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Pulmonary Inflammation in Cystic Fibrosis: Impact of Innate Immunity and Estrogen

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Citation
Cystic fibrosis (CF) is a multisystem disease, affecting many organs including the liver, intestines, respiratory and reproductive tracts, bone, heart, spleen, gall bladder, and pancreas (Table 1) [1]. It is the pulmonary manifestations that account for significant morbidity and mortality in patients with CF [2]. The CF transmembrane conductance regulator (CFTR) protein is central to CF disease. CFTR is a cyclic adenosine monophosphate-activated, adenosine triphosphate-binding cassette transporter protein (Figure 1). Expressed in submucosal glands and the apical membranes of epithelial cells in the liver, pancreas, intestines, reproductive tract, and lungs, the CFTR normally functions as a chloride channel. Individuals with CF have mutations in the CFTR gene. More than 1800 CFTR mutations have been identified to date. A subgroup of CFTR mutations are disease-causing and, as CF is an autosomal recessive disease, two alleles with such mutations are required to cause the disease. CFTR mutations can be grouped into six classes (I–VI) depending on whether they affect the expression, processing, or activity of CFTR, or a combination of these [3,4]. For example, the class III glycine to aspartic acid mutation at codon 551 (G551D) leads to a CFTR channel defect, whereas the class II deletion of phenylalanine mutation at codon 508 (ΔF508) results in a CFTR protein that is aberrantly folded and defectively processed in the endoplasmic reticulum. CFTR alleles with the ΔF508 mutation account for approximately 70% of mutated CFTR alleles worldwide; 64% of the Irish CF population is homozygous for ΔF508, while 94% carry the ΔF508 mutation on at least one chromosome [5].

Gender differences in CF
Gender dichotomy is recognized in CF disease, with female patients having poorer survival, worse lung function, and earlier colonization with Pseudomonas aeruginosa [6–17]. The lack of an explanation for this disparity coupled with a narrowing of the gender gap in recent times has sparked
debate about whether such a gap ever existed, or whether the improvements observed can be explained by therapeutic advances or a greater compliance with therapy among female patients. Interestingly, Masterson et al. studied adherence to infection control guidelines and medical therapy in a cohort of patients with CF, and found that although age-related differences exist, gender is not a significant factor associated with treatment adherence [18]. However, it is clear that female gender is a negative prognostic factor in CF, a finding that has been demonstrated in several countries, registries, and CF care centers. Two recent studies found no difference in survival between genders [19,20]; however, Olsen et al. did acknowledge that female patients with CF are at a higher risk of \textit{P aeruginosa} and \textit{Burkholderia} spp. colonization, require more intensive treatment with antibiotics, and have a greater rate of hospitalization compared with male patients [19]. It was noted in that study that a gender gap may appear after adolescence, which is the time-frame within which other published studies have shown such differences to exist [20]. Studies examining the major female hormone 17\(\beta\)-estradiol (an estrogen; \(E_2\)) and its effect on infectious, inflammatory, and immune consequences in the CF airway are ongoing in an attempt to explain these fundamental gender differences. The next decade may, in fact, provide explanations to these long-acknowledged observations in CF disease.

**Clinical management of CF**

CF is diagnosed by the presence of relevant clinical symptoms in at least one organ system together with evidence of CFTR dysfunction. The latter is satisfied by elevated sweat chloride levels measured on two separate occasions (>60 mmol/L), the identification of two disease-causing \textit{CFTR} mutations by DNA analysis, and abnormal nasal potential difference (NPD) or intestinal current measurements (ICM) [21].

Clinical disease is described as “classic”, with patients having multi-system involvement, or “non-classic”, with
patients in this group – despite fulfilling the diagnostic criteria for CF – having intermediate sweat chloride concentrations (40–60 mmol/L) coupled with disease limited to a single organ system. Features of the disease originate from the basic CFTR defect of impaired chloride ion transport and epithelial sodium channel-driven sodium hyperabsorption leading to thick, viscid luminal mucus and a high salt content in sweat. Great strides have been made in CF treatment over the last two decades, and this is attributable to improvements in clinical trials and a better understanding of disease pathophysiology at a molecular level. What remains central to the effective management of CF is a multidisciplinary approach to patient care. Although the disease is characterized by gastrointestinal, respiratory, and other organ involvement, the remainder of this review focuses only on the pulmonary manifestations of CF.

