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(REVIEW ARTICLE)

Unveiling epidemiology and treatment strategies for efficient management of lung infection in cystic fibrosis

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Abstract

Cystic Fibrosis (CF) is a genetic disorder which results in abnormal Cystic Fibrosis Transmembrane Regulator (CFTR) protein. CFTR protein actively transports chloride and bicarbonate out of cells which produce mucus in epithelium of various organs. However, when there is an abnormality in this protein then ion and water transport across airway surface is affected and form viscous mucus due to dehydration. This disease affects lung, pancreases, intestine and hepatobiliary system. Infection of lungs is main reason of high morbidity and mortality. Patients with CF are more prone to bacterial infection of the respiratory tract. Mainly four bacterial pathogens are found in the respiratory tract of CF patient *viz. Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*. Mucoid *P. aeruginosa* is the most dangerous pathogen among them due to ability to form biofilm. There are many conventional and new treatment options available for CF patients. New therapies are used to correct the CFTR protein and antibiotic treatment is used to prevent or early infection or suppression of chronic infection. However, when *P. aeruginosa* turn into mucoid form, formation of extracellular polysaccharide biofilm starts with alginate as major constituent. The antibiotics fail to reach the target as EPS of the biofilm shield the microbe from antibiotics action. Alginate lyase, an alginate degrading has the ability to break the alginate and subsequently increases the efficacy of antibiotics therapy. The co-administration of alginate lyase and Dnase along with antibiotics has proved to be very efficient in the management of patients with CF.

Keywords: Cystic Fibrosis; Alginate lyase; CFTR protein; *Pseudomonas aeruginosa*; Biofilm

1. Introduction

Cystic Fibrosis is single gene autosomal recessive genetic and life threatening disorder which is characterized by damage to the respiratory and digestive system [1]. This disease also affects the lungs, upper and lower airways, pancreas, intestine and hepatobiliary system [2]. CF is frequently genetically inherited chronic disease in Caucasian population of northern European ancestry [3]. Clinical features of CF include salty flavor skin, continuous coughing and dyspnea, viscous mucous (sputum), unhealthy or breathe audibly, non-cancerous growth on the lining of sinuses foul smelling and greasy stools, body weight imbalance, intestine blockage especially in new born and acute constipation. This disease occurs due to mutation in cystic fibrosis trans-membrane conductance regulator protein which is also called CFTR [4]. The CFTR gene is present on the 7th chromosome in humans [5]. The most frequent mutation in the CFTR gene is deletion of phenylalanine at codon 508 [6]. Different mutations in this gene have different effects on CFTR function and can result in various phenotypes of disease. The CF mutation does not show any symptoms of this disease in the carriers which are heterozygous [5]. The normal CFTR gene or protein transports the chloride through the cells which produce the mucous. This is followed by release of water which makes the mucous thin. However, in defective CFTR gene the mucous becomes viscous and leads to the blockage of the pathway and ultimately results in the clogging of lungs and obstruction in pancreas [7]. In addition to this, degranulating neutrophils release large quantities of nucleic acid and cytosol matrix proteins contributing to the mucous hyper-viscosity [8]. Functional CFTR is important for the proper hydration of the Airways Surface Liquid (ASL) in respiratory tract and this is important for ciliary movement which helps in the proper functioning of lungs and removal of inhaled microorganisms and particles. These inhaled

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microorganisms and particles are thrown into the gut via mucociliary clearance and acid present in gut clear these microbes. The people with CF are unable to flush out their dehydrated airways mucous secretions which create the colonization of microorganisms [9]. This thickness of mucous reportedly leads to male infertility by clogging the vas deferens. Regular gene span of the CFTR is 250000 base pairs and encodes for protein of 1480 amino acids. The protein contains two trans-membrane (anchoring) domain, two nucleotide binding domain and a part which are termed as R (Regulatory) domain [10]. CFTR has a feature that is prevalent with a superfamily of protein found in nature which acts as active transporters of macromolecules. It is important because one of the parts of this superfamily, P glycoprotein perform the double function as transporter as well as chloride channel [11].

