Chapter 2
Literature review

Literature review

2.1 Cystic fibrosis

Cystic Fibrosis (CF) is a life threatening deadly disease, most commonly found in the Caucasian population. Worldwide it is estimated that around 1,00,000 to 7,00,000 people are affected by CF, but it is difficult to state an accurate figure, as people in countries without developed healthcare may die before diagnosis of the disease. There are around 30,000 people with CF in USA and EU, and over 7,500 in UK. In India alone, over 80,000 people living with CF are being estimated. Around 1 in 25 of the Caucasian population carries the faulty gene responsible for causing CF (1). The frequency of common mutation ΔF508 in Indian children is between 19% and 34% and the average life expectancy is between 35 to 40 years (2).

Cystic fibrosis is an autosomal recessive genetic disorder, caused by mutation in a single gene located on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR).The most common mutation is the ΔF508 mutation, which occurs on the surface of nucleotide-binding domain 1 (NBD-1) (3, 4). This mutation leads to dysfunction of the apical membrane CFTR protein, which regulates chloride and sodium transport in secretory epithelial cells, thus resulting in abnormal ion concentrations across the apical membranes of these cells (3). Under normal conditions, sodium (followed by chloride counter-ion) is avidly reabsorbed, primarily through apical sodium channels and CFTR. The absence of functioning CFTR in patients with CF causes abnormal electrolyte transport, leading to the production of an abnormally viscous secretion and reduction in mucociliary clearance (4). The clearance of microorganisms in the respiratory tract is reduced due to increased viscosity of mucus and the chronic bacterial infections result in inflammation of lung tissue and obstructed airways. Pseudomonas aeruginosa infection is the most common bacterial infection, playing the greatest role in morbidity and mortality (5).

More than 1900 CFTR mutations causing dysfunction of the CFTR protein and resulting in a cystic fibrosis phenotype have been identified. There is a classic recessive inheritance pattern found in the disease; i.e., a pathological CFTR mutation on each chromosome must be present in an individual to develop the disease phenotype. There are six classes of CFTR mutations based on their effect on CFTR protein production, trafficking, function, or stability (Figure 2.1) (6). Class I mutations are mostly non-sense mutations causing premature stop codons, with no functional CFTR protein being produced, and leading to production of truncated unstable RNA

(eg, Gly542X, Trp1282X, and Arg553X) (7). Canonical splice mutations and chromosomal deletions are amongst the other class I mutations which also result in no functional protein being made (621+1G→T and CFTR del 2, 3) (7). The commonest CFTR mutation type are the Class II mutations, which - often because of protein misfolding—prevent the correct trafficking of CFTR from the cell surface after production due to which , minimum functional CFTR reaches the apical membrane. The most common class II mutation is, a three-base pair deletion (Phe508del) that causes a single amino acid deletion from CFTR, subsequent protein misfolding and failure of the Phe508del–CFTR to be trafficked to the cell surface. Almost 90% of individuals worldwide with CF have this mutation on at least one CFTR gene, and roughly 50% are homozygous for two Phe508del mutations. Other common class II mutations include Asn1303Lys, Ile507del, Arg560Thr, and Gly85Glu (7). In classes III and IV mutations the CFTR reaches the cell surface, but prevent the channel from working correctly. Class III mutations often called gating mutations (as the channel is present at the cell surface which is rarely open), lead to a substantial reduction in CFTR chloride channel opening time. Gly551Asp is the most common class III mutation (7). Class IV mutations also result in the surface occurrence of CFTR protein but the channel function is reduced even when open. Examples of class IV mutations are Arg117His and Arg347Pro (7). In class V mutations, overall CFTR function is inadequate because of reduced amount of normal CFTR at the surface. Class V mutations are most commonly intron mutations, which by affecting splicing, reduce the efficiency of CFTR production. (e.g. 3849 + 10kbC→T and 2789 + 5G→A) (7). Class VI mutations are the rare ones, but which due to the decreased stability of mature CFTR at the cell membrane, result in reduced amounts of functional CFTR at the cell surface (4326delTC).

In prior decades, CF was predominantly a disease of childhood, whereas recent decades have seen the median survival climb from approximately 27 years in 1986 to approximately 37 years as of 2009 (8). Multiple factors, such as the development of pancreatic enzyme replacement therapies, better and more aggressive antibiotic therapies, nutritional support, and the development of healthcare teams and CF centers dedicated to the care of large population of patients with the disease, from birth to late adulthood, have made significant improvement over several decades since the 1950s. There is a paradigm shift over past few decades due to this change in patient spectrum, with 47% of patients being adults (>18 years), requiring the training of pulmonologists with an expertise in CF, and the development of adult CF healthcare teams.

The demo-graphics of these patients is tracked by cystic fibrosis foundation (CFF) through a national registry, similar to other countries. In US, 2009 a total of 33% adults with CF were college graduates, 35% were full-time employees, and 39% were either married or living with a life partner. Although the data generated by the CFF database are largely descriptive, there has been much work in recent years attempting to identify more specifically the various factors influencing the disease expression and survival. The infectious complications are an important factor in the long term disease severity.

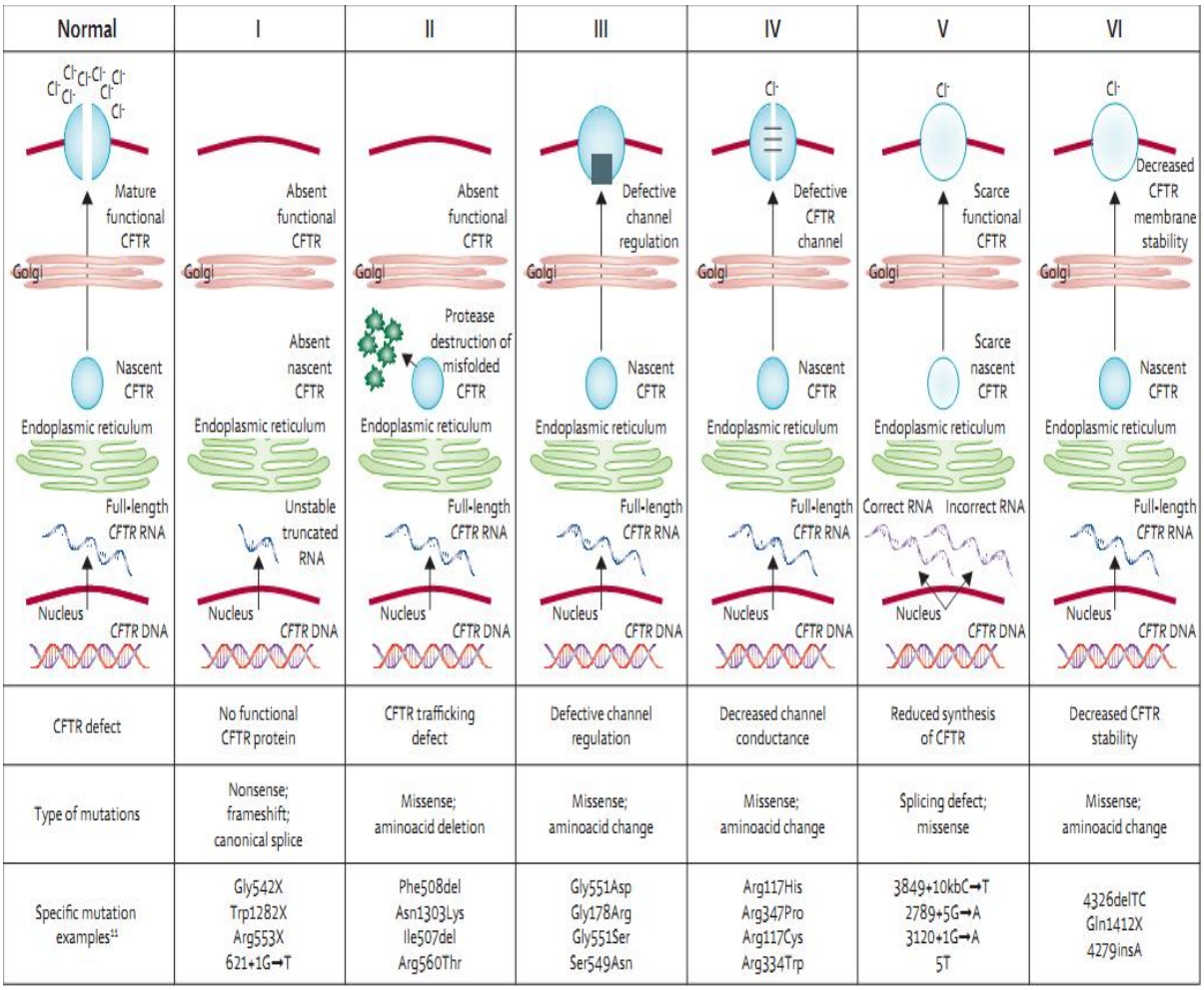

Figure 2.1: Various classes of CFTR mutations (9).

Thus, the severity and progression of disease is influenced by the acquisition of different infectious organisms. For example, acquisition of *P. aeruginosa* early in life is responsible for accelerated decline in lung function and increased mortality (10). Early acquisition of the bacteria in turn relate to several factors, such as use of anti-staphylococcal antibiotics, infectious contacts, hospitalizations due to viral infections, and use of nebulizers (11). This not only shows

the complexity of the relationships, but also highlights points at which intervention might make a long-term difference. There are emerging data about Stenotrophomonas maltophilia leading to worse outcomes, specifically FEV1 and the risk of pulmonary exacerbation (12). The airway fungal organisms specifically Aspergillus fumigatus, may also be deleterious, with various studies showing them to lower lung function (with *P. aeruginosa*), leading to hospitalizations, and use of inhaled antibiotics (13). Finally, non tuberculous mycobacteria (NTM), especially Mycobacterium abscessus, have been linked to the decline in lung function, which is not unexpected given the difficulty this organism can pose clinically (14). Thus, it is clear from multiple studies that the presence of a variety of organisms in the lung may worsen the disease condition. Age at diagnosis is an interesting issue to study, because newborn screening has recently been instituted throughout the United States and in other countries. The severity of disease in children is influenced by factors such as air pollution, ozone, and tobacco smoke exposure. Gender is also related to severity of disease; with females generally doing worse, at least when diagnosed early in life, possibly due to hormonal influences, and other inherent differences in the genders (15). Although most patients are white, minority patients, such as Hispanic and African Americans, report worse emotional and social functioning, which in turn might have a bearing on medical outcomes.