**Respiratory disease in CF**
The pulmonary manifestations of CF are responsible for significant morbidity and mortality and can encompass a spectrum of symptoms ranging from persistent productive cough associated with mucopurulent sputum, to chronic rhinosinusitis with nasal polyposis. Advanced irreversible bronchiectasis is observed with disease progression. The airways become colonized with a range of microorganisms including *Staphylococcus aureus* and *Haemophilus influenzae* early in life, followed by *P aeruginosa* and *Burkholderia* spp., amongst others, in older patients.

Antibiotics are the mainstay in the management of infective pulmonary CF and can be used to suppress and/or treat infection. The route of administration may vary and, to a large extent, depends upon the clinical scenario and microorganism sensitivities; for example, intravenous antibiotics may be combined with inhaled antibiotics in

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**Figure 1.** Schematic illustration of the CFTR. CFTR is a cAMP-activated ATP-binding cassette protein that functions as a chloride channel. It has two MSDs that form the channel. Activation occurs via the R domain, facilitating ATP binding to the two NBDs, allowing the channel to open and close and transport chloride ions.

ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; CFTR: cystic fibrosis transmembrane conductance regulator; MSD: membrane spanning domain; NBD: nucleotide binding domain; R: regulatory.
the hospital setting. The largest proportion of antibiotics used in CF management is prescribed for acute infective exacerbations. An exacerbation is determined by worsening clinical symptoms and parameters of lung function associated with increased or changing sputum production or purulence.

In the context of an infective exacerbation, oral antibiotics can be appropriate if the exacerbation is mild and the organisms are fully sensitive to the antibiotics used. Oral azithromycin can also be prescribed on alternate days in patients chronically colonized with *P. aeruginosa*. Belonging to the macrolide group of antibiotic compounds, azithromycin possesses anti-infective, immunomodulatory, and anti-inflammatory properties. Although not all proven in the setting of CF, these multiple properties have prompted some CF centers to use azithromycin empirically in patients with deteriorating lung function and clinical evidence of airway inflammation. In addition to azithromycin use in CF patients who are chronically colonized with *P. aeruginosa*, a recent clinical trial was conducted in patients not colonized by *P. aeruginosa* [22]. Although there was no effect on pulmonary function, a significant delay in the interval between exacerbations was found.

Hospital- or home-based intravenous antibiotic therapy is reserved for severe exacerbations, where bacterial resistance to oral therapy has been identified and/or a failure of oral antibiotics to resolve infection has occurred. In the setting of an exacerbation in a patient chronically colonized with *P. aeruginosa*, at least two agents (one with broad Gram-negative and anti-pseudomonal cover) are selected and administered for ≥10 days and up to 4 weeks, or longer if necessary. Antibiotic choices are directed by sputum culture and *in vitro* susceptibility testing results, and, in the majority of cases, include one or more of the following: tobramycin, colistin, ceftazidime, piperacillin plus tazobactam, meropenem, and aztreonam. If methicillin-resistant *S. aureus* is cultured, vancomycin or linezolid are employed; if an isolated *P. aeruginosa* strain shows resistance to tobramycin, amikacin remains an option.

In addition to their occasional use in the setting of an acute exacerbation, nebulized antibiotics are generally reserved for the suppression of infection. Tobramycin improves both forced expiratory volume in 1 s (FEV₁) and reduces exacerbations when used for chronic *P. aeruginosa* colonization in a “1 month on, 1 month off” regimen [23]. The monobactam aztreonam lysine has shown similar clinical benefit to tobramycin in chronic *P. aeruginosa* colonization [24], and trials are underway comparing the two. An alternative choice, usually reserved for patients intolerant to or performing poorly with tobramycin, is colomycin. Although effective, it appears to be inferior to tobramycin [25], while no comparisons with aztreonam have been made to date.