Patients with CF are more prone to bacterial infection of the respiratory tract especially pneumonia and bronchitis. So continuous and acute antibiotic medication is compulsory for the proper functioning of the lungs and increases the life span. Antibiotic treatment is important because *Pseudomonas aeruginosa* is the main bacterial pathogen in the CF [12]. *P. aeruginosa* is a Gram negative opportunistic bacterium that resides in a broad range of surrounding and humid places and naturally unaffected by antibiotics. Most strains of *P. aeruginosa* generate one or more pigments namely pyocyanin, pyoverdin and pyorubin. Studies have shown that pyocyanin not only help in persistence of *P. aeruginosa* in the lungs of CF patients but also involves in many mammalian cell functions such as cell respiration, cilliary beating, epidermal cell growth, calcium homeostasis and prostacyclin release from endothelial cells of lung [13]. In the lung of CF patients, *P. aeruginosa* have favorable environment with sticky mucous layer for the biofilm formation [14]. This biofilm provides shielding against antibiotics and immune responses to the *P. aeruginosa*. This shielding also leads to the conversion of non mucoid strain of bacteria into a mucoid strain of bacteria [15, 16, 17]. An oxygen gradient is present in biofilm [18, 19]. In early CF infection most of the *P. aeruginosa* is characterized as non mucoid strain but in the deficiency of oxygen bacteria come under stress and non-mucoid strain transformed into mucoid strain [20]. This stress generates the mutation to happen on the genes which are related to the alginate production and controlled activation of the excess of the capsule like alginate. The biofilms comprising alginate is formed by the mucoid strain leads to less antibiotic entry and clog the phagocytosis of bacteria, although treatment with alginate lyase has improved antibiotic response [21]. In addition to this, in few patients pathology is manifested through digestive defective CFTR through the pancreas and up to 50% of people cured with CF also live through CF-related Diabetes (CFRD). CFRD generally presents type-1 and type-2 Diabetes Mellitus in non-CF individuals [22]. Glucose-stimulated electrical conductance also participates in insulin excretions from pancreatic β-cells [23]. This CFTR results in the dysfunction of glucose-mediated electrical activity and stops the insulin excretions from β-cells in some patients of CF [24]. Liver disease also presents the comprehensive pathology of CF. CFTR in the liver confined to the luminal surface of intrahepatic epithelial cells of bile duct, where it pours biliary secretions with bicarbonate in analogous to its function in the pancreas and also brings the flow of bile acids [25]. The absence of bicarbonate secretions leads to accumulation of harmful bile acids within liver cells. This generates an inflammatory response resulting in fibrosis analogous seen in pancreas and ultimately cirrhosis and portal hypertension [26]. CF is diagnosed through the estimation of sweat through sweat test, as high content of salt is present in sweat [27]. Many antibiotics are used in treatment of CF. Tobramycin is one of the antibiotic which is prescribed for the patients of CF who have an infection of *P. aeruginosa*. Tobramycin is commercialized as inhalator under registered name TOBI®. According to the Cystic Fibrosis Foundation (CFF) antibiotics are essential for curing the CF because patients with CF have high ubiquity of respiratory infections from *P. aeruginosa*. TOBI® an aminoglycoside; broad spectrum antibiotic produced by *Streptomyces tenebrarius* is widely used against Gram-negative bacterial infections and particularly useful in the treatment of *P. aeruginosa* in patients with CF. When the antibiotic and alginate lyase were bound together, a noticeable elimination of mucoid bacteria from biofilm was achieved. This fact may enhance the edge of antibiotic in the control of respiratory tract infection. Ceftazidime medication is effective against the non-mucoid strain of *P. aeruginosa* [28]. Newborn Screening is also helpful for CF treatment. Early identification supports the future aspects of the families which do not screen [29]. CFTR modulators theratyping is an innovative and fast growing field that has power to recognize rare CFTR variants that are reactive to drugs in development [30].

2. Alginate and alginate lyase

Alginate is a high molecular weight, negatively charged polymer which is obtained from brown seaweed [31]. Commercially alginate is obtained from brown algae (Phaeophyceae) namely *Laminaria hyperborea*, *L. digitata*, *L. japonica*, *Ascophyllum nodosum*, *Microcystis pyrifera* and *Saragassum muticum* [32]. Alginate composed of compartment of (1, 4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues [21]. These compartments are composed of G residues, M residues and GM residues [31]. It was also extracted from bacteria such as *Pseudomonas* and *Azobacter* [33]. Alginate is a biomaterial that has great applications in biomedical science and engineering. Alginate is one of the most used dental materials as an impression material, used in orthodontic models, sports mouth guards and bleaching trays [34]. Alginate hydrogels are also used in wound healing, drug delivery and tissue engineering applications [31]. Extraction of alginate from seaweed is easy but multistage process. It starts treating the raw dried material with dilute

mineral acids which are water soluble [35]. Among the various alginates, sodium alginate is commonly used alginate in pharmaceutical and biomedical field [36]. In *P. aeruginosa*, alginate act as a carbon source, protect the bacteria and increases the adhesion to solid surface which results in biofilm formation [37]. Alginate producing *P. aeruginosa* interestingly associated with the surrounding of CF affected lungs [38]. Alginate which is secreted by patients with CF has potential to show resistance to both the host immune system and antibiotics. In the pathogenesis of CF, this alginate or mucoid exopolysaccharide show potential role in the bacterial adherence mechanism, hinders the phagocytosis and affects the leucocyte functioning [15]. Most proteins which enhance the alginate biosynthesis are encoded by 12-gene alg-D operon; this operon also encodes AlgL, a lyase which degrades the alginate [39]. Alginate lyase is an enzyme which brings the breakdown of alginate. It breaks the alginate through the β-elimination of glycosidic bond and produces the unsaturated oligosaccharides with double bond at the non-reducing ends [40]. Alginate lyase also has great application in the treatment of cystic fibrosis by breaking the alginate which is produced by mucoid strain of *P. aeruginosa* [41]. A recombinant paAlgL is also used for the enhancement of antibiotics against the *P. aeruginosa* [42]. Alginate lyases are also important for the breakdown of acidic polysaccharides using their endo and exo activity which in turn can be used for the production of the bioethanol [43].

3. Machinery involved in non-mucoid to mucoid transition of *P. aeruginosa*

The CFTR of epithelial cells help the defense system of the host by binding reversibly to the bacteria under normal circumstances. Hence, mutation in the CFTR gene hinders the normal eradication of the pathogen from the respiratory tract [44]. As among all the microbes responsible for CF, the major contributor leading to 2.6 time higher fatality rate is *P. aeruginosa* [45]. It is of significant importance to understand the machinery involved in the transition of non-mucoid strain into mucoid one. Infection of *P. aeruginosa* is accompanied by two developmental stages progressing from sporadic to chronic infection [46]. The stage of sporadic infection can be eliminated by vigorous antibiotic therapy [47]. However, as soon as the mucoid form is observed in sputum sample of the patients, antibiotics fail to eliminate the pathogen from the lungs [47]. Alginate production in excessive amount signals the initiation of the mucoid conversion [48]. Presence of this polysaccharide possibly facilitates in bacterial adherence to the surface, resists phagocytosis, counteract oxygen radical, affect the immune system regulation via secretion of pro-inflammatory cytokines and repression of lymphocytes transformation [49, 15].