2.2 Pathophysiology of cystic fibrosis

Since the discovery of the CFTR gene in 1989, major efforts have been directed to explain the relationship between alterations in the epithelial ion transport and the development of airway inflammation and infection in the lung. Although the structure and function of the CFTR protein were uncertain before cloning of the gene, characterization of the protein rapidly followed. CFTR belongs to the adenosine triphosphate (ATP)-binding cassette transporter protein family, which regulates transmembrane transport of small molecules. CFTR expression is found in the apical region of the airway epithelial cells plasma membrane and in the serous cells of submucosal glands of the lungs. CFTR is known to work as a regulated chloride (Cl^-) and bicarbonate channel, affecting the activity of other plasma membrane channels (e.g. the epithelial sodium channel [ENaC]) too. Although the structure, function, biosynthetic processing, intracellular trafficking, and five classes of gene mutations of the CFTR protein have been extensively described, there is a big challenge to understand how the absence of functional CFTR protein translates into the spectrum of the CF disease phenotype (16).

2.2.1 Airway surface liquid (ASL) and Ion Transport Defects

Since the ion transport defects in CF were described in the early 1980s, it had long been believed that the abnormalities in salt and water transport across airway epithelia, with disruption in mucociliary clearance, resulted in diseased condition. What was more difficult was the proof of concept. In the late 1990s, two opposing theories arose to explain the development of lung disease. The first theory suggested that a high salt content on airway surfaces led to inactivation of airway defensins (broad-spectrum anti-microbial peptides), making susceptible to chronic infection (17). An alternate hypothesis suggested involvement of ion transport defects in depletion of ASL, resulting in a significant reduction in mucociliary transport, hence leading to a major breach in airway host defense at mechanical level (18). The major defense mechanism of the airways against inhaled microorganisms, is the mechanical clearance of mucus. ASL, a two-layer liquid system, is known to mediate the normal mucociliary clearance. Mucus layer, which is the upper phase is composed of high molecular weight secreted mucins, and the lower layer is known as the periciliary liquid layer (PCL). Effective ciliary function and airway clearance is facilitated by the interaction between these two layers. Adequate hydration of this airway surface fluid is dependent on the balance between sodium (Na^+) absorption by ENaC, and Cl^- secretion by CFTR, augmented by non-CFTR chloride channels. In this way, ion transport is found to exert osmotic forces regulating the water content of ASL, more specifically the height of the layer, which seems to be critical for function of the entire system.

In CF airways, there is an excessive Na^+ reabsorption due to abnormal CFTR function, with failure of Cl^- secretion, leading to a reduction in salt mass on the airway. Osmotic forces are in favor of less water moving into the airway lumen, hence the term “dehydration hypothesis” for this model concept. The PCL water content depletion makes this layer collapse and lose its lubricant activity that separates the mucus layer from epithelial cells, produces adhesion of mucus to airway surfaces, impaires airway clearance of thick mucus secretions, and facilitates the colonization with respiratory pathogens, thus generating chronic infection and inflammation of the airways (19). Although studies of ASL *in-vivo* are technically challenging, much work has been performed in the past several years to lend credence to the dehydration hypothesis, including studies of the composition of ASL (non-CF and CF are both isotonic), the height of the ASL layer (reduced in CF vs. normal), and studies *in-vitro* and *in-vivo* where addition of an osmotic load to the airway restores normal ASL height by “hydrating” the airway, which can

temporarily at least improve mucociliary clearance (20). Alternative (non-CFTR) chloride transport channels in CF have also been described, which further complicate the matter. CFTR and the calcium-activated chloride conductance channel (CaCC), are the two chloride secreting pathways in respiratory airways (19).

2.2.2 Human Cell-Culture Models

Given the complexity of airway structures and the very shallow depth of ASL (~25 mm) and PCL (~7 mm), the measures of ASL physiology are very difficult in humans. However, much information has been provided about the bioelectric and fluid transport properties of airway epithelial cells, and the interactions with the ASL volume, due to the development of human cultured bronchial epithelial cells. Different methods have been developed to measure the composition and properties of airway gland secretion in human bronchial–tracheal cells, and submucosal gland secretions, in non-CF airway human cell cultures compared with CF (20). Most work over the past several years using these model systems indicate that CFTR is predominantly expressed in ciliated cells of airway epithelia and in submucosal glands. The regulation of ASL volume is also influenced by other mediators' actions on ENaC and other chloride channels, such as proteases and protease inhibitors, which further complicates the matter. The effectiveness of alternative secretory channel pathways in modulating the ASL secretion in CF is still under investigation. The CaCC, an alternative ATP-activated chloride channel, contributes to the mucociliary clearance (MCC) and is positively affected by shear stress conditions (by ATP-mediated shear stress signaling). Studies in human epithelial cells have demonstrated that chloride secretion mediated by CaCC leads to the activation of P2Y2 receptors, located in the apical membrane of airway epithelial cells and are activated by ATP, rising intracellular calcium concentrations, and enhancing chloride secretion. Other proteins (e.g. TMEM16A), have been identified in the calcium-dependent chloride transport arena and are under further research (21). Production of mucus with decreased MCC, is also a hallmark of CF airway disease. Mucins, the major macro-components of normal mucus, are high-molecular-weight glycoconjugates, secreted by the superficial epithelial cells and the submucosal glands of the respiratory airways, providing viscoelastic properties to the airway mucus secretions. Similar mucin composition has been found in human and CF airway epithelial cell culture models.

However, there is an overproduction and hyper-secretion of mucin in the CF epithelial cultures in response to chronic infection and inflammation (22).

In addition, there is a link between defective bicarbonate secretion and abnormal mucus hydration. It is evident from the experiments in cultured-airway cells that abnormal bicarbonate secretion leads to decreased mucin expansion and hydration. Bicarbonate was found to decrease calcium cross-linking in mucins, reducing mucin viscosity and mucus production in dehydrated airway. Impairment in bicarbonate secretion has been reported in many organs in CF; suggesting that not only the CFTR-dependent electrolyte abnormalities are implicated in the pathogenesis of CF, but also the diminished epithelial bicarbonate transport and abnormalities of mucus biology may play an important role in mucus formation and development of CF disease (23). Finally, the role of endoplasmic reticulum stress and the intracellular calcium mobilization in the CF airway epithelia is demonstrated in cultured CF bronchial epithelial cells. Intracellular calcium mobilization has been implicated in regulating airway defense functions such as calcium-dependent chloride secretion, ciliary activity, mucin secretion, and inflammatory responses. It seems that the up-regulation of pro-inflammatory pathways are a key component in CF airway inflammation and there still remains a debate on whether the increased calcium mobilization is triggered by chronic infection, or is an intrinsic property of the CF epithelial cells. The unfolded protein response is a form of endoplasmic reticulum stress, which is involved in the calcium-mediated amplification of the airway inflammatory response (24).

2.2.3 Animal Models

The early development of CF animal models, initially the mouse model, together with the more recent development of pig and ferret models, have given opportunities for a better understanding of CF pathophysiology (25). Other non-CF models (e.g. the transgenic mouse model with airway-specific over expression of βENaC) have also shed insights into normal and abnormal airway ion transport and its contribution to health and disease, at an *in-vivo* level. Studies in mice with mutant CFTR protein showed that there is a difference between the ion transport abnormalities as seen in the airways of animals and in humans with CF. Furthermore, the phenotypic manifestations are different: the CF mouse model does not develop significant lung disease, but tends to show early mortality with gastrointestinal disease. Because of these research limitations, Lobo et al have been generated new animal models having specific characteristics of human CF, or CF-like disease. In the transgenic bENaC mouse, for example, the β-subunit of the

epithelial sodium channel was overexpressed, in order to determine how the various ion transport activities (Na^+ vs. Cl^-) relate to disease pathophysiology (26). This model exhibits increased epithelial Na^+ absorption, similar to CF, causing ASL volume depletion and reduced mucociliary clearance. These changes resulted in airway mucus obstruction, goblet cell metaplasia, mucus hyper-secretion with chronic airway inflammation, reduced clearance of bacterial pathogens, and a high pulmonary mortality in β ENaC mice. Although not being a CFTR-knockout model, β ENaC mice address the hypothesis that Na^+ channels play a significant role in the development of CF and CF-like airway disease. Initial studies in CFTR knockout pigs demonstrated defective Cl^- epithelial transport with the subsequent development of meconium ileus, exocrine pancreatic dysfunction, and focal biliary cirrhosis, all being the clinical manifestations seen in humans with CF (27). However, there were no signs of airway inflammation, remodeling, mucus accumulation, or infection, in the lungs of new born pigs with mutated CFTR genes. However, a few hours after birth, there was rapid colonization of lungs of CF pigs, with multiple bacteria, suggesting an impairment in host defense to eradicate airway pathogens in absence of inflammation. This suggests that chronic bacterial infection precedes inflammation and initiates the progression of the CF lung disease. Moreover, there is significant reduction in size and circularity of the tracheal lumen, abnormal smooth muscle, and hypoplastic submucosal glands in the respiratory airways. These changes can be seen in both newborns and young children with CF. The progression of the CF disease in postnatal life, might be influenced by the airway obstruction. Recent data suggest that electrolyte transport in porcine CF epithelia showed reduced Cl^- and HCO_3^- transport, but no increase in Na^+ or liquid absorption in the nasal, tracheal or bronchial epithelia. The prevailing hypothesis of the specific roles of various ion transport mechanisms in the pathophysiology of CF is challenged by these findings. Similar results have been obtained in the recently created ferret CFTR-knockout model of CF (28). Sun and colleagues (28) described the phenotype of the CFTR-knockout neonatal ferrets, which shares many organ abnormalities, such as meconium ileus, pancreatic disease, liver disease, pulmonary disease, absence of the vas deferens, severe impaired nutrition, and malabsorbtion, as seen in human CF disease. Tracheal epithelia from newborn CF ferrets showed defective cAMP-induced Cl^- transport and reduced submucosal gland fluid secretion. Cultures of bronchoalveolar lavage (BAL) fluid in newborn CF ferrets show a predisposition to develop pulmonary infections soon after birth, caused by S. aureus, Streptococcus pneumonia, and enterobacteria. Outside the lung,

liver function was noted to be elevated in newborn CF ferrets that were normalized by the administration of ursodeoxycholic acid, a situation similar to humans. The CF ferret model is another useful model that may improve understanding of the pathophysiology of CF disease, because of the similarities in anatomy and physiology with the human CF disease.

2.2.4 Human Studies

Research studies in human subjects with CF have been provided an important information about the abnormalities in ion transport, mucus secretion, and MCC in the pathogenesis of CF airway disease, due to the research studies conducted in human subjects with CF. By now classic studies in the respiratory epithelia of patients with CF, found an increase in transepithelial electric potential difference, suggesting an abnormality in Na^+ and Cl^- ion transport (29). Interestingly, some years later another human disease involving Na^+ channel dysfunction shed insight into the relative contribution of this ion channel to epithelial dysfunction in human disease. Defective ENaC function was found in patients with systemic pseudo hypo-aldosteronism, with absence of Na^+ transport in the respiratory airways and increased ASL fluid content, and enhanced mucociliary clearance (30). These observations in a human disease (not CF) involving ion channels reveal crucial role of Na^+ channel in the regulation of ASL height, and the important role of ASL fluid hydration in maintaining adequate airway mucus clearance. There was a recent demonstration of the relationship between airway inflammation and worsening of lung function in the pediatric population. Neutrophilic airway inflammation present in BAL of infants with CF was associated with lower pulmonary function tests. At the same time, there was a more rapid decline in lung function due to infection with *S. aureus* and *P. aeruginosa*. These results suggest that the key pathogenic features in the development and progression of CF lung disease are the airway inflammation and infection (31). Other theories have arisen as to the development of disease. The abnormalities in the membrane lipids, have been proposed to explain the predisposition to inflammation and infection on epithelial surfaces (e.g. low linoleic acid and docosahexaenoic acid). More recently, the role of ceramides in the pathophysiology of CF lung disease has been explored. Ceramides are molecules belonging to the sphingolipid family and are an essential component of the plasma membranes, the high levels of which have been found in the airway epithelial cells of knockout mice deficient in CFTR with the subsequent development of inflammation and susceptibility to *P. aeruginosa* infection (32). However, there has been conflict in data to date, with high and low levels of ceramide found in mice and humans.