Agents that promote airway clearance are used for the respiratory management of CF. Dornase alfa reduces sputum viscosity, improves FEV₁, and reduces hospitalization time [26], while hypertonic saline hydrates inspissated mucus by drawing water into the airway lumen. This can help to reduce pulmonary exacerbations [27]. Bronchodilators such as β₂ agonists or anticholinergics are further therapeutic options that may be employed in CF patients with varying disease severities.

In addition to macrolides, ibuprofen has been shown to prevent FEV₁ decline with limited side-effects in CF; however, it is uncommonly prescribed because of concerns about the high doses required and its long-term impact on renal function [28]. Other critical backbones in the respiratory care of patients with CF include chest physiotherapy, exercise regimens, and routine prophylactic influenza and pneumococcal vaccinations.

The development of new therapeutic approaches for CF continues at a rapid pace that is best demonstrated by the large number of potential treatments under investigation according to the US CF foundation pipeline [29]; these include antioxidants, protease inhibitors, and anti-
inflammatory therapies. Targeting the basic genetic defect with CFTR potentiators offers significant promise. VX-770 is an agent that increases the activity of surface CFTR channels in patients with the G551D mutation. In Phase II clinical trials, VX-770 was well tolerated and showed improvements in NPDs, sweat chloride levels, and pulmonary function over a 2-week period [30]. With such encouraging early data, the results of Phase III trials are eagerly anticipated, as is the development of similar compounds aimed at the correction of other and more common gene variants seen in CF.

**Pulmonary inflammation in CF**

Pulmonary infection and inflammation in CF are multifaceted processes. Defective chloride ion conductance by airway epithelial cells causes a reduction in the airway surface liquid (ASL) height, an increase in mucus expression and viscosity that facilitates chronic microbial colonization, and an influx of activated neutrophils (Figure 2). Imbalances in the normal protease–antiprotease balance occur because of the presence of excess neutrophil- and bacterial-derived proteases within the CF lung, which can interfere with normal innate immune responses [31]. Pulmonary inflammation in CF is also mediated by proinflammatory molecules such as ceramide, chemotactic tripeptides [32], C5a, and the leukotriene LTB4, which together contribute to the highly proinflammatory milieu in the CF lung. Because of the high numbers of infiltrating neutrophils in the CF lung and their ability to release reactive oxidant species, the redox balance within the CF lung is also altered. This is further exaggerated as a result of reduced glutathione levels on the respiratory epithelial surface in CF [33], and together these imbalances lead to oxidative damage. Additional idiosyncratic factors such as CFTR...
genotype, gender, *Pseudomonas* spp. mucoidy, and co-colonization with *Staphylococcus* spp., *Burkholderia* spp., or anaerobic bacterial species are further contributory factors. These protein factors coupled with the consequence of treatment regimens and exacerbation history all combine to generate a disease requiring very specific personalized medical treatment for each CF case.

Pulmonary innate immune mechanisms include, amongst others, the mucociliary escalator, proteases, antiproteases, antimicrobials, and pattern recognition receptors. The purpose of cilia is to sweep mucus and dirt out of the lungs. The luminal surface of the airway is coated with mucus, which acts as a protective barrier against toxins and pathogens, and normally clears particles and infectious agents from the airways via the mucociliary escalator. Mucus is composed of water, ions, proteins, lipids, and polymeric glycoproteins called mucins. To date, 19 human mucin genes have been identified. The most important airway mucins are the secreted mucins, Muc5AC and Muc5B, which are produced by goblet cells of the superficial airway epithelium. Their expression is increased in the CF lung, and the overall composition of CF mucus is altered as a result of an increased content of macromolecules such as DNA, filamentous actin, lipids, and proteoglycans. Together these contribute to mucus plugging within the CF lung [34,35].

Toll-like receptors (TLRs) are a family of pattern recognition receptors that act as a first line of defense in the innate immune response. Expressed by immune and epithelial cells within the lung and belonging to the TLR/interleukin-1 (IL-1) receptor superfamily, TLRs can recognize and respond to conserved molecular patterns in microbial factors and endogenous danger signals (Table 2) [36]. These pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) provide rapid and potent stimulation of innate immune signaling pathways, which culminates in proinflammatory cytokine expression and communication with the adaptive immune system to effectively eliminate invading pathogens and coordinate the ensuing inflammatory process. The CF lung is a PAMP- and DAMP-rich milieu, represented by microbial-derived factors (bacterial, viral, and fungal) and neutrophil elastase, respectively. Current understanding accepts that the chronic inflammatory phenotype evident in CF airway epithelial cells is due, in large part, to activation of TLRs [37].

