patients chronically colonized by *P. aeruginosa* to attempts at early eradication therapy as soon as *P. aeruginosa* is detected (330, 331).

Historical overview of early eradication treatment

The initial observations of the favourable effect of eradication treatment for early *P. aeruginosa* isolates dates back to the eighties and early nineties.
Littlewood *et al*. (332) observed that treatment with inhaled colomycin in 7 patients with recent *P. aeruginos* acquisition resulted in a significant reduction of *P. aeruginosa* positive cultures.
Steinkamp *et al*. (333) treated 28 CF children with recent colonization with iv anti-pseudomonas antibiotics (azlocillin + tobramycin) during 2 weeks. Eighteen (64%) of these were free of *P. aeruginosa* at the end of the treatment. This number gradually declined over time, leaving just 1 patient (3.5%) free of *P. aeruginosa* after 15 months. This high number of recurrence might be explained by the long time lapse between detection of the first positive *P. aeruginosa* respiratory culture and the start of IV treatment (1-11months, median 5 months).
Valerius *et al*. (334) compared treatment with oral ciprofloxacin and inhaled colomycin for 3 weeks with placebo for 3 weeks in 26 CF patients with a new *P. aeruginosa* episode. At the end of the 27 months observation period, the treatment group had significantly less *P. aeruginosa* positive cultures and a significantly lower rate of chronic infection.

Eradication trials

As a result of these early observations, several eradication studies were undertaken, both randomized controlled trials (RCT) and cohort studies. The different eradication trials are summarized in Table 4 (see attachment). Comparison between the different studies however is difficult because of different methodologies, different eradication treatment regimens,

different outcome measures and different definitions of eradication and chronic colonization and/or chronic infection (see Chapter II). Below we will use both terms (colonization/infection) equivalently.

- Studies on delay in chronic infection

The Danish group published extensively on their long-lasting experience with eradication treatment. Details on the eradication treatment given in the studies can be consulted in Table 4. In the absence of treatment for initial *P. aeruginosa* colonization and of segregation measures, approximately 10% of the CF patients will become chronically infected directly after the first positive culture. The remainder develops chronic infection after a median time of 12 months (314). Valerius *et al*. (334) observed that a significantly lower number of the treated patients evolved into chronic infection after 27 months compared to the placebo group (14% versus 58%). The experience continued and Frederiksen *et al*. (335) reported that only 16% of the treated patients developed chronic infection after 3.5 years compared to 72% of the historic control patients. The "Early aggressive eradication therapy for intermittent *P. aeruginosa* airway colonization in CF patients: 15 years experience" was published in 2008 (336). Of the 99 patients with a first ever *P. aeruginosa* isolate in the study, 12 patients developed chronic infection after a median time of 3.7 years after the first ever isolate (0.5-10.3 y). A Kaplan Meyer estimate showed that up to 80% of the patients were protected against development of chronic infection for up to 15 years. Patients developing chronic infection had a significantly shorter *P. aeruginosa* free time interval after treatment of the first isolate compared to patients remaining intermittently colonized. Treatment failure, defined as a *P. aeruginosa* positive culture immediately after ended treatment of the first ever *P. aeruginosa* colonizaton episode, was a strong risk factor for development of chronic infection after 3 to 4 years (OR 5.8).

- Studies on microbiological effect: eradication, recurrence of *P. aeruginosa* and time to recurrence

These studies on the microbiological effect of eradication treatment are difficult to compare because definitions of eradication and/or *P. aeruginosa* free time period differ between studies.

Eradication is in some studies defined as "three negative respiratory culture results within 6 months of treatment" (337, 338); some studies also include negative *P. aeruginosa* antibodies in their definition of eradication (339, 340). Other investigators delineate successful eradication as negative *P. aeruginosa* culture at the end of the eradication treatment (336, 341) or at various time points after the end of treatment (113, 333, 342, 343). Still others use different definitions (312, 344, 345) or in some studies, eradication is not defined (346).

The *P. aeruginosa* free time period includes in some trials the treatment period (344, 345), other start counting from the end of the treatment onwards. In some studies, the treatment group taken as a whole (336, 337) and in others the *P. aeruginosa* free time period is calculated only for those who have a recurrence of *P. aeruginosa* (347). In other trials, the *P. aeruginosa* free time period is calculated but not defined (346).

Different treatment regimens have been studied. The majority of the studies uses inhaled antibitiocs (mainly tobramycin) or the combination of oral ciprofloxacin and inhaled antibiotics (tobramycin or colomycin) for varying lengths of time. All studies are outlined in Table 4. Whatever early eradication treatment is used, all trials report successful eradication and/or significantly less positive *P. aeruginosa* cultures during treatment (332, 334, 348). The more recent eradication trials (2000-2010) also report the percentages of eradication immediately after or within 6 months after treatment , these vary between 80 and 100% (113, 337, 338, 342-346, 349).

Data on the recurrence of *P. aeruginosa* are extremely diverse, with percentages of recurrence varying between 12% (343) and 100% (346). Recurrence rate estimates are

influenced by the follow up time of the study: it is logical that chances of recurrence increase with lengthened observation periods.
Munck *et al.* (346) reported a new *P. aeruginosa* episode in all patients after a median time of 18 months, Tacetti *et al*. (337) in 51% of the treated patients after a median time of 18 months. In the Elite trial (347), the median time to recurrence of *P. aeruginosa* was 26 months for patients in the 28 day treatment group and 25.8 months for the patients in the 56 day treatment group (ns). Thalhammer and Eber *et al.* (344, 345) notified a 29% recurrence after median time period of 8 months, but in this study the time while still on inhaled antibiotics was included in the calculation of the *P. aeruginosa* free time period.
Unfortunately, no follow-up data are provided for the studies of Thalhammer and Eber *et al*. (344, 345) and Tacetti *et al*. (337). The results of Douglas *et al*. (313) suggest an extremely low percentage (12%) of *P. aeruginosa* recurrence. This can be explained by the use of only annual BAL cultures to detect *P. aeruginosa*, without culture of other respiratory samples in between. The *P. aeruginosa* free time period in "the 15 year Danish experience" (336) was significantly less in non-treated versus treated patients, with a (statistically not significant) trend to longer *P. aeruginosa* free time periods in the 3 month regimen (10.4 m of *P. aeruginosa* free time) compared to the 3 week treatment regimen (5 months) and the nontreated group (1.9 months). The less successful results of eradication of Steinkamp *et al*. (333) and Nixon *et al*. (312) can presumably be explained by late diagnosis (annual BAL for detection, also indicated by the high number of mucoid *P. aeruginosa* at detection in the study of Nixon) and by late start of IV treatment after detection of *P. aeruginosa* (1-11 months, median time 5 months) in the study of Steinkamp *et al*..

- Studies on effect of eradication treatment on clinical parameters

Effect on clinical data seems harder to prove.
Frederiksen *et al*. (335) demonstrated a favourable effect of the early treatment on lung function parameters. Pulmonary function was maintained or increased during the year after treatment in comparison with the control group, in which pulmonary function declined ($p < 0.01$). Although some of the treated patients developed chronic *P. aeruginosa* infection, these had significantly better pulmonary function at the onset of chronic *P. aeruginosa* infection compared to the control patients ($p < 0.001$). Frederiksen *et al*. used a historic control group, but apart from the intensive antibiotic protocol, all other aspects of infection control and treatment were the same for the study group and the historic control group.
Other trials investigating the effect of early eradication on pulmonary function (112, 333, 340, 347) failed to detect a favourable influence. Unfortunately, these studies have a short duration of follow-up (12 to 27m).
Steinkamp *et al*. (111, 333) reported a significantly better weight gain immediately after intravenous antibiotic treatment.
Taking into account however the deleterious effect of chronic *P. aeruginosa* on the clinical outcome, it seems logical that preventing or delaying chronic infection should have an advantageous effect on clinical parameters. Effect on clinical data however is currently scarce, this may be due to the short observation time in most studies.

- Head-to-head comparison of different treatment regimens

As result of these observations and trials, the use of eradication treatment for *P. aeruginosa* in patients not chronically colonized/infected by *P. aeruginosa* has become the mainstay of treatment. The optimal route of delivery of anti-*P. aeruginosa* antibiotics (IV/oral/inhalation or combination) nor the optimal duration of eradication treatment for long lasting eradication (and also minimizing the risk of side-effects) is currently unknown.
To the best of my knowledge, up till now, only 5 studies compared different treatment strategies. Three of these have been presented at the NACFC 2009, detailed results haven't been published yet.
The "Danish experience" learned that the 3 month eradication regimen with oral ciprofloxacin and inhaled colistin tends to be superior than the 3 week regimen. This observation led to a