Nonetheless, the concept of ceramide in the immunomodulation of CF lung disease, as a possible therapeutic target remains under investigation.

2.2.5 Extrapulmonary CF Disease Pathophysiology

Liver disease occurs in 27% to 41% of patients with CF, with approximately 5% of patients suffering from severe disease (33). Bile duct obstruction is responsible for hepatobiliary complications of CF liver disease, resulting in periductal inflammation, bile duct proliferation, and periportal fibrosis leading to cirrhosis and portal hypertension. The CFTR protein which is localized in the apical membrane of the cholangiocytes and cholecystocytes, regulates the Cl^- , Na^+, and bicarbonate transport and the fluid content of the bile. In absence of functional CFTR, there is an abnormal secretion of Cl^- and bicarbonate, generating thick bile secretions and favoring the bile duct plugging. Recent studies in the CF mouse model showed evidence of increased bilirubin conjugates and unconjugated bilirubin, lower gallbladder bile pH levels, and elevated levels of calcium bilirubinate, all of which favour supersaturation of bile and formation of black pigment gallstones (34). Studies in the CF pig model demonstrated signs of biliary cirrhosis like , cellular inflammation, ductal hyperplasia, and fibrosis (35). There were elevations of alanine aminotransferase and bilirubin in the ferret model that normalized with ursodeoxycholic acid, similar to the response seen in infants with CF, suggesting similar pathophysiologic mechanisms. Recent studies in patients with CF with and without liver involvement suggest that CF liver disease represents a phenotype, due to altered nutrition, carrying a poorer prognosis and a higher risk of developing CF-related diabetes (36). The pathophysiology of exocrine pancreatic disease in CF is related to abnormal pancreatic secretions, decreased intraluminal bile salt and increased bile salt fecal loss, abnormal composition of bile secretions, and intestinal mucosal abnormalities, leading to chronic fat malabsorbtion, hypoalbuminemia, fat-soluble vitamin deficiency, and mal-nutrition (37). In a large retrospective cohort study, from the perspective of endocrine pancreas function, CF-related diabetes was found to be associated with a higher mortality rate in CF patients (38).

Studies in the CF knockout mouse model showed improvement in the lipolytic activity and lipid absorption after pharmacologic gastric acid reduction. In the CF pig model, severe exocrine tissue destruction was evident at birth, with the endocrine pancreatic tissue apparently intact. Relatively lower degree of exocrine pancreatic insufficiency is found in CF ferret model, with

dilation of acini and ductules and eosinophilic zymogen secretions. Of note, compared to the mouse model, the CF pig and ferret models have more similarities in the pathophysiology of the CF pancreatic disease in humans, and will likely be useful for further research to understand the mechanisms of CF pancreatic insufficiency. Difficult complications like, Meconium ileus, distal intestinal obstruction syndrome, and significant constipation are found in the gastrointestinal tract in CF (39). In the intestinal epithelia, CFTR dysfunction leads to decreased Cl^- and fluid volume secretion and increased Na_1 absorption by ENaC (followed by fluid absorption), producing more viscous intestinal contents and predisposing to obstruction (36). Meconium ileus occurs in 13% to 17% of patients with CF, with significant morbidity and occasional mortality (40). Recently new evidence of CF-related enteropathy has been described by direct observations of the intestinal mucosa in subjects with CF, showing evidence of intestinal mucosa inflammation, causing chronic intestinal malabsorbtion (41). The CF mouse, pig, and ferret models have demonstrated gastrointestinal obstructive pathology; however, the pig and ferret models exhibit more meconium ileus than in human infants with CF. Other intestinal abnormalities seen in CF disease, such as intestinal atresia, diverticulosis, and micro-colon have been found in CF pigs and ferrets as yet not commonly noted in humans with CF (42).

2.3 Bacterial infection

The mainstay in defining microbiology of CF airways is the bacterial culture from sputum and antibiotic susceptibility testing. In the adult CF population, *P. aeruginosa* is the most common pathogen cultured chronically (43). Some of the pathogenic strains of *P. aeruginosa* are hyperproducers of pyocyanin, which affects the cilia of the respiratory epithelium and induces neutrophils apoptosis. It is regulated by quorum sensing (QS), a mechanism by which individual bacteria communicate with each other, to alter gene expression in response to changes in population density by secreting molecules referred to as “auto inducers” (44). In *P. aeruginosa*, these genes encode for pathogenicity, biofilm formation, and motility. Due to this mechanism there is precise regulation of genes in response to environmental stimuli and cell density. Bacteria isolated from the chronically infected patient with CF differ from the usual *P. aeruginosa* cultured from acute infections in the non-CF population. For example, colonies with differing morphotypes, can be formed by clonal *P. aeruginosa*. Mucoid is the classic CF isolate, caused by the overproduction of alginate; coliforms, nonpigmented forms, and slow-growing small-colony variants (SCVs) are among the other morphotyes. SCVs are hydrophobic, poorly

motile, biofilm formers and are frequently missed in routine culture because of their prolonged culture time. Other phenotypes, such as type III secretors, exhibiting lipopolysaccharide modification may enhance pathogenicity and antibiotic resistance (45). Thus, susceptibility testing may be relatively inaccurate, due to wide range of *P. aeruginosa* phenotypes and morphotypes (46). Several small studies suggest improved outcomes with eradication of *P. aeruginosa* from the airways of newly or intermittently infected patients with CF. Taccetti and colleagues (47) treated 58 newly infected patients with CF with inhaled colomycin and oral ciprofloxacin for 3 weeks. Eradication was achieved in 81% of these patients and was maintained for a median of 18 months with the annual decline in FEV1 compared with well-matched controls.

Apart from *P. aeruginosa*, other pathogens like *Staphylococcus aureus* (48), *Haemophilus influenzae* (49), *Burkholderia cepacia* Specie (50) are also involved in cystic fibrosis. *P. aeroginosa* infections are the most common bacterial infections playing greatest role in morbidity and mortality in CF patients (5).

2.4 Treatment of CF

The treatment of cystic fibrosis is directed towards alleviation of symptoms and correction of organ dysfunction. The basis of treatment is the clearance of lower-airway secretions, treatment of pulmonary infections and pancreatic-enzyme replacement. With the increase in knowledge in genetics and pathophysiology of the disease, new therapeutic approaches have been emerged in recent years. The increased life expectancy in the CF population is mainly thought to be related to the development of pancreatic enzymes and antibiotics, initially and more recently to the development of a multifaceted approach to the management of CF. The median predicted survival age for people with CF has risen steadily over the last 25 years, due to improvements in treatments. Since 2002, the median predicted survival age has increased by 10 years- from age 31.3 in 2002 to age 41.1 in 2012. For period of 2008-2012, the median predicted survival was 37.8 years (51).

2.4.1 Ion Transport Therapies

Defective Cl^- secretion and Na^+ hyperabsorption lead to airway dehydration with impairment of mucociliary transport. Airway hydration therapies include those activating Cl^- secretion by non–CFTR-dependent pathways, inhibiting Na^+ absorption, or adding osmotic agents directly to the airway surface. Hypothetically, these therapies alone or in combination should enhance

mucociliary clearance of bacteria, mucus, and inflammatory products, with a hope to preserve lung function (52). Osmotic agents may also enhance ciliary beat frequency and cough clearance. Inhaled hypertonic saline (7%), is the best known and widely used class of this therapy (53). Twice daily hypertonic saline, has been shown to improve mucociliary clearance and lung function and is currently used in pediatric and adult patients with CF. Inhaled mannitol, another osmotic agent, was shown to improve FEV1 over a 2-week period in phase II trials and is currently under study in CF and non-CF bronchiectasis (54). Denufosol tetrasodium, a puridinine triphosphate derivative and P2Y2 receptor agonist, activates alternate chloride channels in the respiratory epithelium. Initial studies using aerosolized denufosol showed significant differences in FEV1 compared with placebo controls, but follow-up studies failed to show an improvement in the primary outcome of lung function thus dropping the compound from further study (55).

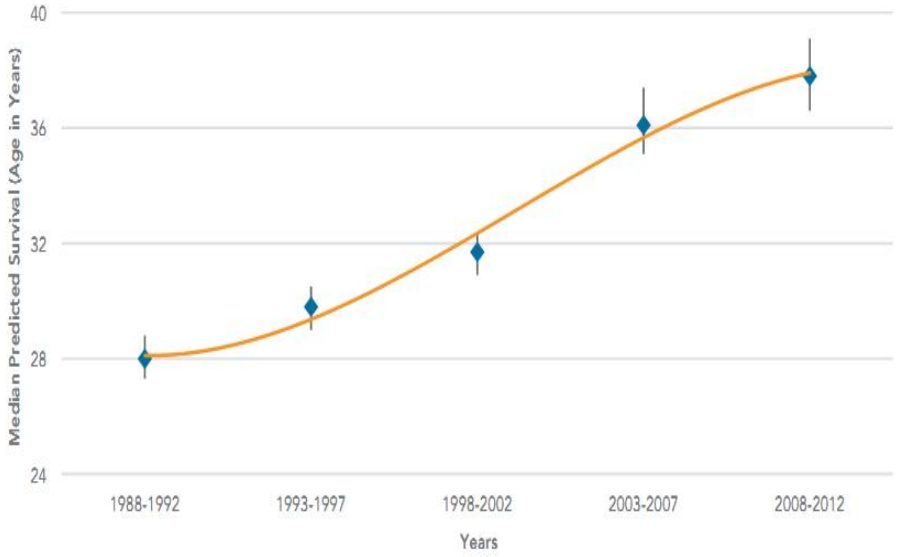

Figure 2.2: Median survival age in cystic fibrosis from 1988 until 2012. The 95% confidence intervals for the survival estimate are denoted by the vertical bars (51)

2.4.2 Antiinflammatory therapies

Standard anti-inflammatory regimen for patients with CF is not available. Initially oral glucocorticoids were used, but adverse effects outweigh any potential benefits for chronic therapy. The macrolide therapy significantly improved the survival of patients with diffuse panbronchiolitis, a lung disease quite similar to CF but unaccompanied by multi-organ involvement, has led to the widespread use of azithromycin in CF patients (56). *P. aeruginosa*

inter-communicate for biofilm formation via a system of lactones, a process known as quorum sensing. Macrolides inhibit mobility of *P. aeruginosa* and quorum sensing. They decrease mucus production by epithelial cells and pro-inflammatory cytokine biosynthesis by monocytes and epithelial cells (57).