Non-mucoid strains present in the primitive infectious stage cause less distress in the lung environment. However, the advent mucoid strain can be associated with the formation of bacterial biofilm, development of anti-*P. aeruginosa* antibodies and inflammation [49]. The host's protective responses against the mucoid strain are mainly governed by PMNs (polymorphonuclear leukocytes) and antibodies [50]. The biofilm of *P. aeruginosa* trigger the oxidative burst of PMNs [51] and the complement system [52]. There are possibilities that the host's own inflammatory response to the infection is responsible for the conversion of non-mucoid strain [53]. A study was conducted to examine the importance of PMNs and toxic oxygen by products released in the selection of mucoid variants. The natural inflammatory host response was imitated by growing *P. aeruginosa* PA01, non-mucoid strain in a biofilm followed by treatment with low levels of H_2O_2, which was otherwise released by the PMNs under in vivo conditions and as a result, development of mucoid variants has been observed [53].

4. Mutation in gene encoding MucA leads to mucoid conversion

The reason behind occurrence of chronic stage of CF infection is the phenomenon involving switching of *P. aeruginosa* infecting strain into alginate overproducing mucoid strain which leads to worsening clinical outlook [15]. This switch mainly occurs due to loss of function of MucA which acts as an anti-sigma factor. Transcriptional activation of AlgD leads to mucoidy. AlgD heads biosynthetic pathway of alginate production as it encodes for GDP mannose dehydrogenase, which is alginate specific enzyme. It catalyses double oxidation of GDP mannose into its uronic acid and this reaction provides a route to sugar intermediates to participate in alginate production. AlgU is an alternative sigma factor required for initiation of AlgD transcription. In cases of AlgU is inactivation, AlgD transcription discontinues and mucoidy got revoked [54]. Genetic evidence suggests that *P. aeruginosa* MucA and MucB suppress the regulation of alginate production by AlgU [55]. The AlgU gene along with negative regulators MucA and MucB controls the conversion of non-mucoid into mucoid strain. Mutation in MucA sets AlgU uninhibited which in turns causes activation of AlgU(σ E)-dependent promoters of AlgD and AlgR. AlgR binds to 3 sites RB1, Rb2, Rb3 in the AlgD promoter [56] and in association with AlgU causes strong transcription activation of AlgD resulting in alginate overproduction. An experiment was performed. to investigate the presence of MucA mutation in the H_2O_2 treated strains Same mutation was observed in them which involves deletion of a guanine residue in a strand comprising 5 guanine residue situated at 426-430 bp in the MucA ORF, leading to early termination of translation followed by formation of truncated MucA

protein which is unable to binds to AlgT (also called AlgU) and result in continuous expression of alginate biosynthesis operon [53].

5. Microbial environment of lungs in case of CF

Humans are generally habituated with the microflora of their body and in this scenario there is no occurrence of disease such a CF. However, this relationship gets disturbed as a result microbes evolve into pathogens. The condition becomes favorable for bacterial intrusion and colonization in the particular organ (lungs in case of CF) as soon as the disease affects the body organs [57]. CF is marked by the huge diversity of microflora in lungs. The cosmopolitan nature of microbial environment can be correlated to the presence of antibiotics, stratified oxygen environment in the lungs, inflammatory products including eDNA released during host immune response etc. which favors the growth of variety of bacterial species [58, 59]. Microbial diversity was observed on the basis of different zones of oxygen in the lung environment and it was found that microbial species not only vary with patients but also varies within the same patient at different point of time.

A large number of studies have been conducted to identify the CF causing pathogens. Each study has its own selection basis and concentrated on broader categories of microbes but most of the studies have focused on bacterial pathogens. The four major pathogens which are the main focus of all clinical studies include *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Presence of *S. aureus* and non-encapsulated *H. influenzae* indicate the early stages of infections during infancy. Invasion by *P. aeruginosa* has been observed later in life of the patient which is often the chronic stage of infection [57]. Other contributors which aid in elevating the fatality rate include *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans* and *Klebsiella* sp. [16, 60]. Apart from bacterial pathogens, the range of the species extends to viruses for example respiratory syncytial virus, Adenovirus, influenza [61] and fungi which include *Aspergillus* sp. and *Candida* sp. [62].

This evident microbial diversity led the researchers to focus and identify more bacterial species from sputum of CF patients. A study aimed at comparing the biofilm formation ability, reported two new bacteria *Inquilinus limosus* and *Dolosigranulum pigrum* found in phlegm of patients [63]. *I. limosus* is an aerobic Gram-negative Bacillus [64] which is a potential threat because of its ability to exhibit mucoid phenotype, multiple drug-resistance and prolonged existence in the airway. However, the pathogenesis of *D. pigrum* is not very clear because of no or little evidence in favor of its antibiotic resistance ability, but it is strongly correlated with nosocomial pneumonia and blood poisoning [65].

6. Major players in pathogenesis of CF

6.1. Staphylococcus aureus

This Gram positive bacterium is the first and foremost detected pathogen in the case of CF, possibly due to the poor immune defense system in infant population detected with CF [66]. Among 48% of all the healthy children in the USA and 36% in Netherlands *S. aureus* was found to be present mainly in the pathway connecting pharynx to the nasal cavity [67, 68]. The *S. aureus* exhibit basically two types of phenotypes; Small Colony Variants (SCV), persisted in the lung environment because of their ability to circumvent the host immune response by decreasing the expression of virulence factors associated with them [69] and Methicillin-Resistant *S. aureus* (MRSA) which is a more serious clinical threat [66]. Although it's existence is uncommon in comparison to the latter but the treatment of SCVs can lead to eminence of the MRSA.