change in early treatment policy to a 3 month regimen from the first *P. aeruginosa* culture on (336).
The Early *Pseudomonas* Infection Control (EPIC) (350) program consists of a randomized multicenter trial in 304 CF patients ages 1–12 years at first isolation of *P. aeruginosa* from a respiratory culture. Trial participants are assigned for 18 months to either anti-pseudomonal treatment on a scheduled quarterly basis (cycled therapy) or based on recovery of *P. aeruginosa* from quarterly respiratory cultures (culture-based therapy). The study drugs include inhaled tobramycin (300 mg bid) for 28 days, combined with either oral ciprofloxacin (15–20 mg/kg bid) or oral placebo for 14 days. The primary endpoints of the trial are the time to pulmonary exacerbation requiring IV antibiotics or hospitalization for respiratory symptoms, and the proportion of patients with new *P. aeruginosa* -positive respiratory cultures during the study. Eradication was achieved in > 82% of patients receiving inhaled tobramycin 300mg bid ± oral ciprofloxacin. There was no significant difference in endpoints between culture-based therapy and prophylaxis (cycled therapy) and no significant difference in endpoints with the addition of oral ciprofloxacin to inhaled tobramycin versus inhaled tobramycin monotherapy.
A prospective study in Belgium (349) randomized 50 CF children at first isolation of *P. aeruginosa* to inhaled tobramycin 300 mg bid for 28 days, or inhaled colistin 2 MU bid plus oral ciprofloxacin 10 mg/kg tid for 3 months. According to the interim analysis of 32 patients, eradication was achieved in 14 of 17 tobramycin-treated patients (82.4%) compared to 14 of 15 patients treated with the colistin/ciprofloxacin combination (93.3%). The difference in outcomes between the 1-month and 3-month treatment regimens was statistically not significant (p=0.14).
A comparative trial of tobramin 300mg/ciprofloxacin and colistin/ciprofloxacin for 28d in 136 CF children (338) reported no difference in the rate of eradication with the two regimens.
Although these studies were not able to detect a superior regimen, inclusion of more patients and a longer follow-up period might help to reveal differences.
Keeping this in mind and having a critical look at the study design of the EPIC trial, one can suggest that a 2 week duration of oral ciprofloxacin might be too short to detect any differences in eradication. In the study of Tacetti and colleagues (338) the difference between inhaled tobramycin and colomycin might be too small to detect differences in treatment outcomes. From that point of view, the study of Proesmans and colleagues (349) is very interesting of design, comparing two widely used but totally different eradication schedules (inhaled tobramycin 300mg bid versus oral ciprofloxacin and inhaled colomycin for 3 months), although the study group of this trial might be too small to detect any differences in outcome.
Conclusion: Head-to-head comparison of different treatment regimens has until now not defined a superior treatment regimen.

- Can the acquisition of *P. aeruginosa* be prevented by antibiotic therapy?

This question remains unanswered till now, as few studies addressed this topic. Long term antibiotics are always used with caution because of fear for side effects and development of antibiotic resistance.
Heinzl and colleagues (351) reviewed 14 year data on the effect of nebulized gentamicin bid in “high risk “ patients on long term treatment and compared these with high risk patients who stopped the treatment prematurely. No one of the 12 patients on long term treatment (mean treatment duration 77 months, range 26-416 months) acquired *P. aeruginosa* during the study period, compared to 7 out of 16 patients who stopped treatment prematurely (mean treatment duration 41.8 months, range 3-106 months).
In the 18 months EPIC trial, as mentioned above, there was no significant difference on endpoints between culture-based therapy and prophylactic cycled therapy (350). It must be noted that patients entered this study after a first ever *P. aeruginosa* colonization episode and consequently were not *P. aeruginosa*-naïve.

Tramper-Stranders *et al*. (352) studied the value of prophylactic antibiotic therapy in 65 children with CF without *P. aeruginosa*. In this 3 year triple-blind RCT, patients were treated 3 monthly with a 3 week course of oral ciprofloxacin (10mg/kg bid) and inhaled colistin (1MU bid) or placebo. There was no difference in acquisition of *P aeruginosa* infection between the control and treatment groups (annual incidence 14% vs 11%). Chronic infection was observed in 19% of the control patients and 12% of the treated patients.
Conclusion: Up until now, no studies have demonstrated the favourable effect of prophylactic antibiotic treatment in prevention of infection with *P. aeruginosa*.

- Does early eradication treatment result in eradication or suppression of *P. aeruginosa*?

So far, it is clear that early eradication treatment reduces the number of positive *P. aeruginosa* cultures, extends the *P. aeruginosa* free time period and delays the onset of chronic infection. However, the main question whether the initial *P. aeruginosa* is really eradicated after a treatment or whether the eradication treatment only results in temporary reduction of the bacterial load escaping the sensitivity of the detection techniques used (culture, PCR), is not yet touched upon. An alternative explanation of the finding of an identical *P. aeruginosa* genotype at follow-up samples might be reinfection from the same initial source. This question on eradication versus suppression can be boarded by studying the initial *P. aeruginosa* genotypes and comparing them with the genotypes of subsequent *P. aeruginosa* isolates. This difference might be very important in the evaluation of the efficacy of eradication treatment. Genotyping of *P. aeruginosa* isolates was previously applied in epidemiological studies of *P. aeruginosa* in chronically colonized CF patients (255, 260, 353) but longitudinal data on the genotype of *P. aeruginosa* isolates after initial colonization and eradication treatment are limited:
In the study of Munck *et al*. (346) 14 patients (74%) were colonized by a *P. aeruginosa* strain with a genotypic profile different from that of the first isolate and the other 5 patients had subsequent *P. aeruginosa* isolates with identical genotypes. No difference between both groups in terms of mean interval between first and second *P. aeruginosa* isolations was found.
Taccetti *et al*. (337) reported data on genotyping for 16 patients with at least one subsequent *P. aeruginosa* isolate and found that acquisition of *P. aeruginosa* isolates with a different genotype occurred in 73% of the 50 episodes. These data were not presented in relation with subsequent chronic colonization status.
Whereas these studies suggest that a minority of the *P. aeruginosa* recurrences are regrowth of a partially suppressed *P. aeruginosa* strain, others investigators revealed the opposite: Gibson (342) examined *P. aeruginosa* genotypes of BAL and OP cultures in those patients with 2 or more *P. aeruginosa* positive cultures during the study. In the 6 patients with failed eradication (defined as a positive culture for *P. aeruginosa* in follow-up BAL), genotype of *P. aeruginosa* in both BAL samples (baseline and follow-up) were identical. Six out of 24 patients (25%) who initially cleared the infection but later on had a new positive OP culture, had a different follow-up *P. aeruginosa* genotype compared with the baseline BAL or OP culture.
In the ELITE trial (347), 12 out of 21 patients (57%) had the same *P. aeruginosa* genotype at follow-up cultures.
Conlusion: Comparison of genotypes of *P. aeruginosa* in initial and subsequent *P. aeruginosa* isolates give a clue as to whether eradication treatment really was succesfull. The currently available data vary; with as many as 25% to 80% of identical *P. aeruginosa* genotypes at follow-up samples, indicating eradication failure or reinfection from the same initial source. Ideally, every study on eradication treatment and those comparing treatment regimens should include genotypic characterization of *P. aeruginosa*.

- Is eradication of mucoid *P. aeruginosa* strains possible?

It is often stated that mucoid strains of *P. aeruginosa* cannot be eliminated from the lower airways, but recent studies have suggested that eradication of these mucoid strains can be achieved with early, aggressive therapy. Gibson *et al*. (113) have reported eradication of mucoid and nonmucoid *P. aeruginosa* with inhaled tobramyin 300mg. A recent study in the UK (354) indicates that eradication of mucoid *P. aeruginosa* is a realistic clinical goal. A retrospective chart review of pediatric patients seen at one centre in the period 1999-2008 identified 116 subjects with at least one episode of mucoid *P. aeruginosa* infection and at least one year of follow-up. A total of 67 of 116 patients (57.8%) cleared mucoid *P. aeruginosa* for more than 1 year; 38 of these 67 patients (32.8% of the total group) remained *P. aeruginosa* free for a median time of 55 months (range 12-103 months) and a median number of 30 clear cultures (range 2-106 cultures). Unfortunately, no information is given on the eradication treatments used.

Conclusion: Recent data indicate that eradication of mucoid *P. aeruginosa* is possible.

General conclusions on eradication treatment for P. aeruginosa in patients not chronically colonized by this pathogen.

The different studies on eradication treatment all agree on the favourable effect of antibiotics on eradication of *P. aeruginosa*, on the frequency of detection of *P. aeruginosa* and on the delay in onset of chronic *P. aeruginosa* infection. Early treatment for *P. aeruginosa* colonization/infection episodes is now considered the standard of care in the CF centres. More studies however are needed to determine the optimal treatment regimen and duration of eradication treatment. Ideally, every study on eradication treatment and those comparing treatment regimens should include genotypic characterization of *P. aeruginosa*.

Early diagnosis of *P. aeruginosa* infection

INTERMEZZO: **Phenotypic versus molecular/genotypic methods for detection and typing of *Pseudomonas aeruginosa***

- Detection of *Pseudomonas aeruginosa*

Methods used for the detection of *P. aeruginosa* can be divided into phenotypic and genotypic procedures. Phenotypic procedures take advantage of biochemical, physiological and morphological phenomena such as cell and colony morphology, cell wall staining properties and the ability of a microbial species to grow under a given set of environmental conditions (e.g. temperature, oxygen dependency, osmolarity and the need for certain nutrients).