2.4.3 Antimicrobial Drugs

Treating infection in the CF airways is a standard therapy; oral and intravenous regimens are considered standard of care, and the advent of implantable devices has continued to improve making this a safe, feasible option for hospital and at home (58). Inhaled antibiotics offer an alternative method of delivery that delivers high concentrations of drug to the site of infection, while minimizing systemic exposure and toxicity. Current inhaled agents target *P. aeruginosa*, which is the most common pathogenic microbe in the adult airway. There are new innovative antibiotic formulations, such as dry powder formulations and lipid nanoparticle aerosols, and there is improvement in delivery devices also. The first of this class to be studied and approved for use in CF was inhaled tobramycin (300 mg/mL). It improved FEV 1, decrease P aeruginosa density, and decrease hospitalization rates. More recent development is the inhaled aztreonam, and initial trials have shown that patients with P aeruginosa with moderate to severe lung disease improved respiratory symptoms and FEV1 (59). Another inhaled antibiotic, colistin, has long been available but is not studied well till date in formal double-blind controlled trials. Inhaled antibiotics also under study include amikacin, levofloxacin, ciprofloxacin, and a fosfomycin–tobramycin combination. Inhaled amikacin is a liposomal nanoparticle being developed as a once-daily medication, having ability to penetrate into the bacterial biofilm. Recent phase II trials have shown a significant improvement in FEV 1, *P. aeruginosa* density, and reduction in sputum production. Inhaled ciprofloxacin is formulated as a dry powder, with obvious advantages in delivery and ease of use and portability. The ELITE study showed that a 28-day regimen of inhaled tobramycin 300 mg/ml is effective in treating early P aeruginosa infections (60). Treatments aimed to eradicate MRSA should also be considered after first isolation.

Thus, the administration of antibiotics by inhalation is an attractive alternative, delivering high concentrations of antibiotic directly to the site of infection while minimizing systemic bioavailability. Pulmonary administration of various antibiotics has been found to improve lung function in CF patients with chronic pulmonary pseudomonas aeruginosa infection and to reduce

the frequency of hospitalisation. When given by inhalation, the antibiotic is delivered directly to the target organ, increasing the therapeutic index of the drug (61). Inhalation is recommended in the European Consensus document on antibiotic treatment against *P. aeruginosa* (62).

2.4.4 Airway Clearance

Chest physiotherapy has been the chief non-pharmacologic approach in enhancing clearance of pulmonary secretions. Standard chest physiotherapy involves postural drainage with chest percussion in several anatomic positions to favour gravitational clearance of secretions from all lobes of the lung. The daily maintenance chest physiotherapy is believed by many clinicians and patients, to have long term benefits in improving airway clearance (63). Further, mucolytic reduce the abnormal viscosity of airway secretions in patients with cystic fibrosis. A purified recombinant human deoxyribonuclease I (rhDNase I) (Pulmozyme) that can digest extracellular DNA reduces the viscoelasticity of sputum specimens from patients with cystic fibrosis. Classic mucolytic such as N-acetylcysteine or ambroxol have minor effect on lung disease in CF patients (64). Moreover, the efficacy and safety of N-acetylcysteine at high doses has not been established. Therapy with β-adrenergic bronchodilators like β2 adrenergic agonists, anticholinergics and corticosteroids improved pulmonary function in patients hospitalized for exacerbations of pulmonary infections (65).

2.4.5 Lung transplantation

Double lung or heart-lung transplantation is a treatment option for patients with cystic fibrosis and end-stage lung disease. Compared to other organ transplantations, the overall survival of lung-transplant patients is poorer , with 3-year survival of about 60% in CF patients (66).

2.4.6 Pancreatic enzyme replacements

Poor nutritional status has been linked to worsen the prognosis of CF patients (66). Introduction of enteric-coated microencapsulated enzymes (Creon®, Solvay Pharma) has resulted in great improvement in the weight of CF patients. As patients with pancreatic insufficiency are prone to malabsorption of the fat-soluble vitamins (i.e., A, D, E and K), a supplementation of these vitamins is often given to CF patients (65).

2.4.7 Molecular Therapies

The possibility of gene targeted therapy emerged as early as 1989, when the genetic basis for the disease was firstly described. However, the reality of the challenges involved in successful gene transfer in CF, tempered the early excitement. Initially, viral vectors using adenoviruses and

adeno associated viruses were tried but were unsuccessful, due to adverse events (67). In the UK Cystic Fibrosis Gene Therapy Consortium, a plasmid-DNA and non-viral vector approach is presently been studied and phase 1 trials have been started. Other new therapies affecting CFTR transcription, processing, or functioning are in the development pipeline. These molecules may be ingested orally, and range from agents that allow the ribosome to read through premature stop codons in CFTR mRNA, "correctors" that help CFTR fold properly, and "potentiators" that increase chloride channel activity at the cell surface. CF_{508}, the most common CF mutation, is a class II mutation, in which the misfolded CFTR is degraded before reaching the apical cell membrane. However, the misfolded protein retains enough function that if guided to the apical membrane, it could show a clinical impact. VX-809 is a possible corrector assisting in the delivery of CFTR to the airway apical epithelium. Current phase II studies in homozygote D F508 patients show promising results with significant improvement in sweat chloride levels. 10 % of CF mutations are class I nonsense mutations that lead to premature termination of mRNA translation and result in an abridged, nonfunctional CFTR protein. PTC124 is a small molecule, found to be safe and well tolerated in phase I trial, that allows ribosomal read through of premature stop codons and formation of a functional CFTR protein. Phase II studies in adults with CF showed enhancement in Cl^- efflux and a large phase III study aimed at FEV1 is currently underway (68). Class III mutations are associated with reduced opening of the CFTR channel and thus a reduction in Cl^- movement into the airway lumen. Potentiator therapy directed towards these mutations, increases the function of a correctly placed CFTR, and a current potentiator, VX-770, and is currently in phase II studies in patients with G551D mutations. These trials have shown improvement in nasal potential differences and, more importantly, in the lung function parameters (69). There are also interesting studies examining the combination of potentiators and correctors, specifically VX-770 and VX-809 in DF508 homozygote patients with CF (70).

2.4.8 Gene therapy

Cystic fibrosis was one of the first diseases considered for gene therapy (71), and most efforts are directed on transferring the normal CFTR cDNA. Recently, antisense or RNA interference-mediated gene silencing has also been pro-posed (71). Cellular uptake of nucleic acids is hindered by their size, charge and extra cellular instability. Therefore, genetic material is delivered by means of virus derived vectors or as synthetic polycations complexes. Bronchiolar

epithelium being the main target of gene delivery , most formulations are delivered directly to the airways as aerosols (72). Several exploratory and clinical studies have been performed as summarized below, but presently no gene delivery system is available clinically.

2.4.8.1 CFTR cDNA delivery

Modified viruses have been widely exploited for CF gene delivery. Although viral vectors are relatively efficient, they are difficult to produce on a large scale, and may induce a potent immune reponse and resistance on repeated administrations. A major setback to the viral vector-based gene therapy was the death of a patient receiving adenovirus-based gene therapy in the 1999, during trial of gene therapy for ornithine transcarbamylase deficiency (non-CF related) (73) (74). Non-viral vectors based on cationic lipids and polymers have been developed as a relatively safe alternative, as they are less immunogenic, easier to modify, and can be mass-produced. Since the initial isolation and cloning of the CFTR gene, 25 Phase I/II clinical trials involving ~ 400 CF patients have been carried out using a variety of viral and non-viral vectors (71, 75). Most of the trials were done using adenovirus, which showed significant adverse effects such as inflammatory response, radiographic pulmonary infiltrates and development of humoral immunity (76). Although the use of the adeno-associated virus was better tolerated, the resulting efficiency of gene delivery was unsatisfactory (77).

2.4.8.2 RNA interference-mediated gene silencing

More genetic targets are emerging as alternative therapeutic options, with the recent advances in RNA interference technology. The inhibition targets include: NF- k B, a transcription factor regulating the expression of pro-inflammatory cytokines (78); B-cell antigen receptor-associated proteins (BAPs), inhibits the normal trafficking of CFTR protein (79); epithelial Na^+ channel (ENaC) (71), is the one whose over-expression is linked to the CF lung disease (80); and valosin-containing protein (VCP), which complexes with the CFTR protein during translocation from the endoplasmic reticulum and facilitates its cytosolic degradation (81). Inhibition of VCP by small interfering RNA (siRNA) results in partial rescue of functional Cl^- channels to the cell surface, improving secretion and decreasing the level of the inflammatory marker, interleukin-8 in the primary CF tracheal cell culture model (81). Suppressing the production of BAP31 protein p also restores Cl^- secretion in various cell types (79). Although RNA interference is a promising therapeutic option for CF therapy, several challenges are faced by the cellular delivery of siRNA. SiRNA is more prone to degradation by serum nucleases than DNA (82), owing to the extra

hydroxyl group in the ribose backbone. Moreover, a compact complex formation between siRNA and a cationic non- viral vector is difficult owing to the small size, low charge density and stiffness of the siRNA (82, 83). Recently this problem has been overcome by forming multimerized siRNAs, which can be condensed with conventional gene carriers and cleaved into monomeric siRNA when taken up by cells (84).

2.5 Challenges in drug delivery to the cystic fibrotic lungs

Airways being a major therapeutic target in CF, many drugs are delivered via inhalation. This mode of delivery ensures deposition of medications at the site of action, increasing their local availability and decreasing their systemic absorption and thus the side effects. Presently, tobramycin (TOBI, Basel, Switzerland) and DNase (Pulmozyme) are available in the US as nebulized solutions. Inhalable dry powders of netilmicin (85), gentamicin (86), tobramycin (87) and colistin (88) have been studied in clinical trials. Inhalable dry powder of tobramycin (89) is expected to be soon in the US market. On the other hand, several challenges remain to be overcomed for efficient inhalational drug delivery to the CF lungs. For example, the tenacious CF sputum functions both as a physical and a chemical barrier to drug delivery into and/or across the sputum. A framework is also presented by CF sputum for development of bacterial resistance. On the other hand, concomitant uses of mucus thinning agents are known to enhance the mucociliary clearance of inhaled medications. Although not discussed in this review, several more challenges such as stability and aerodynamic properties of the formulation further complicate inhalational drug delivery to CF lungs. Moreover, the effectiveness of inhaler device, critically determines the performance of an inhalable drug delivery system. Several efforts are continued to improve the available inhaler devices (90).