6.2. Haemophilus influenzae

H. influenzae usually present in the nasopharynx region is the second most common microbe found in infants suffering from CF. Probability of occurrence of *H. influenzae* in children's is estimated to be 20% .The percentage may increases to 50% in the case of children to 75% in the case of adults [70].Various serotypes of *H. influenzae* have been categorized mainly on the basis of presence or absence of capsules. However, it has been reported that non-capsulated bacterium referred to as *H. influenzae* (NTHi) is mostly correlated with chronic lung infection and severe aggravation in CF subjects [71, 72]. NTHi own various adherence factor which comprises HMW1/HMW2 and Hia protein plays a significant part in bacterial colonization [73].

6.3. Burkholderia cepacia

It is the most dangerous opportunistic human pathogen associate with CF [74]. Most of the severe respiratory complications CF in are caused by *P. aeruginosa* whereas *B. cepacia* contributes to only a small size of population [75]. However, *B. cepacia* infections are highly intimidating because of its fluctuating nature as well as worsen prognosis ranging from no symptoms of illness to a vehement septicemia with a critical respiratory collapse known as cepacia syndrome [76]. The potential of high antibiotic resistance and capacity to survive under adverse environmental conditions creates a major threat to its eradication difficult to nearly impossible from the lung environment [77]. C.F infections involving *B. cepacia* are marked by the presence of either single strain or co-infection with two or more distinct strains or species. Further succession of early strain with another one in a later phase of infection can also occur [78].

6.4. Pseudomonas aeruginosa

P. aeruginosa is the most dangerous opportunistic pathogen due to the presence of a wide assemblage of virulence factors, biofilm forming ability which confers antibiotic resistance and tight regulation of quorum sensing mechanism [79]. The quorum sensing modulates 10% of the genome of *P. aeruginosa* which includes of its swarming motility, biofilm maturation and antimicrobial resistance. The quorum sensing mechanism also regulates the production of various virulence factors like exotoxins, elastases and pyocyanin [80]. Phlegm samples from CF patients have been diagnosed with quorum sensing signals molecules (QSSMs) [81]. It was suggested that QSSMs can possibly be used as an indicator molecule of *P. aeruginosa*, to target it for the treatment.

7. Role of quorum sensing in biofilm formation by *P. aeruginosa*

Quorum sensing in *P. aeruginosa* is a complex circuit and generally comprising of two systems. The first in the hierarchy to be expressed is the las system which consists of genes encoding lasI and las R [82]. The lasI has ability to produce N-3-oxododecanoyl-L-homoserine lactone (3O-C12-HSL)[83] which is required to activate the las R. The las R binds to promoters of quorum sensing regulated genes to govern the production of elastase, staphylolysin, alkaline protease, exotoxin, hydrogen cyanide synthase as well as lasI itself, thereby enhancing the signaling process [84]. The las system exerts a positive feedback for enhanced production of 3O-C12-HCL and also instigates the second system. The second system comprises of RhII and RhIR transcription activators [85]. This system requires a different signaling molecule. RhII produces N-butanol-homoserine C4-HSL [86]. The rhamnolipid synthesis genes, the stationary phase sigma factor, type-1 lectin, type-2 lectin, hcn ABC and pyocyanin production genes are expressed by the RhI system. Another important signal of quorum sensing is quinoline signal (PQS), which relies on the fair production of N-3-oxodo-decanoyl-l-homoserine lactone and N-butryl-l-homoserine lactone [87]. PQS molecules (2-heptyl-3-hydroxy-4-quinolone) play an important role in the transcription of virulence genes mainly involves in production of pyocyanin and rhamnolipid of *P. aeruginosa*. A type four secretion also exists in *P. aeruginosa* which in combination with membrane vesicles eases the transport of effector molecules to target organisms [88]. N-acyl homoserine lactones secreted by *P. aeruginosa* biofilm are sensed by the neighboring microbes in response to which they start constructing their own biofilm. This phenomenon is indicative of the existence of intraspecies signaling in the infectious lung environment [89].

8. Microbial association in lungs of the CF patients

Lungs of CF patient have heterogeneous mixture of microbial species which colonies the airways exhibiting wide variety of interactions among them and this can alter their persistence in the lungs as well as the clinical outcomes. Two or more bacterial species are present in 31% of the CF patients [90] and co-infections were also observed [91, 92]. Old standardized culture techniques fail to recognize the microbial diversity in CF lungs [93]. Identification methods and culture techniques are augmented with 16s rRNA sequencing [94] and RFLP [95] to obtain a comprehensive and clear picture of the lung microbiota. The microbiota of CF lungs advances with age of the subject and different age groups are colonized with different population of bacterial species [96]. However, the diversity decreases with persistence of infection and as the infection becomes old *P. aeruginosa* species predominates [97]. 167 clonal isolates has been found in the sample from a single patient and such a vast diversity within a single patient can be a major reason for *P. aeruginosa* dominance in the later stage of infection [98]. The different microbial species present in the airways of CF patients can interact mainly in two ways: synergistically and antagonistically:

8.1. Synergism

The colonisation by bacterial species in CF patients follow succession [92]. *S. aureus*, mainly found in the lungs of infants make the environment favorable for further infection by *P. aeruginosa* and this is one of the historic evidence of synergism [99, 16]. Co-infection of mucoid strain of *P. aeruginosa* along with rare bacterial species exacerbated the

infection as compared to the mucoid strain infection alone [92]. *P. aeruginosa* promotes infection of *B. cepacia* by upregulating the expression of its virulence factor [100].