P. aeruginosa, a gram negative rod, has some typical morphologic and biochemical characteristics, allowing identification when cultured on solid media. *P. aeruginosa* produces pigments (pyoverdin and pyocyanin), resulting in typical green to green-blue colonies on culture media. However, morphologically, *P. aeruginosa* colonies are very diverse, especially for strains isolated from CF patients. Environmental strains, in contrast with chronic infecting strains, are often flat with a metallic sheen and may have a gelatinous or slimy appearance, particular in areas of heavy growth. Other colonial variants include the small colony variants and the mucoid morphotypes, the latter often seen in CF patients chronically colonized by *P. aeruginosa*. *P. aeruginosa* is able to grow on a wide variety of media and can metabolize a large array of carbon sources: it produces acid from sugars such as glucose, fructose and xylose, but not lactose and sucrose. *P. aeruginosa* is strongly oxidase positive and can grow at 42℃. Commonly used sel ective media are cetrimide agar and MacConkey agar, which inhibit the growth of many other organisms. The Laboratory Bacteriology Research (LBR) of our research group compared the sensitivity for detection of

P. aeruginosa of different culture media and found no difference between McConkey agar, Cetrimide agar and Cetrimide broth

In the case of CF respiratory samples, most routine laboratories use culture techniques to detect bacterial species in the respiratory samples. However, it has been shown that conventional culture methods of sputa from CF patients frequently fail to identify the pathogens, which were shown to be present by means of PCR (355). Misidentification by culture can occur due to the *P. aeruginosa* phenotypic variants seen in chronically colonized CF patients such as the pyoverdine negative mutants, the slowly growing variants, the small colony variants and the auxotrophs, which do not grow on standard media (356). For the molecular detection of *Pseudomonas* species, several assays have been described (355, 357-365). Comparison of conventional culture methods and PCR for detection of *P. aeruginosa* are discussed later on this chapter. A brief explanation of the PCR technique is given here. PCR is a technique which can generate, in just a few hours, thousands to millions copies of a particular piece of DNA, starting from a single piece of DNA or a few copies. Amplified DNA can subsequently be visualized by gel electrophoresis. Using this technique, the negatively charged DNA fragments migrate toward the positive electrode through a prepared agarose gel. After electrophoresis and addition of an ethidium bromide stain, which forms a complex with nucleic acids, exposure of the gel to ultraviolet light demonstrates the amplified fragment as a single fluorescent band. Advantages of the PCR technique are rapidity, good sensitivity and specificity. However, real-time PCR reduces the work-load and the time-to-result, because no gel electrophoresis is needed, and moreover and most importantly, because it enables the quantification of targets (*e.g. P. aeruginosa* cells) present in the sample. Real-time PCR is a method in which the accumulation of PCR products over time is measured directly, by means of a fluorescent signal, increasing proportionally to the amount of generated PCR product. Using an external standard, of which the DNA concentration is known, the concentration of DNA in the unknown sample can be quantified. Several real-time PCR-formats (SYBR Green, hydrolysis probe, hybridization probes) are already developed. In 2009, the LBR at the Ghent University Hospital compared the sensitivity of conventional PCR, combined with two different detection methods, with the sensitivity of four real-time PCR-formats for the detection of *P. aeruginosa* in sputum and concluded that the probe based real-time PCR-formats allowed for the most sensitive detection of *P. aeruginosa* in sputum (366).

- Typing of *Pseudomonas aeruginosa*

Until the early nineties, typing of *P. aeruginosa* for epidemiologic purposes relied on bacterial phenotypic characteristics, such as serospecificity of lipopolysaccharides (LPS), susceptibility to bacteriophages, bacteriocin production and antibiotic susceptibility. Although effective in certain clinical settings, some of these methods have been found to be inadequate under conditions in which *P. aeruginosa* undergoes phenotypic conversion. Moreover, *P. aeruginosa* strains of CF patients are frequently endowed with rough LPS, which renders them refractory to typing with systems that rely on agglutination with antisera or on phage susceptibility. Therefore, genotyic procedures were further developed. The starting point of these analyses is that the genome of each individual (and also each germ) is unique. A multicentre comparison of methods for typing strains of CF patients showed that the chromosomal DNA restriction fragment length polymorphism analysis (RFLP) had the greatest discriminatory power, in comparison with 10 phenotypic techniques (367). The major drawbacks of these restriction digestion based genotypic typing techniques are the need for a high degree of technical skills, and for a large quantity of high-quality DNA or RNA. Therefore enzymatic amplification of nucleic acid sequences has been increasingly applied for genotyping.

PCR has been extensively evaluated for genotyping (368). This technique has evolved from a laborious and relatively insensitive assay into an extremely sensitive and highly flexible procedure, since the discovery of thermotolerant DNA polymerases and the development of automated thermal cyclers.

The basis of PCR fingerprinting is the amplification of polymorphic DNA through specific selection of primer annealing sites. Either constant primer sites bridge a single variable sequence domain or primers detect consensus sequences with variable distribution in the DNA. Differences in the distance between primer-binding sites or in the presence of these sites lead to synthesis of amplified DNA fragments which differ in length and can be detected by electrophoresis. Different PCR fingerprinting techniques such as amplification fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD) and multi locus sequence typing (MLST) have been developed. However, a major problem of these techniques is that these methods are often not equally discriminatory.
In a letter to the editor in the Journal of Clinical Microbiology in 2003, several CF physicians and microbiologists therefore emphasized the need for harmonization of techniques and technique designations for genotyping clinical isolates of *P. aeruginosa* from CF patients (369). Epidemiological research, comparable to our studies, has been done in the UK, Canada and Australia (250, 251, 255). Most of these studies were based on Pulsed-Field Gel Electrophoresis (PFGE).
Since our laboratory had already built up experience to genotype other species with RAPD and fAFLP, we have chosen to use these techniques (370, 371). This choice was supported by a publication of Speijer *et al.* (372), who showed that AFLP analysis was the most discriminatory method. D'Agata *et al*. (373) concluded that AFLP is comparable to PFGE for *P. aeruginosa* isolates.
The culture and genotyping procedure used for *P. aeruginosa* isolates is described in article 1 (see chapter III) (374). We assessed the genotypic diversity of *P. aeruginosa* colonies, initially by RAPD-analysis, and further with fAFLP-analysis for representative strains of the different RAPD-types observed for each patient. In all cases studied here, isolates with identical RAPD-fingerprints also had identical fAFLP-fingerprints, ensuring that no unrelated isolates were grouped into the same RAPD-genotype. For each patient, all of the RAPD-products were always obtained during the same thermal cycling and electrophoresis run, to avoid differences due to the limited inter-run reproducibility of the technique. fAFLP is generally known to be more reproducible and - due to automated digitisation of the fingerprints – it enables large-scale comparison of hundreds of fingerprints, which is impossible with RAPD-analysis. This combined approach of a rapid and cheap initial screening technique (RAPD-analysis) and a more sophisticated, more reproducible and digitized, but also more expensive and laborious technique (fAFLP-analysis), enabled us to genotype a large number of isolates in an affordable, reasonably convenient and a highly reliable and discriminatory manner. Moreover, the established library of fAFLP-fingerprints of CF *P. aeruginosa* strains can be used for further comparisons and long-term studies.

Early detection of *P. aeruginosa* in CF patients

Early detection of *P. aeruginosa* in CF patients is of utmost importance because aggressive antimicrobial therapy at first *P. aeruginosa* detection can prevent or postpone chronic *P. aeruginosa* colonization and the subsequent disease progression (see chapter II) . The standard method for assessing respiratory infection with *P. aeruginosa* is selective culture of sputum, oropharyngeal (OP) swabs or nasopharyngeal (NP) aspirates. Culture of broncho-alveolar lavage fluid (BALF) is considered the gold standard for detection of *P. aeruginosa*, because it reflects colonization of the lower respiratory tract, but since BAL is an invasive procedure it is not often performed. It has been demonstrated that sputum culture shows good correlation with BALF culture (375). First *P. aeruginosa* episodes however occur in young children who are not able to produce sputum. In this age group, diagnosis of *P. aeruginosa* infection is mainly based on culture of oropharyngeal swabs or nasopharyngeal aspirates. These techniques nevertheless have shown to be inferior in detection of *P. aeruginosa*. Therefore, there is an increasing interest in newer non-culture based techniques

for early detection of *P. aeruginosa*. These techniques include serologic testing and molecular methods (PCR).
This section elaborates on the value of the different diagnostic methods for early detection of *P. aeruginosa* in young children with CF.

Diagnostic value of OP cultures for the detection of lower airway *P. aeruginosa*
The diagnostic accuracy of OP culture has important clinical implications, because it is the only non invasive way of assessing respiratory tract specimens in non sputum producing patients. OP cultures are taken by vigorously swabbing the posterior pharyngeal wall and tonsils. Several studies extend on the diagnostic value of OP cultures for detection of lower airway *P. aeruginosa* infection. They all come to similar conclusions.
Ramsey *et al.* (376) collected simultaneous bronchial and OP specimens in 26 non-expectorating CF patients and demonstrated that OP cultures have relatively good specificity (>90%), but a poor sensitivity (46%) for detection of lower airway *P. aeruginosa*. The positive predictive value of OP cultures for detection of *P. aeruginosa* was 83%, whereas the negative predictive value of OP culture was 70%.
Armstrong *et al*. (377) investigated 75 infants at a mean age of 17 months (range, 1-52 m) with CF diagnosed by neonatal screening by collecting 150 simultaneous BALF and OP specimens for quantitative bacterial culture. The sensitivity of throat swabs for detection of *P. aeruginosa* was 71%, the specificity was 93%, the PPV 57% and the NPV 96%. In 8 out of the 9 patients with *P. aeruginosa* isolated simultaneously from BALF and OP cultures, *P. aeruginosa* of OP and BALF were identical, as proved by genotyping, indicating a high concordance of upper and lower respiratory cultures.
Rosenfeld and colleagues (378) compared OP cultures with simultaneous BALF cultures (286 bronchoscopies) in 141 CF children younger than 5 years. In the youngest age group (< 18 months), OP cultures had a sensitivity, specificity, PPV and NPV for detection of *P. aeruginosa* of respectively 44%, 95%, 44% and 95%. In those older than 18 months sensitivity and PPV rose with sensitivity, specificity, PPV and NPV being respectively 68%, 94%, 76% and 91%. The sensitivity of OP cultures improved as the lower airway bacterial density, cultured from simultaneous BALF samples, increased: sensitivity of OP culture was 82% when > 10^5 cfu/ml *P. aeruginosa* were present in BALF.
Burns *et al*. (49) performed 108 BAL procedures in 40 CF patients younger than 3 years. Bronchoscopy with BAL was performed annually, OP cultures were taken every three months. The PPV and NPV of OP cultures were 69% en 85%. Diagnostic accuracy could be increased by combining the results of two OP cultures, *i.e.* the OP culture concurrent with and the one preceding the BALF culture. The PPV of this combination of OP cultures was 93% and the NPV was 97%. Concurrent OP and BALF cultures both yielded *P. aeruginosa* in 18 visits of 14 patients, each time genotypes OP and BALF isolates were identical. This confirms again the high concordance of upper and lower airway cultures.
Conclusion: In young children, *P. aeruginosa* positive OP-cultures do not reliably predict the presence of *P. aeruginosa* in the lower airways. However, a negative OP-culture indicates that *P. aeruginosa* is unlikely to be present in the lower respiratory tract. In older children (with a higher prevalence of *P. aeruginosa*), positive predictive value is better than in younger children.