2.5.1 Cystic fibrosis sputum

The thick, tenacious CF sputum presents a significant challenge for effective inhalational delivery of many therapeutic agents (91). It is important in inhalational delivery of antibiotics that the drug penetrates the sputum and evenly distributes within the sputum. Also, for the delivery of drugs influencing the epithelium, such as ion-channel regulators or gene therapeutics, it is crucial to traverse the sputum and deliver the drug payload to the underlying cell layer. CF sputum is mainly responsible for the failure of CF gene therapy in the past 15 years of pre-clinical and clinical research (92).

2.5.1.1 Physical barrier

Normal mucus in the trachea has thickness of 10 - 30 μm, and 2-5 μm in the bronchi. Whereas gas, ions, nutrients and proteins easily diffuse through mucus, particulate substances can be entrapped and immobilized by the mucus and removed before they contact the underlying epithelial cells (93). In this manner, mucus protects the body from invasion of foreign substances such as toxins, pathogens and environmental ultrafine particles. A typical mucus sample contains 90 - 95% of water by mass (94). The remaining mass is consisted of mucins (~ 2%), DNA, lipids, electrolytes, proteins, cells and cell debris. Mucins are high-molecular-mass glycoproteins with alternating glycosylated and cysteine rich regions (94), produced by the epithelial goblet cells and submucosal glands. Mucins are negatively charged owing to the abundant carboxyl groups at the termini of glycan and form networks via internal disulfide bonds, physical entanglement and non- covalent interactions (95). Viscoelasticity of normal mucus is mainly due to mucins (93), predominantly MU C5AC and MUC5B (22). On the other hand, there is less water (90%) contained in CF sputum. Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung and intact mucins (22) and more DNA and actin, secreted by necrotic neutrophils, epithelial cells and pathogens in the course of chronic inflammation (92). DNA and actin co-polymerize to form a polymer network, thus increasing the viscoelasticity of sputum (96). Mucin fibers are bundled together to form thick cables creating large spaces on the order of hundreds of nanometers (97). Depending on the observation methods, the spaces between mucin cables (mesh spacing) in human cervical mucus are estimated as being from 100 nm to 1000 nm. On the other hand, the local rheology of mucus at nanometer scales (micro scale rheology) is quite different from the bulk estimates (93, 98). It is indicated by the heterogeneous structure of mucus that it is possible to transport NPs through the mucus layer. However, mucus represents a significant steric barrier to most NPs, especially in the case of CF sputum, when the mesh scale is smaller than normal mucus. NPs > 500 nm are almost always immobile in CF sputum (99, 100).

2.5.1.2 Chemical and biological barrier

Owing to the negative charges and hydrophobic regions of the constituent biopolymers, mucus interacts with the charged and/or hydrophobic surfaces of NPs. That's why, the capsid virus-like particles (50 nm) without exposed hydrophobic surfaces diffuse freely through mucus, whereas hydrophobic polystyrene NPs of the same size do not (101). Moreover, antibodies and other

soluble factors in the CF sputum may function as molecular traps. For example, an adenovirus gene vector premixed with the sol phase of the CF sputum showed reduction in gene transfection efficiency due to the presence of adenovirus-specific antibodies (102). CF sputum also inhibits gene transfection by non-viral liposomal vectors by destabilizing the gene complex (103). When mixed with linear DNA, a polycations abundant in the CF sputum, the surface charge and size are drastically changed by DNA-liposome complexes (lipoplexes) and plasmid DNA is released prematurely (103).

2.5.1.3 Bacterial resistance

The tenacious, stationary CF sputum sets the stage for bacterial resistance. *P. aeruginosa* develops an impressive armamentarium of strategies in order to evade antibiotic therapies. In one of the strategies, there is a change of *P. aeruginosa* into mucoid strains forming biofilms that are resistant to phagocytosis and to penetration by antibiotics (104). In addition, the biofilm center contains very little O_2 and nutrients, which slows down the growth of the bacteria and reduces their susceptibility to some antibiotics (105). Biofilm formation is significantly enhanced by the presence of DNA and actin polymers in the CF sputum, which leads to the consideration of using anionic polymers and DNase to oppose biofilm formation (106).

2.5.1.4 Mucociliary clearance

In normal airways, the respiratory cilia transports the mucus at a rate of 2.5 - 5 mm/min (107) towards the oropharynx, where it is either swallowed or expectorated. Mucociliary clearance of mucus-trapped foreign substances is an important pulmonary defense mechanism against inhaled pathogens and particles (108). However, there lies a big challenge in delivery of drug/gene to the airway epithelia, as the delivery vehicles are cleared similarly. Sinn et al. reported that gene delivery by means of viral vectors to normal Balb/c mouse airways is significantly improved due to inhibition of the mucociliary clearance using methylcellulose gels (109); as the mucociliary clearance is reduced in CF patients (110), inhalational medication delivery in that population is less of a challenge. However, when mucus -thinning agents are co-administered to enhance transmucus diffusion of other medicines, the effect of improved mucociliary clearance should be considered.

2.5.2 Cellular challenges

2.5.2.1 Bacterial drug resistance

As mentioned, bacteria seen in the CF airway often develop antibiotic resistance, making it difficult to eradicate them. In addition to forming biofilms and developing a mucoid phenotype, drug-resistant strains of Pseudomonas lack outer membrane porins, through which some of the antibiotics would normally diffuse (111), or develop active drug-efflux machinery (112). Moreover, if treated with a single antibiotic, many Gram-negative CF pathogens may develop resistance; therefore, a combination of two or more agents of different classes is often used (113). Recent studies report the production of inhalable particles co-encapsulating multiple antibiotics, such as ciprofloxacin and ceftazidime (114) or ciprofloxacin and doxycycline (115), to assure their airway co-deposition at the intended doses. The combination particle system is a promising approach as it can treat patients harboring several types of microorganism that may not be killed by a single antibiotic (116). If a synergistic effect is shown by the antibiotics, it can also reduce the amount of particles to be inhaled.

2.5.2.2 Challenges in gene therapy

The failure of gene delivery to the airway epithelia is largely attributable to the cellular barriers. For example, apical surface glycocalyx is a significant barrier to adenovirus-mediated gene transfer (117). The sialic acid in the glycocalyx on the apical surface of airway epithelial cells interferes with gene delivery by affecting the interactions of adenovirus with its receptors (117). In addition, there is requirement of specific receptors on the surface of the epithelial cells for the uptake of viral vectors; therefore, viral gene delivery can be challenging if required receptors are unavailable (118). Moreover, repeated administration of adeovirus or adeno associated virus induces humoral and cellular immune responses to the viruses (119). Although these problems can be avoided by non-viral vectors, they still face other cellular challenges, like degradation of the genetic material during intracellular trafficking (71). Unprotected DNA, for example, is degraded in the lysosomes or by cytoplasmic nucleases. Nuclear envelope is another intracellular barrier, especially in quiescent cells, which limits the entry of exogenous DNA (120-122). Achievement of a balance between extracellular protection and intra-cellular unpacking of DNA is also important for efficient gene transfection (123).

New drug delivery approaches to treat cystic fibrosis, have been developed by researchers to overcome this challenge.

2.6 New drug delivery approaches

2.6.1 Antibiotics encapsulated Liposomal formulaions

Bacterial resistance to antibiotics has been addressed by encapsulating the antibiotics in liposomal formulations. Liposomal gentamicin shows significantly lower minimal inhibitory concentration (MIC) values against drug-resistant strains of *P. aeruginosa* than free gentamicin (124). Growth of various bacteria at concentrations equivalent to sub-MIC levels of free tobramycin is inhibited by liposomal tobramycin (125). Such enhancement of antibacterial activity is attributed in part to the ability of liposomes to penetrate the bacterial membrane (125, 126). Halwani et al. demonstrated through TEM and flow cytometry that liposomal aminoglycosides can penetrate B. cenocepacia, effectively killing highly resistant strains (127). Moreover, liposome encapsulation protects tobramycin in the polyanionic environments such as DNA, actin and bacterial endotoxin thus preserving its bioactivity (128). In animal models, locally administered liposomal antibiotics achieve a higher concentration and longer lung exposure compared to the free drug (129).

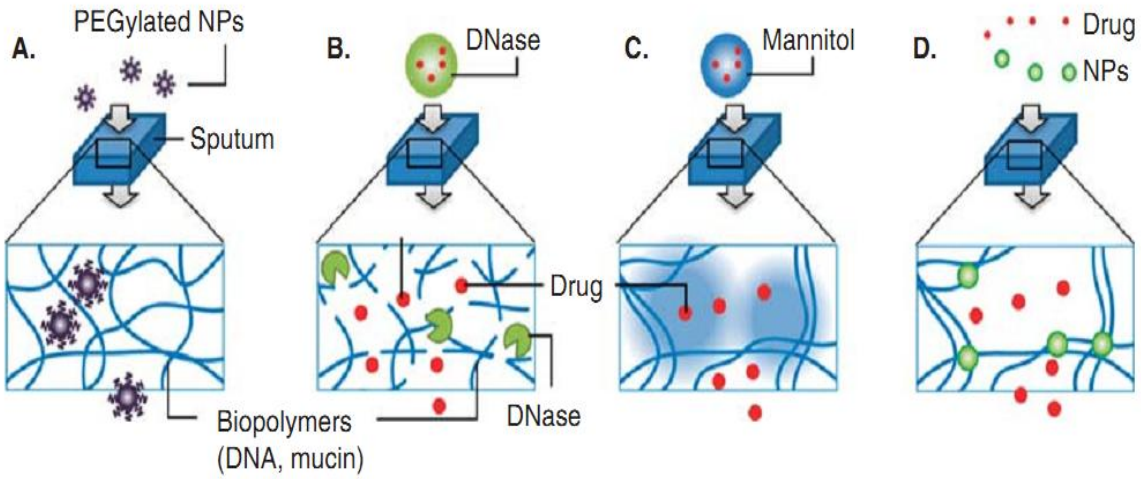

Figure 2.3: New drug delivery approaches to enhance transport of therapeutics through cystic fibrosis sputum. A) Surface modified nanoparticles; B) Co-administration with DNAse; C) Co-administration with mannitol; D) Co-treatments with drug and nanoparticles (130)

2.6.2 Application of external forces to enhance Nanoparticles penetration

The lung delivery of nanoparticles (NPs) have been improved by the magnetic forces (131). Application of external magnetic field gradients during inhalation, significantly increased the deposition of aerosol droplets containing super paramagnetic NPs in the desired lung regions of

mouse (131). This suggests that the magnetic force can aid in the diffusion of NPs through the airway mucus layer. However, scaling up the magnetic gradient field to the human scale would be challenging in clinical application of this technology. A study that used low-frequency ultra sound (20-100 kHz) has been mentioned for NPs delivery through the airway mucus (92). The transport of negatively charged polystyrene NPs (500 nm) was enhanced by 10-fold due to the application of low-frequency ultra sound. However, ultra-sound application to the lungs may not be simple owing to interference by the air in the lungs. Moreover, the duration of ultrasound application should be controlled carefully to avoid generation of excessive heat.