8.2. Antagonism

A kind of hostile relationship has also been evident between *S. aureus* and *P. aeruginosa*. It has been found that *P. aeruginosa* emanates certain factors which hinder the growth of *S. aureus* [101] or sometimes *P. aeruginosa* use the iron released by breakdown of *S. aureus* cell for their own growth [102]. On the other hand, *S. aureus* utilizes 4-hydroxy-2-heptylquinline-N-oxide to protect itself from aminoglycosides based antibiotics [103]. Two kinds of antagonisms behavior have mainly been observed. First one includes the formation of bacteriocins. Bacteriocins are chemical substances released by host bacteria to inhibit the growth or completely kill the bacteria belonging to another strain or species [104]. However, bacteriocin production requires input from the bacterium own machinery and due to this it results in diminished growth of the host bacterium, which can affect its virulence capacity [105].

The second one includes the progression of cheats, which led to the collapse of existing cooperative relationship between two bacterial species. When two microbial species are in communal relationships, they secrete certain products like degradative enzymes, iron-foraging sideophores, and toxins etc which are beneficial for growth of both [106]. Further, one of the microbial species became a hoaxer and utilizes the product for its own growth without contributing in its formation. If the population density of this kind of cheat reaches to a certain concentration, it will have significant impact on the growth of its counterparts and the amount of metabolite produced [107].

The metabolite produced during cooperative relationships may be required for the virulence and such antagonism can lead to decrease in pathogenicity of infection [108]. Antagonistic relationships has also been observed between the CF pathogens and natural microflora of host present in different part of the host body (Example- Gut-associated microflora, skin, oral cavity) etc. It was found that a healthy gut microflora hinder the growth and helps in elimination of *P. aeruginosa*. The feeding of mice with natural yoghurt or augmented with *Lactobacillus casei*, enhance the phagocytic activity of alveolar macrophages, which in turn aid in the elimination of *P. aeruginosa* present in the airways [109].

9. Conventional treatment of the CF

The CF disease mainly involves clogging of the respiratory tract with mucus which can lead to the repetitive infections and inflammations [110]. Most of the conventional treatments of CF target the clearance of the mucus from the respiratory tract. Drugs and therapies used are categorized on the base of the mode of their action.

9.1. Agents targeting mucus clearance

The lysis of neutrophils occurs in the lungs leading to the release of large quantity of eDNA, as a part of the human immune response to CF infections and this leads to a more viscous sputum. Deoxyribonuclease helps in the digestion of this extracellular DNA in turn aiding the process of clearance of the respiratory tract [111]. It further helps in reducing the severity of infection as well as improves lung functions. The early administration of Dnase in children of age less than 2 years helps in improving their nutritional well-being with respect to body mass index [111]. The CF leads to hyper inflammation of airway epithelial cells which in turns secretes increased amount of chemo attractants IL-8 for neutrophils which seems to worsen neutrophils mediated inflammation [112]. Airway epithelial with inadequate CFTR secretes smaller amount of glutathione [113] which is essential for counteracting the reactive oxygen species by oxidative processes produced in response to infection. The N-acetyl-L-cysteine elevates the amount of antioxidant glutathione and helps in preserving the inflammation of tissues caused by hyperactivity of neutrophils.

9.2. Approaches to rehydrate CF airways

One of the main pathology associated with CF is the thickening of mucus in the airways of patients because of the defect in CFTR as it plays an important role in hydration of airway surface liquid [9]. The defective CFTR leads to increase epithelial sodium channel mediated hyperabsorption of sodium [114] and causes increased dehydration of airway surface liquid leading to accumulation of thick mucus [115]. This thickening of mucus provides a favorable environment for bacterial colonization [116]. Hypertonic solutions help in rehydrating the airways by restoring ion balance and maintaining mucus osmolality [117]. Another mechanism by which hypertonic solution aids in mucociliary clearance involves induction of coughing episodes which help in removal of deposited material in the tract [118]. It has been indicated via a study that 6% hypertonic saline solution given for 16 weeks helps in improving lung function [119]. However, the treatment continued for long failed to show any further improvement but some incidences depicted extended intervals in the occurrence of pulmonary exacerbations with the use of hypertonic saline solution [120]. The

dose of hypertonic saline solution depends on the limit of patient's tolerability with an upper limit of 12% and beyond this it causes irritation in the throat region as per the study conducted by Robinson and colleagues [121].

Another reported hydrating agent is mannitol which helps in stabilizing an osmotic gradient on the surface of the airways as well as aids an increase in the volume of surface liquid. Introduction of mannitol in CF patients increases the sensitivity of antibiotics tobramycin on mucoid *P. aeruginosa* forming biofilms [122]. Two comparative phase III trials was conducted of 26 weeks duration which involved inhalation of mannitol by patients and an improved lungs function and reduction in exacerbation episodes were observed [123]. The use of mannitol is considered appropriate by the National Institute for Health and Care Excellence situated in England for those patients who are intolerant or not compatible with treatments of rhDNase or hypertonic saline agent.

9.3. CFTR correctors

Six classes of CFTR correctors (Table 1) have been identified to correct the pathology which arises due to production of defective CFTR protein.