Diagnostic value of nasopharyngeal aspirates for the detection of lower airway P. aeruginosa
Although this is an established technique in many CF centres, the value of nasopharyngeal aspirates (NPA) in detection of lower airway pathogens has hardly been studied in CF patients. Taylor *et al*. (379) compared cultures of throat swabs and of NPA specimens in 47 non expectorating CF children aged 6 months to 10 years. No significant differences were found in the rate of positive cultures for *P. aeruginosa*, *S. aureus* and *H. influenzae*. Concordance between the two sampling methods reached 98% for *P. aeruginosa.*
Conclusion: Although culture of nasopharyngeal aspirate samples is a frequently used procedure, only one small study exists on the value of nasopharyngeal aspirates in de

detection of lower airway pathogens in CF. Relying on these scarce data, there is good concordance between throat swabs and nasopharyngeal aspirates for the detection of *P. aeruginosa*.

Diagnostic value of serological methods in the early dectection of lower airway *P. aeruginosa*.

Plenty of data exist on *P. aeruginosa* serology. The main antibody tests used in these studies are enzyme linked immunosorbent assay (ELISA) against whole cell protein or against single purified proteins (phospholipase C, exotoxin A, alkaline protease or elastase) and counter immuno electrophoresis (CIE) precipitating antibodies. Antibody levels determined by whole cell protein ELISA and levels of precipitins correlate well with each other and have a high sensitivity for chronic infection with *P. aeruginosa* (380). ELISA with single purified proteins has lower sensitivity for the diagnosis of chronic infection (381).

Detection of antibodies to *P. aeruginosa* has been investigated in different settings:

Detection of antibodies against *P. aeruginosa* has initially been used to determine chronic infection with *P. aeruginosa* in CF patients and to monitor the response to antibiotic treatment in CF patients chronically infected with *P. aeruginosa*: patients with chronic infection generally have a firm antibody response and CF patients chronically infected with *P. aeruginosa* who are treated intensively with antibiotics have a lower antibody response (112, 382-384).

Longitudinal follow-up of *P. aeruginosa* antibody titres in children diagnosed with CF at young age shows higher numbers of children with positive *P. aeruginosa* serology than with *P. aeruginosa* positive respiratory cultures: Burns *et al*. (49) followed a cohort of 40 CF patients during the first 3 years of life. They were all diagnosed at young age (mean age at diagnosis 3.9 months), entered the study at a mean age of 11 months and underwent bronchoscopy with BAL at diagnosis and annually from then onwards. OP cultures and *P. aeruginosa* serology were carried out every 3 months. By the age of 3 years, 29 patients (72.5%) had shown positive cultures for *P. aeruginosa* (either from BAL, OP or both), whereas all but one patient (97.5%) had positive serum *P. aeruginosa* antibodies. All 18 patients in whom *P. aeruginosa* was detected in BALF and 10 out of the 11 patients for whom *P. aeruginosa* was cultured only from OP samples demonstrated antipseudomonal antibodies. Ten out of 11 patients who did not have positive *P. aeruginosa* cultures had detectable antipseudomonal antibodies. The authors concluded that by the age of 3 years, only one patient lacked any evidence of *P. aeruginosa* contact, based on results of culture and serology. Unfortunately, no follow-up data of these patients are available.

Longitudinal follow-up of *P. aeruginosa* antibodies in children diagnosed by newborn screening and followed from birth up to the age of 16 years shows that there are abrupt elevations in antibody titers with transition from no *P. aeruginosa* to nonmucoid *P. aeruginosa* and second elevations with transition from nonmucoid *P. aeruginosa* to mucoid *P. aeruginosa* (311).

P. aeruginosa antibody titers correlate with the *P. aeruginosa* colonization status; as demonstrated by Pressler *et al.* (385) and Proesmans *et al*.(322). In these studies, the *P. aeruginosa* antibody levels were significantly different between the different groups of *P. aeruginosa* colonization according to the Leeds criteria (chronic, intermittent, free of *P. aeruginosa*).

Serology can help to predict the clearance of *P. aeruginosa* after eradication therapy: Ratjen *et al.* (386) assessed *P. aeruginosa* antibody titers before and after inhaled antibiotic therapy (tobramycine 80 mg bid for 12 months) in a cohort of 56 patients with a first ever *P. aeruginosa* isolation. *P. aeruginosa* antibodies decreased significantly in patients clearing the *P. aeruginosa* infection, whereas the titers increased in patients in whom antibiotic therapy failed to eradicate the organism. Kappler *et al.* (387) calculated the value of *P. aeruginosa* antibodies in the prediction of evolution to chronic infection in 27 CF patients intermittently infected with *P. aeruginosa* and in 68 CF patients free of *P. aeruginosa*. The authors calculated that if the serum antibodies were positive, it was very likely (PPV = 83%)

that the patient remained infected with *P. aeruginosa* after eradication therapy. If the serum *P. aeruginosa* antibodies were negative, the chance of elimination after eradication therapy was 58% (NPV).
More recently, the interest in antibody tests for early detection of *P. aeruginosa* has increased. The studies are difficult to compare because of different patient groups studied (patients diagnosed by newborn screening versus mixed groups of CF patients with mixed *P. aeruginosa* colonization status), the use of different serological methods, different cut-off values and different study designs (e.g. different frequency of respiratory cultures and serology samples). Moreover, most studies on the value of *P. aeruginosa* serology in early detection of *P. aeruginosa* in the young age group compare serology with OP cultures, with known limitations in diagnostic value of early detection (see above). The different study groups come to conflicting conclusions: while some authors strongly advocate the use of *P. aeruginosa* serology for early detection of *P. aeruginosa* (50, 388, 389), others oppose this point of view (343, 386, 390) .
The first to investigate the value of serology as an early predictor of impending *P. aeruginosa* infection were Brett and colleagues (389). They studied 33 CF patients, from whom *P. aeruginosa* was isolated for the first time, during a period of 3 years. The titer of *P. aeruginosa* antibodies was elevated either at the first isolation of *P. aeruginosa* or up to 24 months before this event in respectively 19 and 5 patients. Another 5 patients had titers that were within the control range before isolation of *P. aeruginosa* but that increased above the control range within 2 months following the first isolation of *P. aeruginosa*. In these 29 patients continuing intermittent isolations of *P. aeruginosa* were accompanied by further increases in *P. aeruginosa* antibody titers. In the remaining 4 patients isolations of *P. aeruginosa* were not accompanied by an increase in specific antibodies.
West *et al*. (50) followed 52 CF patients by OP or sputum cultures and *P. aeruginosa* serology at 6 months intervals for up to 180 months (15 years). Titers to cell lysate and exotoxin A were detected a mean time of 11.9 and 5.6 months before the first isolation of *P. aeruginosa.* In contrast, a titer to elastase was detected a mean of 41.1 months after the first isolation of the organism. The rise of anti–cell lysate and anti–exotoxin A titers occurred prior to, or coincided with, the first isolation of *P. aeruginosa* in 63% and 54% of the patients, respectively. On the contrary, not all patients with positive respiratory cultures developed positive *P. aeruginosa* serology.
Milagres *et al.* (388) followed 51 CF patients for two years: blood samples were obtained at an average interval of 6 months and respiratory secretions (OP culture or sputum) were cultured quarterly. In the group of 11 patients free of *P. aeruginosa* in the 2 years preceding and the first 17 months of the study, 9 (82%) had a positive *P. aeruginosa* serology in the first serum sample and all patients had positive serology at the end of the study. These 11 patients showed negative *P. aeruginosa* respiratory cultures until the time of the 3rd blood sample . Eight (72.7%) individuals had their first *P. aeruginosa* isolation by the time of the 4th blood sample was collected. The time elapsed between the first positive serology and the first isolation of *P. aeruginosa* varied between 18 and 25 months (mean of 21 months). Three patients were still free of *P. aeruginosa* at the end of the study. In the group of 5 CF patients with intermittent *P. aeruginosa* isolation (not specified whether this was before and/or during the study), 1 patient had a positive respiratory culture before *P. aeruginosa* antibodies were elevated, the 4 remaining patients demonstrated positive *P. aeruginosa* serology 3 to 12 months before the isolation of *P. aeruginosa* from respiratory cultures.
In a prospective 3 year study of Tramper-Stranders *et al.* (390) in 57 CF patients (age 4-14 years), with 23% chronically colonized, 23% intermittently colonized and 54% free of *P. aeruginosa* according to the Leeds criteria, *P. aeruginosa* serology was followed yearly, and cultures of respiratory secretions (sputum or OP swabs) at least once a year. A mean number of 6 respiratory cultures was taken during the 3 year study. In 5 of the 13 patients with a positive *P. aeruginosa* culture during the study period, positive *P. aeruginosa* serology preceded positive *P. aeruginosa* cultures and one patient had a simultaneous conversion to positivity for both culture and serology. Ten patients showed a transient positive serology