2.6.3 Mucolytics pretreatment

Before NP drug formulation, various mucolytic agents have been administered to reduce the steric hindrance of mucus, but there have been mixed outcomes. It has been reported that, NPs transport across a layer of isolated CF sputum is enhanced by premixing the NPs with DNase (99, 132), and adenoviral gene delivery to normal mouse airways is enhanced by pre-treatment with N-acetylcysteine (133, 134). However, pretreatment of mucous tissues with N - acetylcysteine does not improve the gene transfection efficiency in a CF mouse model (134), perhaps owing to the enhanced removal of the gene carriers secondary to the increased mobility of the mucus. In addition, free biopolymers may be released by the degrading mucus, increasing viscous drag and delaying NP diffusion (92).

2.6.4 Surface-modified nanoparticles

To reduce NPs interactions with mucus components, Hanes and co-workers proposed modification of the NPs. They have modified NPs surface with low-molecular-mass polyethylene glycol (PEG) to prevent the interactions between the NPs and the mucus components (Figure 2.3 A) (100, 135). Densely PEGylated NPs can diffuse through the cervicovaginal mucus or through the CF sputum (100, 136). The PEG molecular mass and the extent of NPs surface PEGylation are critical in controlling the mucus-NPs interactions. Polystyrene NPs densely coated with 2 or 5 kDa PEG penetrate the undiluted cervicovaginal mucus relatively faster, whereas the NPs with 10 kDa PEG do not, because of the PEG - mucin entanglement. For the NPs coated with 2 kDa PEG, a 40% decrease in PEG coverage results in a 700-fold decrease in the average transport rate within the mucus (137). When sufficiently covered with PEG, even relatively large NPs (500 nm) diffuse through the cervicovaginal mucus layer. The diffusion coefficient of PEGylated 500nm NPs in mucus is only 4 times lower than

that in water, and ~ 70% of them are mobile in the mucus, whereas 45% of uncoated NPs of the same size remain immobile. On the other hand, when the surface is not sufficiently PEGylated, smaller NPs (100 nm) cannot diffuse as effectively as larger PEGylated NPs (138). These studies depicts that for the NPs smaller than a certain limits, the interactions between them and the biomolecules in the mucus are the main hurdles to their migration through the mucus (97). PEGylated NPs (200nm) move through undiluted CF sputum at an average speed 90-fold higher than uncoated particles. However, the movement of the 500 nm NPs is significantly hindered, irrespective of the PEG surface (100). The PEGylation NPs also protects them to aggregate and less likely to be taken up by alveolar macrophages. PEGylation protects the lipoplex from destabilization and loss of transfection activity owing to the anionic environment (139). Recently, PEGylated NPs have been used to deliver PS- 341, a D F508-CFTR corrector and chronic inflammation inhibitor, to the lungs of CF mice (140). It has been also reported that, the PEGylated surface can interfere with the NPs - cell interactions and the efficient cellular entry by NPs (141), and can decrease endosomal escape (92). Anionic polymers are often used for cationic non-viral vectors, to mask the surface charge and reduce the interaction with the anionic environment. Hyaluronic acid (142), alginic acid (143) and poly(propylacrylic acid) (144) have been shown to protect the gene carriers from anionic proteins and preserve their ability to transfect cells. The same principle may be applicable for overcoming the interaction between the gene carriers and the biopolymer network in the mucus or sputum.

2.6.5 Co-administration with agents that influence the cystic fibrosis sputum

To overcome the CF sputum barrier insulating bacteria from inhaled antibiotics (106), co-administering antibiotics and agents that degrade the sputum was proposed. For example, there was an enhancement in the antipseudomonal activities of liposomal and free aminoglycosides in CF sputum by the addition of DNase and/or alginate lysate, which decreased the alginate level in the biofilm (145). An inhalable dry powder system co-delivering DNase and ciprofloxacin has been developed in order to enhance the penetration of ciprofloxacin (Figure 2.3 B) (146). These particles has been observed to decrease the viscoelasticity of the artificial sputum, which resembles the CF sputum in chemical composition and rheological properties. Moreover, these particles kill the bacteria contained in the artificial sputum more efficiently than the particles containing ciprofloxacin alone. This study proposes that inhalable particle systems, co-delivering antibiotics and mucus-thinning agents may be a hopeful strategy for local antibacterial therapy in

the CF airways. Yang et al. used mannitol as an alternative agent to influence the sputum (147). They observed that mannitol improves the antibacterial efficiency of ciprofloxacin against *P. aeruginosa* in the artificial sputum, most probably due to its ability to increase the local water content in the sputum, increasing the heterogeneity of the network and thereby enhancing drug transport (Figure 2.3 C) (147). A recent study proposed utilizing of particle biopolymer network interaction to facilitate transmucus drug diffusion. McGill and Smyth observed that penetrations of fluorescein and rhodamine are significantly enhanced through artificial mucus models after treatment of the mucus with particles (200 nm or 1 μm). This effect is attributed to the collapse of the mucin network mediated by particle - network interactions, which leads to an increase of the mesh size (Figure 2.3 D) (148).

Though various approaches have been developed with the recent advances in inhalational drug delivery technologies, a lack of proper CF animal models impose a significant challenge in clinical translation of these new approaches. Several CFTR-knockout mice have been developed; however, most of them rapidly develop CF-related bowel problems and die in infancy owing to cecal obstruction without ever developing lung disease (149). Therefore, for routine evaluation of inhalable formulations, the existing CF mouse models are not appropriate. Several more differences between the CF mice and humans with CF need to be pointed out. Firstly, the murine airway epithelium expresses an alternative Cl^- channel, which complements the CFTR deficiency and saves mice from severe lung disease (150). In addition, interspecies differences such as lung architecture, physiology and airway cell composition may be physiologically and pharmacologically important contributors to the difference.

Murine model with chronic *P. aeruginosa* lung infection, is an alternative animal model widely used for evaluation of microbial virulence and host defense mechanisms , which develops the mucopurulent matrix seen in the lungs of CF patients (151). In this model, agar or alginate beads containing a mucoid strain of *P. aeruginosa* are implanted via intratracheal instillation into the airways of mice or rats (151). The infection is usually established 3 - 4 days after inoculation. On a histological level, lesions similar to those of chronically inflamed CF lungs are seen in infected lungs (152). This model is relatively inexpensive and useful for evaluating formulations designed for trans- or intramucus drug delivery, but its utility in testing medications affecting the genotype or bioelectric phenotype of the airway epithelium is limited. A CFTR-knockout mouse model with nasal epithelium mimicing ionic transport of the airway epithelium has been used

widely for the proof of concept studies of CFTR gene delivery (71). However, according to a recent study, expression of human CFTR in the nasal epithelia fails to change the nasal bioelectrics of the transgenic mice, raising questions about the validity of the nasal epithelium as a model for airway gene delivery (153). Transgenic mice overexpressing ENaC subunits develop a CF-like lung disease (80). The animals develop several phenotypes pertinent to CF lung disease, such as viscoelastic mucus, delayed mucus transport, lung infection and inflammation. This model may be useful for the evaluation of new drug formulations designed to address the mucus barriers (71). Other larger animal models such as pigs and sheep are considered due to the similarity of their airways to the human airways (149).

2.7 Research envisioned

Although many drugs are delivered by means of inhalation to increase their local availability, several physiological barriers interfere with effective delivery of medications. Several drug delivery approaches have been proposed to overcome these barriers but are responsible only for the symptomatic treatment, and the cause of CF still remains untreated. It was observed that, mainly the bacterial infections (*P. aerugenosa*) causes the morbidity and mortality associated with CF. Aminoglycoside antibiotics are found to be most effective against the *P. aerugenosa*. They are used for treatment of *P. aeruginosa* infection in cystic fibrosis due to their concentration-dependent antibacterial activity, with long term post-antibiotic effect (154). But, the polar cationic nature of the aminoglycoside antibiotics is responsible for their poor penetrability into sputum of CF patients and reduction of aminoglycoside activity due to binding with sputum components like glycoproteins, monovalent and divalent cations; high systemic doses are required to achieve therapeutic concentrations of the drug at the infection site (155, 156). This results in development of the bacterial resistance against antibiotics. However, such high doses results in nephrotic and ototoxic effects, triggering permanent renal insufficiency and auditory nerve damage, with deafness, dizziness and unsteadiness (156). Although the inhalation therapy has reduced the dose, but still their activity for short period requires repeated administration of these antibiotics. In recent studies, it has been demonstrated that nanoparticulate drug delivery is a promising approach to deliver antibiotics (99, 100). NPs provide controlled drug release, which is useful to maintain a constant plasma drug concentration above minimum inhibitory concentration (MIC) for a prolonged duration, thus reducingthe

dosing frequency, maximizing the therapeutic effect of antibiotics while minimizing antibiotic resistance and improving patient compliance (157).

Gene therapy is an approach to cure the disease. For gene therapy to be effective in patients with cystic fibrosis, the cDNA encoding the cystic fibrosis transmembrane conductance regulator protein must be delivered effectively to the nucleus of the epithelial cells lining the bronchial tree within the lungs (71). Although it is believed that gene-based therapies hold tremendous potential for the treatment, it is hindered by the failure to deliver therapeutic gene safely and conveniently. Various vectors like viral and non-viral vectors have been used for this purpose. Viral vectors (Adenovirus, Adeno associated virus, Lentivirus) are attractive in terms of the scientific strategy of exploiting natural mechanism and are efficient to overcome the physiological barriers but, such system suffers from inherent difficulties of effective pharmaceutical processing, immunogenicity, scale up and possibility of reversion of engineered virus to wild type (67, 75). In addition to the cellular immune response, humoral immune responses against the gene transfer agents are a major problem, severely restricting the use of viral vectors for chronic diseases such as CF. To overcome these drawbacks synthetic vectors such as cationic lipids & cationic polymers, have been developed by researchers. These carrier systems should have the ability to target gene to specific cell type and to overcome extracellular and intracellular barrier with high transfection efficiency and minimum toxicity, which represents a significant goal in the field of gene therapy. These Non-viral vectors have greater control of their molecular composition for simplified manufacturing and analysis, flexibility in size of transgene to be delivered and relatively low immunogenicity. Various vectors for gene delivery, like liposome (Lipoplexes), polycations (polyplexes), physical methods (electroporation and iontophoresis) etc, have been developed (67). Number of extracellular, physical and immunological barriers plays an important role in gene transfer across the surface of the epithelial cells. It has been shown that mucus significantly reduces the transfection efficiency of most viral and non-viral gene transfer agents. Specific and nonspecific immune defences, in addition to the physical barriers are important inhibitors of airway gene transfer (158) . For an effective gene therapy treatment, it is important that the expression of the transgene must be maintained at adequate level throughout the life of the patient, by repeatative dosage of the vector. But repeated administration of these vectors is responsible for the toxic effects.