The neutrophil-dominated phenotype of the CF lung predisposes to a variety of phenomena. Neutrophil-derived oxidants and proteases cause derangement in the CF lung and overwhelm the normal antiprotease defenses of the respiratory epithelial surface. Neutrophil elastase is the major protease released by neutrophils in the CF lung, where it has significant effects [38]. Not only does it upregulate the expression of other proteases including cysteinyl cathepsins and metalloproteases, but it also inactivates the serine antiproteases elafin and secretory leukoprotease inhibitor (SLPI), and interferes with their immunomodulatory and anti-inflammatory properties [38,39]. Along with other pulmonary proteases, neutrophil elastase can generate bioactive molecules from proteins such as collagen and, in concert with the other principal elastolytic proteases present in the CF lung (proteinase-3, macrophage-derived metalloelastases, and elastolytic proteases expressed by *P. aeruginosa*), it can promote the secretion of mucus and degrade surfactant proteins and antimicrobials. Neutrophil elastase can also directly injure epithelial cells and reduce ciliary beat frequency, cleave complement components and immunoglobulins, and interfere with effective neutrophil killing of microbes. In the CF lung, neutrophil elastase-induced activation of TLR signaling is mediated via epidermal growth factor receptor (EGFR)-ligand generation and EGFR activation [40].

CF displays wide variability in its clinical features and mortality. Correlations between the *CFTR* genotype and disease manifestations and progression are weak overall, thus highlighting that other factors are important. Indeed, genetic modifiers have been reported to play a substantial role
in determining FEV₁. The most promising candidate modifier genes for CF include IL-8, mannose-binding lectin, transforming growth factor-β₁, interferon-related developmental regulator-1, and endothelin receptor type A (reviewed in [41]), while the serpin peptidase inhibitor-A1 Z allele is a risk factor for liver disease in patients with CF [42].

**Female gender differences in CF**

Female gender differences in CF may be related to circulating female hormones such as estrogens. Estrogens are the primary female sex hormone and have a fundamental role during the female menstrual cycle. Estrogens circulate in three natural forms: estrone (E₁), estradiol (E₂), and estriol (E₃), with E₂ being the predominant and most active form in non-pregnant female individuals. Despite being less potently estrogenic, E₁ has importance during the menopause, while E₃ assumes its core function during pregnancy.

**Estrogen receptors and physiology**

Following diffusion across the lipid cell membrane, E₂ binds to its major cytosolic receptors, the transcription factors estrogen receptor-α (ERα) and -β (ERβ). Both ER subtypes are composed of five domains (A–F), each possessing distinct functions (Figure 3) [43]. Domains E and F bind estrogen and facilitate receptor dimerization and interaction with coregulators. Both receptors can localize to the nucleus as a result of nuclear localization motifs in domain D. Once in the nucleus, ERs bind to DNA via domain C and promote gene transcription