without any positive culture following. Weaknesses of this study are the low frequency of serology (once a year) and of culture (mean number of 6 cultures over 3 years) and the heterogeneous group of CF patients that was followed prospectively (only half of them not colonized by *P. aeruginosa*, without specification whether these were free of *P. aeruginosa* or never had any *P. aeruginosa*). Although these results are largely comparable with the West data (50) i.e. 46% in this study versus 54 to 63% of patients in the West study with positive serology preceding or concomittant with positive culture results, authors come to different conclusions on the usefulness of serology in early detection of *P. aeruginosa* in CF patients.
Ratjen *et al.* (386) reported that 18 of 43 patients (42%) had positive antibody titers to *P. aeruginosa* at the time of the first *P. aeruginosa* infection. Fifty of the 198 patients (25%) who never had a positive culture for *P. aeruginosa* during the study period had positive *P. aeruginosa* antibodies at one time point during the study. It is difficult to come to a conclusion based on these observation, as few information is given on study design (frequency of respiratory cultures, serum samples, follow-up time) of these patient groups.
Douglas *et al*. (343) recently provided interesting data on the value of *P. aeruginosa* antibody tests to predict lower airway infection with *P. aeruginosa* in two populations of young CF children. One population consisted of 76 predominantly asymptomatic infants and preschool children undergoing annual BAL procedure and serum sampling for *P. aeruginosa* serology, yielding 186 concurrent BAL and serum samples. The second group were 55 children undergoing BAL, OP and serum sampling during infective exacerbations; 162 paired samples were obtained in this group. In the first patient group, depending on which serological test used, serology had a sensitivity, specificity, PPV and NPV for detection of lower airway *P. aeruginosa* of 90-95%; 57-62%; 20-23% and 98-99% respectively. For the second patient group sensitivity, specificity, PPV and NPV were 46-84%; 52-82%; 34-43% abd 84-92% respectively. The diagnostic value of serology was in neither patient cohort sufficiently reliable to diagnose *P aeruginosa* infection with any certainty.
The high NPVs observed suggest that serology may have clinical value in excluding lower airway infection with *P aeruginosa* in young children. However, in a population with a higher prevalence of *P aeruginosa* infection, the NPV of serology using either assay is likely to be too low to reliably exclude the presence of lower airway infection.
To summarize, the results of these studies on the value of *P. aeruginosa* serology in the early detection of *P. aeruginosa* indicate that positive serology can precede the isolation of *P. aeruginosa* by various time periods, but that this phenomenon is not observed in all patients. Some patients even have positive *P. aeruginosa* serology without subsequent positive *P. aeruginosa* respiratory cultures, although follow-up time might not have been long enough. It can also be discussed whether these cases represent false positive *P. aeruginosa* serology results or whether the *P. aeruginosa* infection has cleared spontaneously in these patients. When serology is available, it seems reasonable to follow serology together with conventional culture of respiratory secretions at regular intervals (monthly? quarterly?) and to be vigilant when antibody titers are rising.

Conclusion: Current data indicate that serological methods alone are not helpful in early diagnosis of *P. aeruginosa* infection but can be an adjunct tool, when followed longitudinally and in combination with regular culture of respiratory secretions.

Diagnostic value PCR-techniques in the early dectection of *P. aeruginosa*
Over the last 15 years, several PCR formats targeting different genes for the detection of *P. aeruginosa* have been developed (355, 357-365, 391, 392). These molecular techniques for detection of *P. aeruginosa* are yet not widely used in clinical practice.
Studies show sensitivities between 93 and 100%, depending on the primers used (358, 362, 391, 393). Some groups demonstrated a higher sensitivity of PCR compared to culture and/or serological tests for the detection of *P. aeruginosa* in respiratory samples of CF patients (391, 394, 395) while others found no difference (361, 366) or demonstrated a lower

sensitivity for PCR (363). Theoretically, PCR can be useful to detect the *P. aeruginosa* phenotypic variants that are not easily detected with conventional culture methods, whereas culture however will still be needed for susceptibility testing.
Till 2010, the value of PCR in early detection of *P. aeruginosa* was only addressed in one study: Xu *et al*. compared PCR and conventional culture methods in a long term study for early detection of *P. aeruginosa* in 57 adult CF patients (391). Sputum was cultured for *P. aeruginosa* and in addition genomic bacterial DNA was extracted from the same sample and was amplified employing two species specific targets, *i.e.* the outer membrane protein gene locus and the exotoxin A gene locus. In 39 patients (22 *P. aeruginosa* positive and 17 *P. aeruginosa* negative), a complete agreement between molecular and conventional detection techniques existed. In 10 patients a PCR positive/culture negative result was recorded and for 5 of these patients the sputum culture became positive over a mean period of 4.5 months (range 4-17 months).
Conclusion: The value of PCR techniques in early detection of *P. aeruginosa* is addressed in only one study. In this study by Xu *et al.*, the authors concluded that PCR technique was more sensitive than conventional culture methods for early detection of *P. aeruginosa*.

General conclusion on the different techniques for early detection of *P. aeruginosa*. :
Early detection of *P. aeruginosa* in CF patients is of utmost importance, as early initiated therapy increases the chances of eradication, postponing the evolution to chronic infection with *P. aeruginosa*. Culture of BAL fluid is considered the gold standard for diagnosis of lower airway infection by *P. aeruginosa*, but is too invasive for regular use. OP cultures have a low sensitivity and serology has both a low sensitivity and specificity for detection of lower airway *P. aeruginosa*. It is clear that more sensitive diagnostic techniques are needed. PCR based detection of *P. aeruginosa* on respiratory samples seems promising but more studies are needed.

CHAPTER III: Articles

Introduction to the studies: rationale of this thesis

Cystic fibrosis has always been a special field of interest in the Pediatric Pulmonology Department of the Ghent University Hospital. Since the establishment of CF centres in Belgium in 1999, CF care has become better organized. Paramedical co-workers, such as CF nurses, physiotherapists, dieticians, psychologists and social workers, joined the medical staff, optimizing CF care in many ways. Moreover, CF centre care facilitates the set-up of both inter-centre and multi-centre studies.

As discussed in chapter II, *P. aeruginosa* is one of the most common and clinically important pathogens in CF patients. By the age of 30 years, nearly 80% of the patients are intermittently or chronically colonized by *P. aeruginosa*, which adversely affects lung function and survival.

Although knowledge on epidemiology of *P. aeruginosa* in CF patients, effect on disease progression, transmissibility, diagnosis and treatment has increased considerably over the last decades, several questions remain unanswered.

In order to elucidate some of the many remaining unanswered issues in the field of *P. aeruginosa* in CF, we set up several studies, of which some are the topic of this thesis.

1. The origin of *P. aeruginosa* in CF patients remains unclear for most patients. Patient-to-patient transmission has been described and mainly results from prolonged and close contact. We previously demonstrated that the risk of a persistent patient-to-patient transmission of *P. aeruginosa* is low in a CF rehabilitation centre (396) and that no highly transmissible strains of *P. aeruginosa* circulate in the population of chronically colonized patients in Belgium (260). Consequently, the mode of acquisition of *P. aeruginosa* remains unclear for most patients. As *P. aeruginosa* is widely present in the natural environment, the possibility of acquisition from the environment has been raised. Can the organism be transferred from the home environment to the patient? This subject is investigated in the first study of this thesis "*Pseudomonas aeruginosa* in the home environment of newly infected cystic fibrosis patients " (374)

2. After the first ever isolation of *P. aeruginosa*, patients may go through different episodes of colonization, preceding chronic colonization by months to years, eventually resulting in chronic infection. Several studies have demonstrated that treatment for early *P. aeruginosa* colonization episodes results in eradication in the majority of the patients and delays the evolution to chronic colonization. However, little is known on risk factors for chronic colonization and whether this therapy really eradicates *P. aeruginosa* or just temporarily suppresses the organism, with *P. aeruginosa* remaining in amounts too low to be detected by the current microbiological techniques. To solve these questions, we analyzed our data on genotypes of *P. aeruginosa* of first and subsequent colonization episodes. These are presented in "Genotype based evaluation of *Pseudomonas aeruginosa* eradication treatment success in cystic fibrosis patients" (397).

3. The best opportunity for successful eradication of *P. aeruginosa* in patients with CF is at the time of initial infection with nonmucoid, antibiotic-sensitive strains. In order to maximize the chance of successful elimination of *P. aeruginosa*, it is important to use sensitive techniques to detect any colonization of the airways as soon as possible. Currently, routine detection and identification of *P. aeruginosa* in respiratory samples is done by conventional methods such as culture on selective culture media and by recognition of biochemical characteristics. Therefore, we were interested whether PCR based detection techniques can advance the diagnosis of *P. aeruginosa* colonization, allowing earlier initiation of *P. aeruginosa* eradication regimen and this resulted in a third study: "Comparison of culture and qPCR for the detection of *Pseudomonas aeruginosa* in not chronically colonized cystic fibrosis patients " (365).

Article 1

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Pseudomonas aeruginosa in the home environment of newly infected cystic fibrosis patients

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ABSTRACT: **The source of acquisition of *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients remains unknown. Patient-to-patient transmission has been well documented but the role of the environment as a source of initial infection is as yet unclear.**

In the present study, the origin of the first *P. aeruginosa* isolate in CF patients was investigated by comparing the *P. aeruginosa* genotype(s) from newly infected patients with genotypes of *P. aeruginosa* isolates from the home environment and from other patients from the same CF centre.

A total of 50 newly infected patients were studied. *P. aeruginosa* could be cultured from 5.9% of the environmental samples, corresponding to 18 patients. For nine of these, the genotype of the environmental *P. aeruginosa* isolate was identical to the patient's isolate. In total, 72% of the environmental *P. aeruginosa* isolates were encountered in the bathroom. Patient-to-patient transmission within the CF centre could not be ruled out for three patients.