Recently, the administration of therapeutic molecules and gene by pulmonary route has gained attention due to the numerous advantages of pulmonary drug delivery over other delivery routes. These advantages include the large alveolar surface area suitable for absorption, low thickness of the epithelial barrier, extensive vascularization, and the relatively low enzymatic metabolic activity in addition to the absence of the first-pass effect (158, 159). But still the pulmonary delivery of drug and gene has been a challenge for the formulation scientist due to its limitations. The defense mechanisms of the lungs are the major limitation. The respiratory tract, being in direct contact with the external environment, possesses a series of defenses against inhaled materials. The deposited particles in the conducting airways are rapidly cleared into the pharynx, by the mucociliary clearance. In the terminal airways (alveoli), absorptive or non-absorptive processes remove the deposited particles. The absorptive process may involve either direct penetration into the epithelial cells or uptake and clearance by the alveolar macrophages. The non-absorptive process involves transport of particles to the ciliated region followed by clearance through mucociliary escalator (160, 161). In addition to these barriers, specific and nonspecific immune response against gene transfer agents are an important problem, severely restricting the use of viral vectors for chronic diseases such as CF. This can be overcome by the use of non-viral vectors.

The challenges encountered during pulmonary delivery can be overcome by using particulate drug delivery system, which is another option for pulmonary administration. Particulate nanocarriers such as liposomes, polymerosomes, micelles, microparticles and nanoparticles can be used for improving the therapeutic index, reducing metabolism, prolonging biological half-life, reducing toxicity, increasing bioavailability, and for better drug & gene targeting and delivery resulting in a reduction of dosage frequency and improved patient compliance (161). However, these nanocarriers are removed due to the binding of plasma complement, immunoglobulins, leading to uptake by macrophages. The grafting of synthetic hydrophilic polymers such as poly (ethylene glycol) (PEG) onto the surface of the vehicles, markedly delays the clearance. PEG forms a hydrated shell hindering protein interaction with nanocarrier or drugs themselves, thereby greatly reducing opsonization and uptake by macrophages. Apart from this, it protects polyplex structure against undesirable interactions with impertinent surroundings. Notably, the stability, nuclease resistance and in-vitro knockdown efficiency of PEGylated polyplexes has been shown to depend greatly on the PEG chain length. PEGylation also helps in

improving the transfection efficiency of the gene in bronchial cells in-vitro. The water solubility of the complexes is increased by PEG, thus improving their physical stability (162). Such "stealth" drug delivery system (DDS) have prolonged pharmacokinetics and lesser side effects, like activation of host defense (immune response, cytokine release, complement activation) (163).

Drug therapies act as the supportive treatment to suppress the secondary effects of the disease. Yet these therapies alone have not been proven to be effective for the treatment of the cystic fibrosis. The combination of these two therapies (gene therapy & drug therapy) will be effective in the treatment of the cystic fibrosis. The gene therapy will help to modify the genetic defects while the antibiotics will help in eradicating the lung infection. The drug therapy will synergize gene therapy and help to the cure the symptoms of the cystic fibrosis. Stealth polymeric nanocarriers composed of the PEG grafted biodegradable polymer can be used for the controlled and targeted delivery of drugs and genetic material. Biodegradable polymeric carrier system has various advantages of being non-toxic, biodegradable and biocompatible, with very interesting biological properties, such as permeation-enhancement and mucoadhesion. Thus, the Stealth polymeric nanocarriers may serve as suitable candidates for the delivery of drugs and gene formulations in the effective treatment of the Cystic fibrosis.

2.8 Objectives of the proposed work

Research work was planned according to following objectives

1. Transformation of Plasmid cDNA into bacterial cells (*E. coli* Strain DH5α or Top 10) for replication, isolation & purification of plasmid.

2. To incorporate plasmid cDNA and drug separately in stealth polymeric nanocarriers and to evaluate the designed drug delivery system for their physicochemical parameters and in-vitro release characteristics.

4. To study in-vivo performance of the developed formulation *in-vitro* on cystic fibrosis cell line (CFBE 41o- cell line).

5. Developing the optimized formulations as dry powder inhaler by freeze drying or formulations ready for nebulization after reconstitution and studying the in-vitro lung deposition pattern of nanoparticles using cascade impactor.

It is hypothesized that stealth polymeric nanocarriers in dry powder formulation for nebulization after reconstitution containing therapeutic gene and drug will provide more efficient and direct

delivery of the genes and drug into the lung cells. PEGylation gives stealth effect to the nanocarriers which prevent it's uptake by macrophages and also aids in improving the transfection efficiency of the gene in bronchial cells in-vitro. Further, co-administration of the drug and gene formulation in treatment of the CF, will increase possibility of better therapeutic response in patients.

2.9 pDNA and Drug profile

2.9.1 pDNA profile

2.9.1.1 piRIS2-EGFP CFTR plasmid (Plasmid used for CFTR correction)

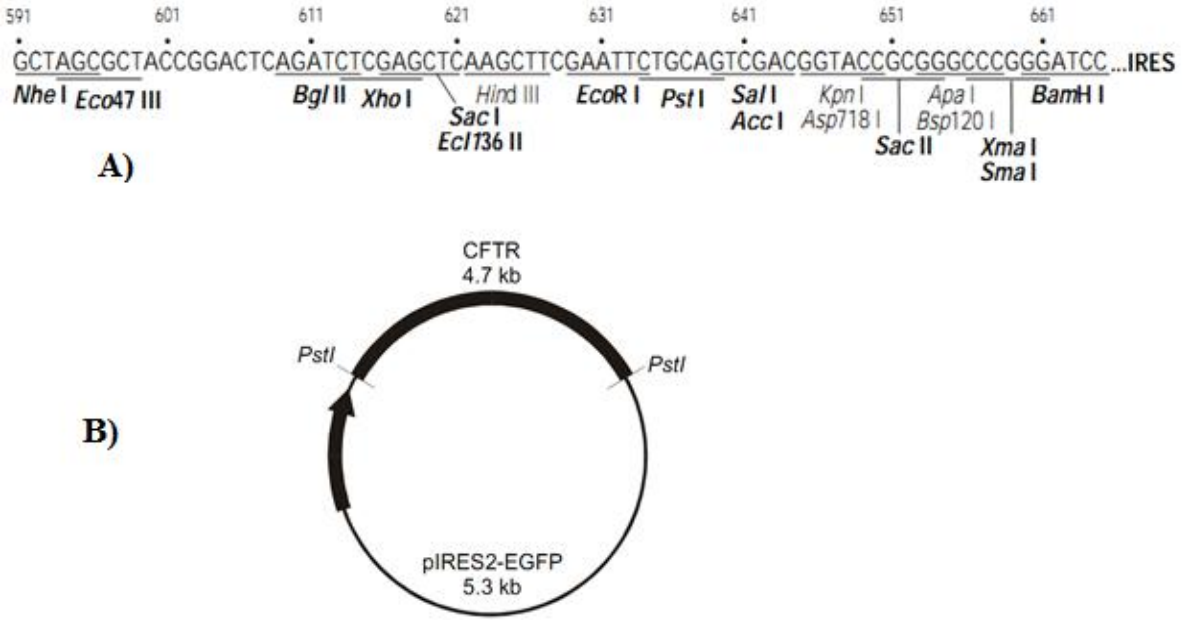

Figure 2.4: a) piRIS2-EGFP vector; b) piRIS2-EGFP CFTR vector

piRIS2-EGFP CFTR plasmid (Figure 2.4) is used for correction of CFTR in cystic fibrosis patients. This plasmid contains CFTR gene encoded in pIRES2-EGFP vectors. This vectors contains the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. This permits the translation of both the gene of interest (cloned into the MCS) and the EGFP gene from a single bicistronic mRNA. pIRES2-EGFP is designed for the efficient selection (by flow cytometry or other methods) of transiently transfected mammalian cells expressing EGFP and the protein of interest. This vector can also be used to express EGFP alone or to obtain stably

transfected cell lines without time-consuming drug and clonal selection. The CFTR gene is inserted at Pst I site. The vector consists of Kanamycin resistance marker. The Kanamycin resistance is a selection marker for transformed cells after growing in the Kanamicin containing LB plates. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. The pIRES2-EGFP backbone also provides a pUC origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

2.9.1.2 pcDNA-3 LUC-WT plasmid (Plasmid used for evaluation of transfection efficiency)

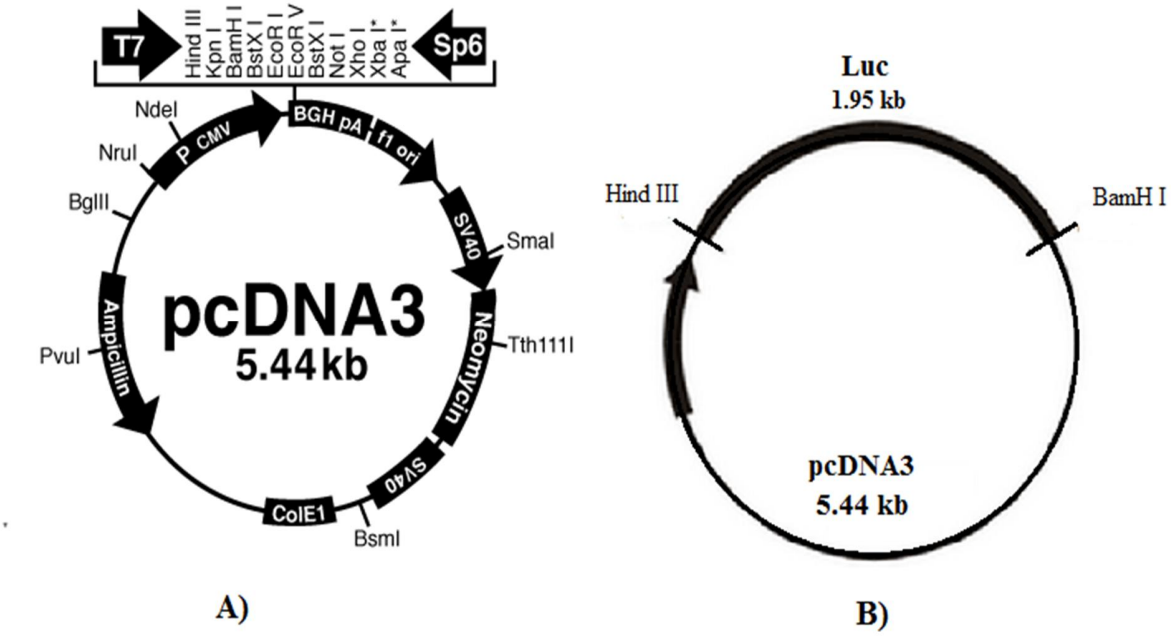

Figure 2.5: a) pCDNA 3 vector; b) pcDNA-3 LUC-WT plasmid vector

pcDNA-3 LUC-WT plasmid (7.4 kb) (Figure 2.5) contains a luciferase gene encoded in vector. It is Mammalian expression vector with the CMV promoter. Luciferase gene is encoded between Hind III & BamH I site. pcDNA-3 LUC-WT plasmid is designed for the efficient selection (by Colorimetry) of transiently transfected mammalian cells expressing luciferase and the protein of interest. The vector consists of ampicillin resistant gene. The Ampicillin resistance is a selection marker for transformed cells after growing in the Ampicillin containing LB plates.