<table>
<thead>
<tr>
<th>PAMP</th>
<th>DAMP</th>
<th>TLR</th>
<th>Adaptor protein</th>
<th>Transcription factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacylated lipopeptides</td>
<td>β-defensin-3</td>
<td>TLR1/2</td>
<td>MyD88, MAL</td>
<td>NFκB</td>
</tr>
<tr>
<td>Triacylated lipopeptides</td>
<td>Serum amyloid A, neutrophil elastase, HSP60, HSP70, GP96, surfactant A and D, eosinophil-derived neurotoxin, biglycan, versican, hyaluronic acid, HMBG1, anti-phospholipid antibodies</td>
<td>TLR2</td>
<td>MyD88, MAL</td>
<td>NFκB</td>
</tr>
<tr>
<td>Double-stranded RNA</td>
<td>mRNA</td>
<td>TLR3</td>
<td>TRIF</td>
<td>IRFs, NFκB</td>
</tr>
<tr>
<td>LPS</td>
<td>Bicyclan, heparan sulphate, hyaluronic acid, neutrophil elastase, serum amyloid A, oxidized LDL, fibronectin EDA, fibrinogen, tenasin-C, lactoferrin, β-defensin-2, saturated fatty acids, HMBG1, HSP22, HSP60, HSP70, HSP72, GP96, lactoferrin</td>
<td>TLR4</td>
<td>MyD88, MAL, TRIF, TRAM</td>
<td>NFκB, IRFs</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Anti-phospholipid antibodies</td>
<td>TLR5</td>
<td>MyD88</td>
<td>NFκB</td>
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<tr>
<td>Guanosine- and uridine-rich single-stranded RNA</td>
<td>IgG-chromatin complexes</td>
<td>TLR9</td>
<td>MyD88</td>
<td>IRFs, NFκB</td>
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via their transcriptional activation domains in the A/B region. While E₂ mediated changes in gene expression take several hours (so-called “genomic” effects), E₂ can also exert rapid influences upon cells within seconds mediated by ERα and ERβ and by the genetically and structurally unrelated G-coupled protein receptor-30 [44]. With respect to genomic signaling, ERs modulate gene expression by hormone–receptor complex interaction with estrogen response elements in the promoter or enhancer region of target genes. Interestingly ER homo- or heterodimers can activate or inhibit gene expression depending on cell type [45]. As both ERs are targets for post-translational modifications including phosphorylation, acetylation, nitrosylation, and ubiquitination, their transcriptional activity can be subtly modulated in a context-specific manner.

**Estrogen receptor localization and function**

The distribution of the ERs varies throughout the body. In some organs, both receptors are equally expressed, whereas, in others, one subtype predominates (Table 3) [43]. ERα predominates in the reproductive tissues, bone, liver, and kidneys, while ERβ is more abundant in the colon, bladder, and lung. The effects of E₂ after exposure are plentiful and extend beyond its well-described reproductive role. For example, it regulates carbohydrate and lipid metabolism, skeletal homeostasis, and functional cerebral integrity. Its role within the pulmonary environment is a subject of intense ongoing investigation by our group and others, particularly in the context of chronic inflammatory lung diseases such as CF [46]. The complex role of estrogens in inflammation is best summarized by its “inflammatory paradox”: inhibitory and anti-inflammatory in some contexts versus proinflammatory at other times (e.g. autoimmunity) [47]. Estrogenic effects in a given environment are dependent on a number of criteria including the immune stimulus, variability of ER expression, target organ microenvironment, the cell type, the timing and concentration of E₂ exposure, and intracellular metabolism.

As estrogen plays an important (albeit complex) role in inflammation, it is not surprising that epidemiological and immunological evidence suggests a role for estrogen in the cause and course of chronic inflammatory diseases such as CF. Increased expression of ERβ has been linked to oxidative stress, hypoxia, and trauma [48–50]. We have shown that ERβ is also dominant over ERα in CF bronchial epithelium. Within this context, it makes sense that ERβ is the predominant ER within the chronically inflamed CF airway [46].

**Contrasting roles of E₂ in CF and asthma**

Our group has demonstrated that, in the female CF airway, high circulating E₂ levels confer TLR hyporesponsiveness to a range of bacterial agonists manifested by an inhibition of IL-8 release. The mechanism by which this phenomenon occurs is through ERβ-mediated upregulation of SLPI, a serine antiprotease expressed in the respiratory tract, which also has antimicrobial, anti-
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Inflammatory, and immunomodulatory properties [46]. SLPI competitively inhibits nuclear factor-κB (NF-κB) p65 subunit binding to DNA, thereby inhibiting the transcription of NF-κB-regulated genes such as IL-8 (Figure 4) [51]. Additionally, NF-κB has been shown to interact with ER pathways in non-CF environments. For instance, in monocytic cells and macrophages, E2 can block lipopolysaccharide-induced NF-κB activity [52]. As E2 can play an anti-inflammatory role in both CF and non-CF contexts, we hypothesized that, although uncontrolled chronic inflammation is damaging over a prolonged time period, surges of acute inflammation in the setting of an acute bacterial exacerbation in CF may, in fact, provide protection. This “protective” acute inflammatory burst is compromised during E2 exposure in female patients with CF and, taken with the fact that E2 compromises the ASL, high circulating E2 levels in female patients can confer both a higher initial risk of infection and a subsequent blunted response to such infection [53]. Whether similar events occur during viral exacerbations remains to be determined.