In summary, a low prevalence of *Pseudomonas aeruginosa* was found in the home environment of the newly infected cystic fibrosis patients. The bathroom should be targeted in any preventive cleaning procedures. An environmental source of the new infection could not be ruled out in nine patients.

KEYWORDS: **Cystic fibrosis, environment, genotyping, *Pseudomonas aeruginosa***

The major pulmonary pathogen in patients with cystic fibrosis (CF) is *Pseudomonas aeruginosa*. Dehydration of mucus during exacerbations and a defective host defence make CF airways prone to chronic infection with *Pseudomonas aeruginosa* [1, 2]. By adulthood, >80% of patients are infected with this pathogen, which adversely affects lung function and survival [3, 4].

To date, the source of initial infection of CF patients with *P. aeruginosa* remains unknown. Possible sources include the environment, person-to-person spread (especially from another person with CF), or through contact with contaminated objects. Patient-to-patient transmission has been well documented. It generally results from prolonged social contact, such as that between siblings [5], close friends or people staying at holiday camps [6, 7] and CF rehabilitation centres [7, 8].

Epidemiological studies have not confirmed the acquisition of *P. aeruginosa* from the environment. As *P. aeruginosa* is widely present in soil, plants and water [9, 10], the environment could be the initial source of infection. The lack of knowledge about the exact role of the environment in the acquisition of *P. aeruginosa* by the CF patient is of concern to parents and physicians, and sometimes leads to questionable preventive measures, such as not drinking tap water unless it has been boiled or not visiting swimming pools [11, 12].

In an attempt to elucidate the source of new strain acquisitions, the current authors carried out a Belgian multicentre study. For all patients who were not initially infected with *P. aeruginosa*, but for whom a recent new infection with *P. aeruginosa* could be documented, cultures of wet surfaces were taken at their homes as soon as possible after detection of the infection. DNA fingerprints of the *P. aeruginosa* isolates from the newly infected patients were compared with fingerprints of *P. aeruginosa* strains recovered from the home environment. Using the Belgian inventory of the DNA fingerprints of most patient *P. aeruginosa* isolates [7], the DNA fingerprints of the *P. aeruginosa* isolates from the newly infected patients were compared with the fingerprints of *P. aeruginosa* isolates from chronically infected CF patients attending the same CF centre as the newly infected patients.

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Article 2

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Original Article

Genotype based evaluation of *Pseudomonas aeruginosa* eradication treatment success in cystic fibrosis patients

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Abstract

Background: Longitudinal data regarding the genotypes of *Pseudomonas aeruginosa* isolates after eradication treatment are limited. We followed cystic fibrosis patients after a first ever isolation of *P. aeruginosa* and evaluated the *P. aeruginosa*-free time period after eradication therapy.
Methods: Between January 2003 and December 2008 respiratory samples were cultured prospectively from 41 patients with a first ever *P. aeruginosa* isolate. Twenty five patients had at least one subsequent isolate. Treatment efficacy was assessed based on the time to a second isolation and on comparison of the RAPD genotypes of the *P. aeruginosa* isolates.
Results: Eleven patients became chronically colonized during the study period. For ten of these the second isolate had the same genotype as the first isolate. Moreover, these patients had a significantly shorter *P. aeruginosa*-free time interval between the first ever and the second isolate compared to the 14 not chronically colonized patients (median 0 months versus 7.5 months, $p<0.05$).
Conclusion: Our results indicate that the presence of a genotypically identical subsequent *P. aeruginosa* isolate and/or a short *P. aeruginosa*-free time interval after treatment are ominous signs and might be useful additional tools to predict impending chronic colonization. Current routine bacteriological methods for the detection of *P. aeruginosa* may lack the sensitivity to discriminate between true eradication and low bacterial persistence.

Keywords: *P. aeruginosa*; Eradication treatment efficacy; Genotyping

1. Introduction

Pseudomonas aeruginosa is the major pulmonary pathogen in patients with cystic fibrosis (CF) [1–3]. By adulthood, over 80% of the patients are chronically infected with this pathogen, a condition which adversely affects lung function and survival [4,5]. Chronic lung infection by *P. aeruginosa* increases inflammation and destruction of lung tissue. CF patients chronically infected with *P. aeruginosa*, show a greater loss of lung function [4,6] and a higher overall morbidity [7,8] in comparison with non chronically infected CF patients. After a first ever isolation of *P. aeruginosa*, patients may go through different episodes of colonization, preceding chronic colonization by months to years, eventually resulting in chronic infection.

Early treatment of initial *P. aeruginosa* colonization can postpone the onset of chronic colonization [9–11] and maintain or increase pulmonary function [12]. Genotyping of *P. aeruginosa* isolates was previously applied in epidemiological studies of *P. aeruginosa* in chronically colonized CF patients [13–15], but longitudinal data on the genotype of *P. aeruginosa* isolates after initial colonization and eradication treatment are limited [16–18].

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Article 3

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RESEARCH ARTICLE Open Access

Comparison of culture and qPCR for the detection of *Pseudomonas aeruginosa* in not chronically infected cystic fibrosis patients

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Abstract

Background: *Pseudomonas aeruginosa* is the major respiratory pathogen causing severe lung infections among CF patients, leading to high morbidity and mortality. Once infection is established, early antibiotic treatment is able to postpone the transition to chronic lung infection. In order to optimize the early detection, we compared the sensitivity of microbiological culture and quantitative PCR (qPCR) for the detection of *P. aeruginosa* in respiratory samples of not chronically infected CF patients.

Results: In this national study, we followed CF patients during periods between 1 to 15 months. For a total of 852 samples, 729 (86%) remained *P. aeruginosa* negative by both culture and qPCR, whereas 89 samples (10%) were positive by both culture and qPCR.
Twenty-six samples were negative by culture but positive by qPCR, and 10 samples were positive by culture but remained negative by qPCR. Five of the 26 patients with a culture negative, qPCR positive sample became later *P. aeruginosa* positive both by culture and qPCR.

Conclusion: Based on the results of this study, it can be concluded that qPCR may have a predictive value for impending *P. aeruginosa* infection for only a limited number of patients.

Background

Cystic fibrosis (CF) is one of the most common genetic disorders, caused by mutations in the CFTR gene, coding for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein [1]. Mutations in this gene lead to inactivity of the CFTR protein and/or reduced expression of the protein at the cytoplasmic membrane [2]. Improper functioning of the CFTR results in the production of viscous mucus and in a defective innate immunity [2,3]. The reduced functionality of the mucociliary system and the ongoing inflammation result in an increased sensitivity of the CF airways to infection by bacterial pathogens, of which *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most important.

Chronic lung infection with *P. aeruginosa* is a major cause of morbidity and mortality among the CF patients [4]. It is now well-established that early aggressive antibiotic treatment of new infection with *P. aeruginosa* is successful in postponing chronic infection. Hence, it is important to detect new infection with *P. aeruginosa* as early as possible so that eradication treatment can be started as soon as possible [5-7]. Currently, routine detection and identification of *P. aeruginosa* in respiratory samples is done by conventional methods such as culture and biochemical characteristics. Misidentification can occur due to the variable phenotypic characteristics of this species [8]. Moreover, the sensitivity of culture might be limited, especially when compared to DNA amplification based techniques. Thus far, however, only one group has compared both approaches in a long term study for early detection of *P. aeruginosa* from CF patients [9].

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Chapter IV: Conclusions:

In this thesis we studied different unknown aspects of acquisition, diagnosis and persistence of *Pseudomonas aeruginosa* in the respiratory secretions of CF patients in an attempt to answer the following questions:

Can the home environment be a possible source of *P. aeruginosa* acquisition in CF patients?

The origin of *P. aeruginosa* colonization in CF patients is currently unknown for most patients. Patient-to-patient transmission has been described and generally results from prolonged and close contact (260). As *P. aeruginosa* is widely present in the natural environment, the possibility of acquisition from the environment has been raised. The observation that CF patients can be protected from acquiring mucoid *P. aeruginosa* but not from acquiring nonmucoid *P. aeruginosa* through patient segregation (268) adds to this suspicion. Can the home environment of CF patients be a source of transmission? This topic was investigated in the first study: "*Pseudomonas aeruginosa* in the home environment of newly infected cystic fibrosis patients" (374). Here, we examined the degree of contamination with *P. aeruginosa* in the houses of CF patients and the possible environment-to-patient transmission by comparing environmental *P. aeruginosa* isolates with the patient isolates of 50 CF patients with a first ever *P. aeruginosa* colonization episode. To our knowledge, a similar study has not been carried out before. We demonstrated that *P. aeruginosa* is not widely present in the homes of CF patients. *P. aeruginosa* was detected in only 6% of the samples, originating from 36% of the houses. The data are comparable to data found in non-CF houses in previous studies (277, 280, 281). A lower environmental contamination with *P. aeruginosa* was observed though, compared with the former studies in CF houses (277-279). The different findings might be explained by the following differences in procedures: 1) different sampling sites; 2) different sampling methodology; 3) different cleaning procedures carried out in the houses; and 4) the different *P. aeruginosa* colonisation status of the CF patients investigated. As *P. aeruginosa* is known to multiply overnight in the sinks, taking samples in the morning before use of taps and drains in the morning might have increased the positive *P. aeruginosa* cultures in our study.
In nine patients (18%), the genotype of the environmental *P. aeruginosa* isolate was identical to that of the patient's isolate, suggesting possible environment-to-patient transmission. It remains debatable however, whether the environmental isolates are the source of infection or conversely, whether contamination of the environment was caused by the patient. The only way to answer that question is by setting up a longitudinal study, taking regular cultures in the homes of CF patients before the first ever *P. aeruginosa* infection. This kind of setup is difficult, as it is possible that one has to sample years before the first ever infection is documented.
In our study, 72% of the environmental *P. aeruginosa* isolates were detected in the drains of the bathroom. This confirms the results of previous studies on the prevalence of *P. aeruginosa* in both CF and non CF houses indicating that drains are important reservoirs of *P. aeruginosa*. Therefore, bathroom drains should be targeted in the preventive cleaning procedures.
These results indicate that the home environment is not the major source of *P. aeruginosa* infection in CF patients and as a consequence, the exact source seems to remain unknown. Based on this study and on literature data however, we can conclude that probably no major source of acquisition of *P. aeruginosa* for CF patients exists. Some patients acquire it from the environment, while others contract *P. aeruginosa* through patient contact. The latter can be avoided by patient segregation but as *P. aeruginosa* is ubiquitously present in nature, acquisition in most patients seems unavoidable. Trying to keep away from all possible sources puts a high psychological and social burden on the patients. Therefore, too strict

and poorly validated hygienic guidelines should be avoided. Further investigations should focus on determining factors facilitating acquisition of *P aeruginosa* in CF patients.