2.9.2 Netilmicin sulfate

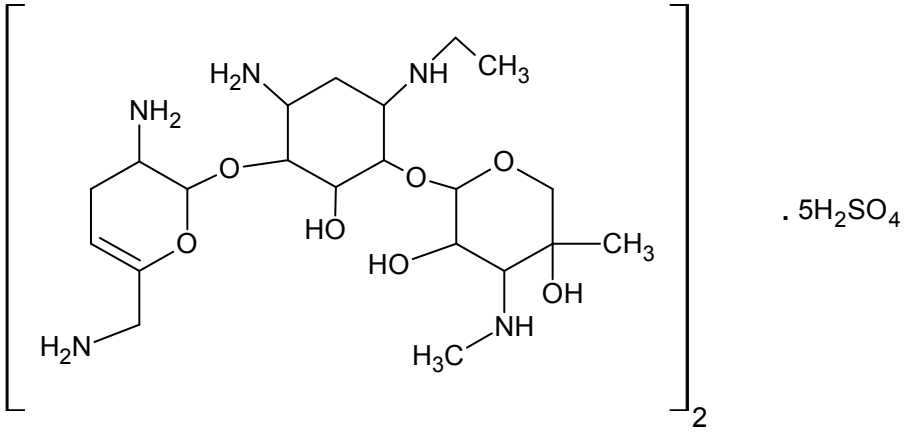

Figure 2.6: Structure of Netilmicin Sulfate

2.9.2.1 Chemistry

Netilmicin sulfate is chemically (2R,3R,4R,5R)-2-[(1S,2S,3R,4S,6R)-4-amino-3-[[(2S,3R)-3-amino-6-(aminomethyl)-3,4-dihydro-2H-pyran-2-yl]oxy]-6-ethylamino-2-hydroxycyclohexyl] oxy-5-methyl-4-methylaminooxane-3,5-diol (Figure 2.6). It is a semi-synthetic 1-N-ethyl derivative of sisomycin, an aminoglycoside with action similar to gentamicin, but with less ear and kidney toxicity (163). Its molecular formula is $(C_{21}H_{41}N_5O_7)_2.5H_2SO_4$ and its molecular weight is 1441.56 g/mol (164). Netilmicin sulfate is an aminoglycoside which exists in dimer form. Chemically, aminoglycosides consists of two or more amino sugars joined by a glycosidic linkage to a hexose nucleus. The hexose is either streptidine or 2-deoxy streptidine. Netilmicin sulfate consists of two amino sugars. It is highly charged molecule and generally contain more than one charge on its surface and therefore exists as a salt form with a strong acid or base (165).

2.9.2.2 Physical properties

Netilmicin sulfate is a White to off- white powder. It is a basic molecule, highly soluble in water irrespective of pH. It is practically insoluble in methanol and ether. It is acid – labile and is generally denatured at pH below 3. Log P value is -3. 981 (164).

2.9.2.3 Pharmacodynamics

2.9.2.3.1 Mechanism of action

Aminoglycosides bind to specific 30S-subunit proteins and 16S rRNA. Specifically, netilmicin binds to four nucleotides of 16S rRNA and one amino acid of protein S12, interfering with decoding around nucleotide 1400 of 16S, thereby inhibiting the formation of an initiation

complex, this region interacts with the wobble base in the anticodon of tRNA. This leads to interference with the initiation complex, misreading of mRNA ,due to which incorrect amino acids are inserted into the polypeptide leading to nonfunctional or toxic peptides and the breakup of polysomes into nonfunctional monosomes, leaving the bacterium unable to synthesize proteins vital to its growth (166, 167).

2.9.2.3.2 Antibacterial spectrum

It has bactericidal activity against the *Pseudomonas aeruginosa, Klebsiella-Enterobacter-Serratia* group, *Citrobacter* sp., *Proteus* sp. (indole-positive and indole-negative), including *Proteus mirabilis*, *P. morganii, P. rettgrei, P. vulgaris,* and *Neisseria gonorrhea (167)*. Netilmicin is also active *in-vitro* against isolates of *Hemophilus influenzae, Salmonella* sp., *Shigella* sp. and against penicillinase and non-penicillinase-producing *Staphylococcus* including methicillin-resistant strains. Some strains of *Providencia* sp., *Acinetobacter* sp. and *Aeromonas* sp. are also sensitive to netilmicin [4]. Many strains of the above organisms which are found to be resistant to other aminoglycosides, such as kanamycin, gentamicin, tobramycin and sisomicin, are susceptible to netilmicin *in vitro*. Occasionally, strains which are resistant to amikacin but susceptible to netilmicin have been identified. The combination of netilmicin and penicillin G has a synergistic bactericidal effect against most strains of *Streptococcus faecalis* (enterococcus). The combination of netilmicin and carbenicillin or ticarcillin is synergistic for many strains of *Pseudomonas aeruginosa*. In addition, many isolates of *Serratia*, which are resistant to multiple antibiotics, are inhibited by synergistic combinations of netilmicin with carbenicillin, azlocillin, mezlocillin, cefamandole, cefotaxime or moxalactam. (168, 169).

The pharmacodynamic properties of aminoglycosides are concentration-dependent and show significant post-antibiotic effect. Aminoglycosides eliminate bacteria quickest when their concentration is appreciably above the MIC for an organism; this is referred to as concentration dependent activity. The aminoglycosides also exhibit a significant post-antibiotic effect (PAE). PAE is the persistent suppression of bacterial growth following antibiotic exposure. Practically speaking this means that trough levels can drop below the MIC of targeted bacteria for a sustained period without decreasing efficacy (170). For aminoglycosides the ideal dosing regimen would maximize concentration, because higher the concentration, the more extensive and faster the degree of bactericidal activity. Therefore, the Peak/MIC ratio is an important predictor of efficacy. It has been shown that aminoglycosides eradicate bacteria best when they

achieve a Peak/MIC ratio of at least 8-10. Therefore it is important to give a large enough dose to produce a peak level 8 to 10 times greater than the MIC (170). The risk of ototoxicity and nephrotoxicity is increased if peak levels are consistently maintained above 12 to 14 mcg/ml or trough levels consistently exceed 2mcg/ml (167).

2.9.2.3.3 Adverse effects

The main adverse effects are ototoxicity and nephrotoxicity, but the intensity of them is significantly less as compared to other aminoglycoside antibiotics (170). The symptomatic adverse reactions produced by netilmicin Sulfate are more or less tolerable and if they become severe, they can be treated symptomatically; these include nausea, vomiting, maculopapular rash, urticaria, pain, phlebitis, elevated hepatic transaminases, headache, lethargy, drowsiness, paresthesias, tremors, muscle twitching, peripheral neuritis, disorientation, seizures, neuromuscular blockade; musculoskeletal weakness or paralysis, respiratory depression or paralysis, palpitation, hypotension, ataxia, diarrhea, stomatitis, decrease in creatinine clearance; hematuria, proteinuria, urinary frequency, oliguria and polyuria (170).

2.9.2.3.4 Drug interactions

The risk of kidney toxicity is increased when netilmicin sulfate is taken with cefamandole, cefazolin, cefonicid, cefoperazone, ceforanide, cefotaxime, cefotetan, cefoxitin, ceftazidime, ceftizoxime, ceftriaxone, cefuroxime, cephalothin, cephapirin, cisplatin and cefradine (166). The effects of many muscle relaxants, including atracurium, doxacurium, metocurine, mivacurium, succinylcholine, tubocurarine and vecuronium, are increased when taken with netilmicin sulfate. Increased ototoxicity is noted when netilmicin sulfate is taken with bumetanide, ethacrynic acid, furosemide and torasemide. Thalidomide increases renal toxicity of netilmicin (167).

9.2.4 Pharmacokinetics

2.9.2.4.1 Absorption

Netilmicin is rapidly and completely absorbed after IM administration, usually given by IV injection or infusion, peak serum levels were achieved within 30-60 minutes. It is poorly absorbed from the gastrointestinal tract. It follows a 3 compartment pharmacokinetic model. On infusion distribution is not usually observed (85, 169).

2.9.2.4.2 Distribution

The average Volume of distribution (Vd) of aminoglycosides in otherwise healthy adults is 0.26 L/kg (range: 0.2-0.3). Although aminoglycosides do not distribute into adipose tissue, they do

enter the extracellular fluid contained therein. Therefore, obese patients require a correction in the weight used for Vd calculation. Patients with cystic fibrosis have a markedly increased Vd of 0.35 L/kg due to increase in extracellular fluid brought about by the disease process. Patients with ascites have additional extracellular fluid because of accumulation of ascitic fluid, which increases the Vd to approximately 0.32 L/kg. ICU patients may have a Vd 25-50% above normal. Plasma protein-binding of Netilmicin sulfate is low (85).

2.9.2.4.3 Excretion

It is excreted un-metabolized from the body by glomerular filtration. It is said to be reabsorbed from the proximal tubules which is believed to be the reason for its nephrotoxicity. Its excretion is believed to be closely related with creatinine clearance. Cystic fibrosis patients show a 50% decrease in elimination rate. A major body burn increases the basal metabolic rate resulting in a marked increase in elimination. ICU patients are often hyper metabolic and therefore eliminate the drug more rapidly (166, 169).

2.9.2.5 Indications and dosing

Netilmicin sulfate is indicated in treatment of patients with serious or life threatening bacterial infections such as lower respiratory tract infections, urinary tract infections, Skin infections, intra-abdominal infections caused by organisms such as *E.coli, K.pneumoniae, P.aeruginosa,* Enterobacter species, *P.mirabilis* and *S.aureus (167, 169).*

2.9.2.5.1 Dosing Regimen (85, 166-168)

Adult: 4-5 mg/kg once daily or in equally divided doses given every 8 or 12 hr. Life-threatening infections: Increase to up to 7.5 mg/kg daily every 8 hr. All doses may be given as IM, slow IV (over 3-5 min) or as 50-200 ml infusion over 0.5-2 hr. Treatment is usually given for 7-14 days. *Child:* Premature infants and neonates <1 week: 6 mg/kg daily in divided doses every 12 hr. Infants and neonates >1 week: 7.5-9 mg/kg daily in divided doses every 8 hr. Older children: 6-7.5 mg/kg daily in divided doses every 8 hr. Alternative regimen: Neonates <6 week: 4-6.5 mg/kg daily in divided doses every 12 hr. Older infants and children: 5.5-8 mg/kg daily in divided doses every 8 or 12 hr.

2.9.2.5.2 Renal impairment

Dose reduction or lengthening of interval between doses may be necessary.

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