Table 1 Classes of CFTR mutations and their correctors

Mutation types	Description	Devices	Manufacturer
TYPE I	Involves addition of prematurely terminated codon in the sequence which in turn leads to disruption of protein translated from this truncated sequence or the mRNA containing prematurely terminated codon.	PTCI24 (commercialized as Ataluren). Small molecule compound is administered orally.	Translarna® PTC Therapeutics Ltd, New Jersey
TYPE II	Involves the generation of misfolded proteins which are unable to be transported through the membrane. This protein is targeted for premature degradation by endoplasmic reticulum. The mutation ultimately led to decrease in the number of CFTR molecules reaching the cell.	Lumacaftor VX-809, VX-152 and VX-440 are under clinical development. Triple therapy combination studies under clinical phase II includes- VX-152/VX-661/Ivacaftor VX-440/VX-661/Ivacaftor	Orkambi® Vertex Pharaceuticals (Europe) Ltd, UK
TYPE III	Also called gating mutation in which CFTR tunnels got blocked thereby reducing chloride transport efficiency.	Ivacaftor® (VX-770) [USFDA approved]	Kaldeyco®, Vertex Pharmaceuticals Ltd, UK
TYPE IV	Alteration in the transmembrane pore due to substitution of incorrect amino acids in the membrane spanning domain which hinders the conductance of bicarbonate or chloride.	Ivacaftor® has been evaluated for increased survival	
TYPE V	Results in degradation of small portion of mRNA thereby reducing the quantity of CFTR produced.	Treatment under research	
TYPE VI	Increased frequency of recycled CFTR from plasma membrane which is unable to make up with the normal chloride output levels.	Treatment under research	

9.4. Treatment with antibiotics

Antibiotic treatment can be used for prevention or elimination of early infection or suppression of chronic infection or in case of aggravated infection. Tobramycin is manufactured by Novartis Pharmaceuticals, UK and available in the market in two forms either as tobramycin solution given via inhalation or in the form of powder. This antibiotic is mainly used for the suppression or eradication of *P. aeruginosa* infection. Inhaled tobramycin is the first line of treatment in

North America [124]. Colistimethate sodium is manufactured as Colobreathe® by Forest Laboratories Ltd, UK and used mainly in the suppression of severe infection involving *P. aeruginosa*. Aztreonam lysine is manufactured as Cayston® by Gilead Sciences Ltd, USA and used mainly in the case where patients are intolerable to colistimethate sodium or tobramycin. On the basis of 7 controlled randomized trials, this drug was proved to be efficient against *P. aeruginosa* by detecting the change in forced expiratory volume from baseline with an estimated improvement of 3.5% [125]. Other antibiotics used include liposomal amikacin which aids targeted drug delivery and shown to increase forced expiratory volume at both phase II and phase III trials. Solution based levofloxacin has to be inhaled and manufactured as Bronchitol® by Pharmaxis Pharmaceuticals, USA .

10. Drawbacks of conventional treatments

Major challenge faced in conventional treatment is its affordability to the general masses and most of the treatment requires longer duration to show their effectiveness. This leads to the requirement of repeated administration and moreover, in some cases the exact duration and effectiveness of treatment is variable. The severity, with which the disease comes, requires the treatment to be available to each and everyone, but this is a challenging task in reality. The problem of availing recently developed precision medicines become more complex with the increase in the survival rate of patients in Western Europe by 2025 [126]. The traditional treatment in UK include colistimethate sodium, tobramycin etc. [127]. The problem lies in the access of newer medicines to the patients as the lag period between medicine approval, marketing and availability to the patient is very long. Being a matter of international concern, a survey was conducted to estimate both direct and indirect cost in different countries associated with CF treatment, over the past 15 years [128]. The cost of treatment of CF individual was estimated to €19,581.08, €23,330.82 and €68,696.42 in Poland, Bulgaria and the US, respectively.

Ivacaftor® in spite of having great potential and risk capital-based development, reported to be the most expensive treatments. The average cost of Ivacaftor® per patient is reported to be €182,000.The compliance rate for Ivacaftor® in clinical trials came out to be 91% [129]. However, an electronic monitoring conducted by investigators of UK, reported compliance of 61% in comparison to other treatments [130]. These contradictory data puts a big question on its clinical and economic value. Although the precision medicines target the gene defects and are comparatively more successful than gene therapy which are still in clinical trials, but their long-term effects are still unknown and their unaccepted high cost further adds to the problem. Orkambi® which is a combination of Lumacaftor® and Ivacaftor® was scrutinized for its cost and effectiveness in terms of its medical benefits. It costs around € 104,000 per person every year, and the estimated cost covering the entire individual prescribed for Orkambi® would be around €500m every year.

11. Gene therapy for cystic fibrosis

Gene therapy seems to be a convincing approach in the case of CF as it is a single gene disorder which is recessive in nature and can be treated simply by replacement of the defective CFTR gene with the normal one. More than 20 clinical trials have been undergone for gene therapy using both viral and non-viral agents. Viral vectors till date used includes adenovirus, adeno-associated virus, lentivirus and sendai virus. However, various problems have been encountered in viral mediated gene transfer. Neutralizing antibodies are produced by the host immune response with repeated exposure in response to viral capsid. Alternatively, in order to avoid repeated administration of virus, permanently transfect stem cells of the airways with integrating vector. Further, in case of immunodeficient patients it was found that integrated vectors induce oncogenic response. Abnormal toxicity and inflammation have been observed in some case of engineered adenovirus. Virus which don't induce immunogenicity, also don't possess receptors for specific proteins present on respiratory epithelial cells (Lentivirus). Non-viral gene transfer agents like liposome and nanoparticle based encapsulations, seems to generate inflammatory responses by host immune systems as they consist of a high level of unmethylated CpG dinucleotides which are recognized as foreign by the human body [131].

12. Enzymatic treatment for cystic fibrosis

The experiments conducted to recognize the contribution of alginate lyase in disrupting alginate biofilm and reducing sputum viscosity in *P. aeruginosa* brought into light these important conclusion [132].

Microbes responsible for forming alginate biofilm for example *P. aeruginosa* itself express endogenous alginate lyase activity. The reason behind the persistence of alginate even in the presence of alginate lyase can be attributed to the harsh environment in the lungs of CF patients or inhibitory components which either decrease or inhibit endogenous alginate lyase activity.

When the sample sputum of patients were treated with exogenous alginate lyase, about 15% of the sample involved in studies shows disruption of alginate biofilm and regarded as alginate lyase sensitive whereas the rest which were not affected were termed as alginate lyase insensitive.