While the hypothesis described above suggests that estrogen is one of the important factors involved in the gender gap in CF disease, several other gender-specific co-variables may also play roles. Nonetheless, an E2-mediated hyporesponsive inflammatory response helps explain why some anti-inflammatory agents in CF such as the LTB4 antagonist have been unsuccessful during clinical trials, with an increased infection rate seen in the treatment arm, resulting in premature trial termination (ClinicalTrials.gov identifier: NCT00060801) [54]. Conversely, improvements in pulmonary inflammation have been demonstrated with long-term high-dose ibuprofen in milder disease; however, the underlying mechanisms for these effects in CF remain to be further elucidated [28]. Evidently, the context and timing of administration of an anti-inflammatory agent requires careful thought.

Asthma is another chronic airway-based disease in which exacerbations have been shown to follow hormonal variations in female patients. A role for E2 in the pathophysiology of asthma stems from work illustrating an increased risk of the condition in post-menopausal women using hormone replacement therapy [55], and cyclical changes in airflow and gas transfer over the menstrual cycle in affected patients [56]. However, in contrast to CF, E2 exposure appears to have a protective effect on the asthmatic airway; female patients with asthma experience increased exacerbations, hospitalizations, and reduced pulmonary function when E2 levels are low. An explanation for these findings may lie in the fact that E2 relaxes airway smooth muscle via its rapid effects on intracellular calcium [57]. In addition, during natural menstrual cycles, exhaled nitric oxide, a marker of airway inflammation, is markedly reduced, reiterating the anti-inflammatory, protective role of E2 in asthma [58]. Interestingly, these effects were abolished with oral contraceptive (OCP) use and support the concept of “catamenial asthma” (i.e. exacerbations during menstruation, a time of low E2). It would be interesting to know the effect of OCP use in the context of CF lung disease. In the asthmatic lung where the ASL is not compromised, the anti-inflammatory effect of high E2 levels may protect and relax the airway and prevent exacerbations. In CF, the already diminished ASL undergoes further dehydration upon E2 exposure, increasing the risk of infection while also being less well equipped to cope with infection because of TLR hyporesponsiveness.

Table 3. ER distribution. ERs are distributed throughout the body however predominance of one over the other may be found at particular sites. The major estrogen receptor within the lung is ERβ.

<table>
<thead>
<tr>
<th>ER</th>
<th>Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>CNS, CV system, breast, liver, urogenital tract, bone</td>
</tr>
<tr>
<td>ERβ</td>
<td>CNS, CV system, lung, breast, GI tract, urogenital tract, bone</td>
</tr>
</tbody>
</table>

CNS: central nervous system; CV: cardiovascular; ER: estrogen receptor; GI: gastrointestinal.
Figure 4. The effect of E$_2$ within the CF airway. **A:** In states of low circulating E$_2$, microbial agonists stimulate TLRs resulting in the activation of NF-$\kappa$B and subsequent release of the neutrophil chemokine IL-8 attracting neutrophils to the airway with the aim of bacterial clearance; **B:** During high circulating E$_2$ levels and following infection and exacerbation, a blunted response to microbial agonists occurs resulting in diminished luminal IL-8 and a hyporesponsive immune state due to the upregulation of SLPI that inhibits NF-$\kappa$B binding to DNA.

E$_2$: 17$\beta$-estradiol; ER$\beta$: estrogen receptor-$\beta$; IL-8: interleukin-8; NF-$\kappa$B: nuclear factor-$\kappa$B; SLPI: secretory leukoprotease inhibitor; TLR: toll-like receptor.
Unraveling further effects of E2 on airway epithelial and immune cell physiology will lead to a greater understanding of how hormonal control can impact on the pulmonary manifestations of CF. Conceivably, this may lead to new treatment regimens to address the CF gender gap.

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