What is the natural evolution after eradication therapy for a first ever colonization episode with *P. aeruginosa* in CF patients? Does treatment really eradicate the pathogen or does it only result in a temporary suppression of the organism? Can the evolution to chronic colonization be predicted?

Several studies have demonstrated that treatment for early *P. aeruginosa* colonization episodes results in eradication in the majority of the patients and delays the evolution to chronic colonization. However, little is known on risk factors for chronic colonization and whether this therapy really eradicates *P. aeruginosa* or just temporary suppresses the organism, with *P. aeruginosa* remaining in amounts too low to be detected by the current microbiological techniques. In the second study of this thesis, entitled "Genotype based evaluation of *Pseudomonas aeruginosa* eradication treatment success in cystic fibrosis patients" (397), we followed 41 CF patients prospectively after eradication treatment (consisting of oral ciprofloxacin and inhaled tobramycin or colomycin for three months) for a first ever *P. aeruginosa* colonization episode and evaluated the efficacy of eradication treatment by calculating the *P. aeruginosa* free time period after treatment and by comparing genotypes of initial and subsequent *P. aeruginosa* strains. Eradication treatment was successful in 34 (83%) of them, with over half (18 patients) experiencing a new *P. aeruginosa* episode after a median *P. aeruginosa* free time period of 7.5 months. In accordance with the data from the "15 year Danish experience with eradication treatment for *P. aeruginosa*" study by Hansen *et al.* (336), reporting a significantly shorter *P. aeruginosa* free period between the first ever and second positive culture in CF patients becoming chronically "infected", our study confirmed that patients becoming chronically "colonized" have a significantly shorter *P. aeruginosa* free interval between the first ever and second isolation event (i.e. median 0 months in the 11 patients becoming chronically colonized during the study period compared to 7.5 months in the 14 patients not becoming chronically colonized). As a result of these findings, we changed microbiological follow-up procedures at our CF center and now take cultures on a monthly basis after a first successful eradication treatment, decreasing the detection period to subsequent *P. aeruginosa* positivity, c.q. advancing new eradication treatments and probably postponing the onset of chronic colonization.
By incorporating genotyping of the *P. aeruginosa* isolates in the evaluation of eradication treatment, we were able to demonstrate that not only a short *P. aeruginosa* free time interval but also the detection of an identical *P. aeruginosa* genotype in the second isolate was an ominous sign for impending chronic colonization. In our study, a surprisingly high number of second *P. aeruginosa* isolates identical to the first ever isolate (i.e. 68%) was seen, whereas the two other and smaller studies revealed identical genotypes of *P. aeruginosa* isolates compared to the first ever isolate in 5 out of the19 patients (26%) (346) and 27% of 50 episodes in 16 patients (337). Our results point rather to treatment failure, with merely suppressing but not really eradicating *P. aeruginosa* after an eradication treatment. The period with *P. aeruginosa* free samples might be explained to a certain extent by a lack of sensitivity of the routine microbiological detection methods used. The recurrence of isolates with the same genotype could also be explained by reinfection fom the same environmental source. However, in our first study on the contamination with *P. aeruginosa* in the houses of CF patients with a first ever *P. aeruginosa* episode, we found that the home environment of these patients is not heavily contaminated, making reinfection rather an unlikely explanation. The results of this study clearly indicate that there is a high need of more sensitive microbiological diagnostic techniques for the early detection of *P. aeruginosa* in CF patients not chronically colonized by this pathogen.

Can the diagnosis of impending infection with *P. aeruginosa* in CF patients not chronically infected by this pathogen be advanced by the use of PCR based detection techniques instead of or in addition to conventional microbiological culture techniques?

This topic was addressed in the third study " Comparison of culture and qPCR for the detection of *Pseudomonas aeruginosa* in not chronically infected cystic fibrosis patients" (365). In this study, we evaluated whether detection of *P. aeruginosa* by means of quantitative PCR (qPCR) techniques can advance the diagnosis of *P. aeruginosa* infection. Advancing the diagnosis gives the possibility to start eradication treatment earlier, probably increasing the likelihood of successful eradication and thereby postponing or even avoiding chronic colonization by *P. aeruginosa*. In order to investigate this, we prospectively followed 397 CF patients, not chronically colonized by *P. aeruginosa* and examined respiratory secretions of these patients at least four times a year by means of both conventional culture and qPCR methods. Our results indicate that there is a good concordance between the results of conventional culture and qPCR for detection of *P. aeruginosa* in the vast majority of the samples (i.e. 816/852; 96%). Twenty-six samples, originating from 26 patients were culture negative but PCR positive. In these patients, early *P. aeruginosa* infection could be suspected but the follow-up samples became culture positive in only five of them, in four patients after 3 months (i.e. the next culture), in the remaining patient after 9 months, with two negative PCR/culture results in between. In the remaining 21 patients, the positive PCR signal became negative again and did not predict a positive culture at the next follow-up sample. We concluded that PCR positivity may have had a predictive value for impending infection in only a limited number of patients.

These data, gathered on clinical samples, confirm the results of our previous laboratory study in which culture methods were equally sensitive to the combination of the most sensitive DNA extraction method and the most sensitive amplification assay, i.e. probe based qPCR (366). Our results do not confirm the results of two other reports (391, 398) on the value of qPCR in early detection of *P. aeruginosa*. In the first study, Xu *et al*. (391) compared blood agar, MacConkey agar and *Pseudomonas* isolation agar with PCR for detection of *P. aeruginosa* from sputa of 59 CF patients. In this analysis, five of the 10 patients with culture negative/PCR positive results converted to culture positivity after a time range of 4 to 17 months (median 4.5 months). These authors concluded that PCR detected *P. aeruginosa* on average 4.5 months prior to culture. However, this should be interpreted with caution because of the low number of patients.

The second study, a large longitudinal analysis with a similar set up as ours was published in 2010: Logan *et al*. (398) investigated 2099 sputa and throat swabs received from 183 pediatric CF patients over a 29-month period in order to evaluate the efficacy of real-time PCR for the early detection of *P. aeruginosa*. Interestingly, in this analysis, the use of PCR was of more value than in our study for early detection of *P. aeruginosa* in CF patients not chronically colonized by this pathogen. In 26 out of the 80 specimens (32.5%) identified as PCR positive/culture negative for *P. aeruginosa*, the subsequent patient sample was *P. aeruginosa* culture and PCR positive, suggesting that PCR has the potential to advance the diagnosis of impending infection with *P. aeruginosa*. However, the authors do not mention the time lapse between the samples. Although the results of PCR in this report are promising, the authors are cautious in their conclusions. "Whether it is reasonable or acceptable to advise therapeutic intervention based on a PCR result in particular without the aid of antibiotic susceptibility data is unknown. However, early PCR detection has the potential to heighten the levels of awareness regarding potentially impending infection, thereby facilitating increased patient screening potential to detect *P. aeruginosa* earlier than the microbiologic culture."

It is not clear what makes the difference between the results of these two reports and our study. We followed these patients for a median time of 6 months (1-15 months). Extending

the follow-up- time and including more samples will not alter the results; as it can be discussed whether a positive PCR result more than 6 months before a positive sputum culture for *P. aeruginosa* with negative results of PCR and culture in between really reflects early detection. In order to evaluate the value of PCR in early detection of *P. aeruginosa*, it might be more interesting to increase the frequency of PCR and sputum cultures after a first PCR positive/culture negative result .

Differences can not be explained by inferior quality of the PCR technique used, as this has been analyzed comprehensively in a previous study (366). Yet, the different approaches of the studies and the availability of many different DNA-extraction protocols and PCR-formats make it often difficult to compare the different results and to come to unambiguous conclusions.

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Chapter V: Future perspectives

We studied different unknown aspects of acquisition, diagnosis and persistence of *Pseudomonas aeruginosa* in the respiratory secretions of CF patients and demonstrated that the home environment is not the major source of *P. aeruginosa* infection in CF patients, that patients becoming chronically colonized have a significantly shorter *P. aeruginosa* free interval between the first ever and second isolation event, that the detection of an identical *P. aeruginosa* genotype in the second isolate is an ominous sign for impending chronic colonization and that a positive *P. aeruginosa* PCR may have had a predictive value for impending infection in only a limited number of patients.
Although our research tried to answer some specific problems in daily CF care, it raises several new questions, emphasizing the urgent need for continued research.