However, by further analyzing alginate lyase insensitive patients, it was observed that the inhibition of enzyme activity in patient's sample may be due to the following reasons: -

- Production of antibodies in the patient, against the source from which alginate lyase was extracted.
- Proteolytic enzymes may be secreted by pathogens present in infected lungs.
- Molecules like polysaccharides may compete with alginate for the active site of the enzyme.

Sputum samples were also analyzed for the presence of metal ions and many divalent ions, as their presence in the samples seem to affect the activity of the enzyme. The samples were consistent in the presence of Zn^{2+}, Mg^{2+} , Ca^{2+} in alginate lyase insensitive sample though Mg^{2+} reported to help in enhancing the enzyme activity. Electron microscopy images indicate that the presence of increased level of both bound and free divalent zinc and calcium ions in sputum form complex with alginate and brings about changes in its structure thereby inhibiting alginate lyase activity.

12.1. Increased efficacy of antibiotic killing of mucoid *P. aeruginosa* by alginate lyase

The efficacy of treatment involving alginate lyase alone and in combination with antibiotics was compared in a study conducted using 16 biofilms, which were treated with gentamycin and ceftazidime separately. A decline in non-mucoid strain was observed with gentamycin treatment and after 168 h, no bacteria were present in the biofilm. However, bacteria survived in the case of mucoid strains of *P. aeruginosa* under similar conditions [28]. When all biofilm culture were incubated with gentamicin along with alginate lyase, bacterial count falls in those containing alginate lyase and after 120 h incubation, no bacteria was detected in any of the biofilms treated with the combined formulation of gentamicin and alginate lyase [28].

12.2. Coadministration of alginate lyase and Dnase along with free or liposomal aminoglycosides

Presence of actin, mucin, extracellular DNA and alginate biofilm prevents antibiotic diffusion in CF patients. In order to evaluate the potential of DNase and/or alginate lyase with conventional and encapsulated liposomal aminoglycosides (mainly tobramycin, amikacin and gentamycin) in eradicating alginate biofilm mucoid (PA-489121) and non-mucoid (PA-489122 and ATCC 27853) clinical isolates of *P. aeruginosa* were used [133]. The biofilm was formed on Calgary biofilm device (CBD) plates .The non-mucoid ATCC 27853 biofilm was treated with both conventional and liposomal aminoglycosides formulation. Mimium Biofilm Eradication Concentration (MBEC) was observed to increase 16-64 folds in comparison to MIC in case of conventional aminoglycoside whereas the increase was 8-32 fold for encapsulated aminoglycosides. However, the treatment of biofilm by combination of alginate lyase and antibiotics, MBECs for conventional aminoglycosides reduced 2-8 folds in comparison to MIC and 0-4 folds reduction was observed in case of liposomal antibiotics. An increase in MBECs in case of treatment of mucoid PA-489121 biofilms, with encapsulated and conventional aminoglycosides was found to be 64-256 and 128-512 fold in comparison to MICs, respectively. On addition of alginate lyase MBECs for conventional and liposomal aminoglycosides decrease by 4-8 folds. The MBECs of PA-489122 biofilm was increased by 8-32 folds for conventional and 16-128 fold for encapsulated aminoglycosides. However, in this case with the addition of alginate lyase no significant change was observed.

12.3. Effect of DNase and alginate lyase on CF sputum

Although biofilm was not completely eradicated with treatment of DNases and alginate lyase but a noticeable decrease in viable bacterial count was observed. However, on keeping the concentration of DNase and alginate lyase at optimum level and with increase in antibiotic concentration, a decrease in cfu/ml was observed in comparison to antibiotic treatment alone. Further, no difference in bactericidal activity was found with use of free and liposomal antibiotics except with gentamicin and liposomal combination. Co-administration of tobramycin with alginate lyase and DNase found to decrease cfu/ml. Additions of DNase decrease amakacin activity in free or liposomal form, whereas combination of DNase and alginate lyase increases the activity of free amikacin but not with liposomal amikacin.

12.4. Reduction in the immunogenicity and enhancement of catalytic activity of alginate lyase

Alginate lyase has been proved to be an important biotherapeutic agent but being a product of a foreign source (mainly bacteria), its administration in human body induce immunogenicity. Experiments were conducted to develop a polyethylene glycol conjugates of alginate lyase to reduce its immunogenicity inside the patients as well as to enhance its catalytic function [134]. They took A1-III alginate lyase from *Sphingomonas* sp. in study and cysteine residues was

incorporated at 5 different available positions in order to prevent enzyme inactivation. An orthogonal handle was formed to avail the site-specific PEG attachment. PEG is activated by treatment using maleimide for PEGylation.

The 5 of the PEG variants (S32C-his-PEG, A53C-his-PEG, A270C-his-PEG and A328C-his-PEG , A41C-his-PEG were found to exhibit catalytic activity (V_{max}/K_m) greater than or equal to the wild type histine tagged version of the enzyme. The PEG conjugated enzyme has 60% more V_{max} with >2 times increases in catalytic efficiency compared to wild type histidine tagged version. The A53C-his-PEG was found to be 80% more effective with respect to alginate (therapeutic target) as compared to wild type for bacterial biofilm disruption. The PEG variant of the enzyme showed approximately 60-90% decrease in immunogenicity when reacted with anti-A-III Ig antibodies and it bound to considerably lower fraction of human Scfv antibody library in comparison to wild type. The PEG conjugates of the enzyme shown to remove more than 90% of mucoid biofilms which was estimated to be 15% more efficient as compared to wild type. The high catalytic efficiency and low immunogenicity in A53-HIS–PEG version of alginate lyase may prove to be a promising candidate for applications especially for alginate biofilm degradation [134].