The source of *P. aeruginosa* acquisition remains unidentified for most patients. Some patients acquire it from the environment, while others contract *P. aeruginosa* through patient contact. The latter can be avoided by patient segregation but as *P. aeruginosa* is ubiquitously present in nature, acquisition in most patients seems unavoidable. Trying to keep away from all possible sources puts a high psychological and social burden on the patients, as clearly demonstrated in the results of questionnaires published by Steinkamp (275) and Ullrich *et al*. (276). Therefore, too strict and poorly validated hygienic guidelines should be avoided.

Further investigations should focus on determining factors facilitating acquisition of *P aeruginosa* in CF patients. Currently, we are studying the role of modifying genes in the clinical course of CF related lung disease. We examined 82 Single Nucleotide Polymorphisms (SNPs) in 22 genes contributing to the innate immunity and 385 SNPs in 110 genes contributing to the inflammation of the lung in 200 CF patients (Haerynck *et al*.,in preparation). Once facilitating polymorphisms for early *P. aeruginosa* acquisition or fastened respiratory deterioration in CF patients can be recognized, a more strict follow-up schedule should be considered for this group of CF patients.

As successful eradication mainly depends on early treatment, research should focus on early detection of *P. aeruginosa*. Relying on our research and on current literature data, neither PCR nor *P. aeruginosa* serology alone can predict impending infection but any positive test can heighten the awareness. The ideal combination of tests should be determined.

Evaluation of a successful eradication treatment should not only be based on culture results and determination of the *P. aeruginosa* free time period and the delay of chronic colonization but also on the comparison of the genotypes of successive *P. aeruginosa* isolates after eradication therapy. We are investigating the reliability of a genotype based definition of chronic colonization through analysis of long-term follow-up data of CF patients after a first ever *P. aeruginosa* colonization episode. Ideally, this genotype based definition should anticipate the diagnosis of chronic colonization compared to the currently widely used European consensus or Leeds definition of chronic colonization and unequivocally determine in which patients new eradication therapies for *P. aeruginosa* are not worthwhile starting any more.

A batch of possible antimicrobial therapeutic compounds for CF people is under investigation presently. Setting up patient trials is time-consuming and challenging though and the prior efficacy evaluation of the different potential future therapies should be optimized in order to analyze which components are valuable investigating in patient trials. In most of the currently used models only single genotypes of *P. aeruginosa* are studied, whereas in the patients' airways sometimes two or three genotypes of *P. aeruginosa* and moreover numerous other species are present. In addition, the current biofilm models lack the patient mucus, the

patient leukocytes and the different medications the patient is receiving. Briefly, only an ex vivo sputum model can mimic as closely as possible the lung environment of the individual patient and consequently, this model might enable personalized medicine. At present, our research group is developing an ex vivo sputum model in order to study the therapeutic effect of antimicrobial drugs in CF patients with a respiratory exacerbation.

Chapter VI: Summary

Cystic fibrosis (CF) is a life-shortening genetic multi-organ disease, affecting more than 100 000 people worldwide and about 1000 people in Belgium. The disease is caused by a defect in the CFTR protein, resulting in a defective transport of chloride across the cellular membrane. CF affects all racial and ethnic groups, but it is more common among Caucasians. Life expectancy of CF patients has increased considerably over the last decades, due to advances in treatment and better organised patient care. While in the fifties, most CF patients did not survive infancy, over the years the mean survival has steadily increased to a mean predicted survival age of 37.4 years in the US (12).

P. aeruginosa is one of the most common and clinically important pathogens in CF patients . By the age of 30 years, nearly 80% of the patients are intermittently or chronically colonized by *P. aeruginosa*, which adversely affects lung function and survival. *P. aeruginosa* strains causing early colonization or infection usually have a nonmucoid phenotype and are relatively antibiotic-sensitive. Aggressive treatment of these early colonizing strains often successfully eradicates *P. aeruginosa*. Early treatment of initial *P. aeruginosa* colonization can therefore postpone the onset of chronic colonization and maintain or increase pulmonary function. Chronic mucoid *P. aeruginosa* colonization is usually impossible to eradicate, and the goal of antibiotic treatment is then to suppress, rather than to eliminate the pathogen. In this thesis, we studied different unknown aspects of acquisition, results of eradication therapy and early diagnosis of *P. aeruginosa* in CF patients.

The origin of *P. aeruginosa* in CF patients is unclear for most patients. Patient-to-patient transmission has been described and mainly results from prolonged and close contact. As *P. aeruginosa* is widely present in the natural environment, the possibility of acquisition from the environment has been raised. The different studies on the prevalence of *P. aeruginosa* in CF health care settings demonstrate that particularly sinks are heavily contaminated. Genotypes of environmental *P. aeruginosa* sometimes matched patient strains and in one patient CF health-care environment-to-patient transmission was strongly suspected. In contrast to the extensive studies on patient-to-patient transmission and on the presence of *P. aeruginosa* in the hospital environment, there are only a few reports on the prevalence of *P. aeruginosa* in the home environment of both CF and non-CF patients. None of them studied the possibility of environment-to-patient transmission, neither did they compare genotypes of *P. aeruginosa* isolates recovered from either patients or from their home environment. We investigated the possibility of CF home environment-to-patient transmission in the first study of this thesis: "*Pseudomonas aeruginosa* in the home environment of newly infected cystic fibrosis patients". We demonstrated that *P. aeruginosa* is not widely present in the homes of CF patients: *P. aeruginosa* was detected in only 6% of the samples, originating from 36% of the houses. In nine patients (18%), the genotype of the environmental *P. aeruginosa* isolate was identical to that of the patient's isolate, suggesting possible environment-to-patient transmission. It remains debatable however, whether the environmental isolates are the source of infection or conversely, whether contamination of the environment was caused by the patient. In our study, 72% of the environmental *P. aeruginosa* isolates were detected in the drains of the bathroom. Therefore, bathroom drains should be targeted in the preventive cleaning procedures. These results indicate that the home environment is not a major source of *P. aeruginosa* infection in CF patients and as a consequence, the exact source still seems to remain unknown at first sight. Based on this study and on literature data however, we can conclude that probably no major source of acquisition of *P. aeruginosa* for CF patients exists. Some patients acquire it from the environment, while others contract *P. aeruginosa* through patient contact. The latter can be avoided by patient segregation but as *P. aeruginosa* is ubiquitously present in nature, acquisition in most patients seems unavoidable. Trying to keep away from all possible sources puts a high psychological and social burden on the patients. Therefore, too strict and poorly validated hygienic guidelines should be avoided.

Further investigations should focus on determining factors facilitating acquisition of *P aeruginosa* in CF patients.

After the first ever isolation of *P. aeruginosa*, patients may go through different episodes of colonization, preceding chronic colonization by months to years, eventually resulting in chronic infection. Early treatment for *P. aeruginosa* colonization/infection episodes is now considered the standard of care in the CF centres. Although the effect of eradication treatment regimens on the deferral of chronic colonization CF patients has been reported, longitudinal data on the genotype of *P. aeruginosa* isolates after initial colonization and eradication treatment; i.e. the efficacy of the eradication therapy are limited. We analyzed our data in "Genotype based evaluation of *Pseudomonas aeruginosa* eradication treatment success in cystic fibrosis patients" and demonstrated that patients becoming chronically colonized have a significantly shorter *P. aeruginosa* free interval between the first ever and second isolation event and that the detection of an identical *P. aeruginosa* genotype in the second isolate is an ominous sign for impending chronic colonization. In our study, a surprisingly high number of second *P. aeruginosa* isolates identical to the first ever isolate (i.e. 68%) was seen, whereas the two other and smaller studies revealed identical genotypes of *P. aeruginosa* isolates compared to the first ever isolate in just about 25% of the episodes. Our results indicate treatment failure, with merely suppressing but not really eradicating *P. aeruginosa* after an eradication therapy. More studies are needed to determine the optimal treatment regimen and duration of eradication treatment.

Early detection of *P. aeruginosa* in CF patients is of utmost importance, as early initiated therapy increases the chances of eradication, postponing the evolution to chronic infection with *P. aeruginosa*. Culture of BAL fluid is considered the gold standard for diagnosis of lower airway infection by *P. aeruginosa*, but is too invasive for regular use. OP cultures have a low sensitivity for detection of lower airway *P. aeruginosa*. The results of the studies on the value of *P. aeruginosa* serology in the early detection of *P. aeruginosa* indicate that positive serology can precede the isolation of *P. aeruginosa* by various time periods, but that this phenomenon is not observed in all patients. It is clear that more sensitive diagnostic techniques for early detection of *P. aeruginosa* in CF patients not chronically colonized by this pathogen are needed. Therefore, we were interested whether PCR based detection techniques can advance the diagnosis of *P. aeruginosa* colonization, allowing earlier initiation of *P. aeruginosa* eradication regimen and this resulted in a third study: **"**Comparison of culture and qPCR for the detection of *P. aeruginosa* in not chronically colonized cystic fibrosis patients. Our results indicate that there is a good concordance between the results of conventional culture and qPCR for detection of *P. aeruginosa* in the vast majority of the samples but that PCR positivity may have a predictive value for impending infection in only a limited number of patients. These results do not confirm the results of the two other reports on the value of PCR for early detection of *P. aeruginosa*, with respectively in 50% and 32.5% of the samples identified as PCR positive/culture negative for *P. aeruginosa*, a subsequent patient sample positive for *P. aeruginosa* culture and PCR, suggesting that PCR has the potential to advance the diagnosis of impending infection with *P. aeruginosa*. Relying on our research and on current literature data, neither PCR nor *P. aeruginosa* serology alone can predict impending infection but any positive test can heighten the awareness. The ideal combination of diagnostic tests for early detection of *P. aeruginosa* in not chronically colonized CF patients is still to be determined.

Chapter VII: References

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