However, an extensive study was conducted to contradict the mechanism of enzymatic degradation of alginate by alginate lyase followed by destruction of biofilms. It was observed from the study that the alginate lyase action on biofilm and their contribution in increasing the efficiency of antibiotics is not related to their catalytic activity [135]. The alginate lyase from *Sphingomonas* sp. (A1-III) and its hexahistidine tagged variant (A1-III-His) were purified and used. Catalytic efficiency of both the enzyme on the alginate purified from *P. aeruginosa* doesn't show any major difference. 48% reduction in bacterial count was observed when mucoid FRD1 biofilm were treated with A1-III-His along with tobramycin. Whereas, treatment conducted only with A1-III-His enzyme results in 40% reduction which can be correlated precisely to alginate degradation. However when stand alone treatment of A1-III-His was given to non-mucoid *P. aeruginosa* strain SMC406, bacterial cells viability decreased by 40% which were unpredictable because SMC406 was a non-mucoid strain and it does't synthesize alginate. Ideally it should be unaffected by the treatment with alginate lyase, but on the contrary alginate lyase successfully decreased the count of non mucoid *P. aeruginosa* too.

Double point mutations were performed by targeting two of the active sites of A1-III-His and A1-DM-His. The mutant did not exhibit any catalytic activity on brown seed weed alginate, even at higher enzyme concentration. However, the mutant effectively reduce biofilm by 25% and the biofilm signals were reduced to background level when treatment involves mutant enzyme in combination with tobramycin [135]. Hence this study makes a very contradictory suggestion that antibiotic synergy and biofilm disruption are not essentially related with alginate lyase activity.

12.5. **Cross linked enzyme aggregate to overcome the limitations in oral administration**

Attempts have been made to develop an aggregate of alginate lyase cross linked with glutaraldehyde to overcome the limitations faced during oral administration of the enzyme [136]. Any enzymes suffer frequent inactivation due to the high acidic conditions of the gastric environment. The alginate lyase extracted from *Sphingobacterium multivoram* was co-immobilized with ciprofloxacin in the form of a capsule composed of biopolymeric microsphere. This combination helps in reducing viscosity of the CF sputum and promotes rapid antibiotic diffusion [41]. The major hurdles in the oral administration of such formulation include enzyme denaturation above 37ºC and below pH of 3. Enzymes were also found sensitive to the formulation constituents [41]. Cross link enzyme aggregates were synthesized using glutaraldehyde along with bovine serum albumin to minimize the denaturing effect of glutaraldehyde. Cross linked enzyme yield increased by 4.5% with the use of bovine serine albumin, where cross linking enzyme in combination with low methoxy pectin, the cross linked enzyme yield increased by 14.7%. Cross linked alginate lyase formulation with bovine serum albumin and low molecular weight pectin when incubated under conditions, pH 1.2-8.2 which will otherwise denature the native enzyme. Cross-linked enzyme was found to active even at a pH of 3.0 and activity is reduced by only 30%, when incubated at pH 1.2 and 2.0, under the same condition. On the contrary activity of the native enzyme was reduced by 60% at pH 4 and it becomes inactivated when incubated at pH below 3.

On accounts of thermal stability, cross-linked alginate lyase have shown 50% higher activity than native enzyme at 40ºC, 70% higher activity at 45ºC and 10% higher activity when incubated at 60ºC. The activity of cross-linked enzyme, incubated at pH 1.2 (gastric environment) and temperature 40-45ºC (body temperature of patient having fever) was measured in order to mimic the internal environment of patient suffering from CF. These condition caused denaturation of the native enzyme but the cross linked enzyme showed 52.6±5.0% and 43.0±2.1% residual activity at 40 and 45ºC, respectively. The concentration of alginate in CF subjects was reported to be 2% w/v and cross-linked enzyme was efficient in viscosity reduction of alginate solution by 25%. Hence, the cross-linked enzyme can be considered as a potential alternative of native alginate lyase as biotherapeutic agent. It has been also suggested that due to comparatively high stability of cross-linked aggregate than free enzyme, cross-linked alginate layse has the potential

for oral administration taking into consideration various obstacles that can be encountered during oral administration [136].

13. Future prospects

Enzymatic treatment holds a chance to be more effective and beneficial than gene therapy treatment in the near future with certain advancement because the mechanism of action and the results of enzyme based treatment are more predictable in comparison to unpredictable outcomes of genetic treatments. It has an added advantage of being cost effective and do not pose any potent threat to the patient's body if the treatment goes ineffective which is not the case with other treatments used currently. The catalytic and non-catalytic efficiency of alginate lyase has been investigated and proved to be effective against *P. aeruginosa* biofilm which is the main causative agent of chronic infection in CF patients. The problems which need to be addressed in order to make them more effective than the existing one include focusing on reducing its immunogenicity with the development of novel conjugates and efforts should be made to discover new sources with greater enzyme stability which can withstand varying environment condition within the patient's body. Moreover, the current enzyme therapies used in combination with antibiotics can be made the target oriented in order to increase the efficacy of the treatment.

14. Conclusion

The average life expectancy of the CF patient in US is 40 years whereas in Canada and UK it is 44 years or more. Advance treatments no doubts helped in extending the life span, however due to cost related issues and non-availability of advance treatments like CFTR modulator to the patients in under-developed or developing countries makes treatment very difficult and most of the patients die in their early teens. Moreover, in spite of availability of advance treatments at genetic levels, fatality rate accounts for 90% in case of the CF patients. The only measure left in case of chronic stage CF is a lung transplant, which demands for the urgent development of novel treatments, which should increase person's life expectancy complimented with lesser complications, reduced sufferings along with an eye on ethical concerns.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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