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DNA methylation changes in cystic fibrosis: Cause or consequence?

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Abstract

Twin and sibling studies have shown that lung disease severity is variable among cystic fibrosis (CF) patients and affected to the same extent by genetic and nonheritable factors. Genetic factors have been thoroughly assessed, whereas the molecular mechanisms whereby nonheritable factors contribute to the phenotypic variability of CF patients are still unknown. Epigenetic modifications may represent the missing link between nonheritable factors and phenotypic variation in CF. Herein, we review recent studies showing that DNA methylation is altered in CF and we address three possible factors responsible for these variations: (i) overproduction of reactive oxygen species, (ii) depletion of DNA methylation cofactors and (iii) susceptibility to acute and chronic bacterial infections. Also, we hypothesize that the unique DNA methylation profile of each patient can modulate the phenotype and discuss the interest of implementing integrated genomic, epigenomic and transcriptomic studies to further understand the clinical diversity of CF patients (Graphical Abstract).

KEYWORDS

cystic fibrosis, DNA methylation, enhancers, infection, phenotypic variability, reactive oxygen species

1 | CYSTIC FIBROSIS

Cystic fibrosis (CF, OMIM 219700) is a monogenic disease that results from the impairment of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride and bicarbonate channel expressed at the apical membrane of epithelial cells.¹ The defective protein results in thick, sticky and obstructive mucus in various epithelial tissues together with altered innate and adaptive immune function. Accordingly, CF patients are characterized by a multisystem disease affecting the respiratory, digestive and reproductive systems. Morbidity and mortality are mainly due to the progressive loss of lung function.

Twin and sibling studies have shown that lung disease severity is variable among CF patients and affected to the same extent by genetic and nonheritable factors.^{2,4} The genetic factors responsible for this phenotypic variability include the type of mutations in *CFTR* and polymorphic DNA sequence variants at modifier genes, other genes that modulate the patient phenotype.¹ Nonheritable factors

comprise the exposure to environment and stochastic events.² The genetic factors have been thoroughly assessed,¹ whereas the molecular mechanisms whereby nonheritable factors contribute to the phenotypic variability of CF patients are still unknown. Herein, we posit that epigenetic modifications may represent the missing link between nonheritable factors and phenotypic variation in CF and we discuss the role of DNA methylation.

2 | DNA METHYLATION IS ALTERED IN CYSTIC FIBROSIS

Recent studies have shown that the DNA methylation profile was altered in nasal epithelial cell samples, whole blood samples and lung macrophages from CF patients.⁵⁻⁸ Specifically, gain and loss of DNA methylation at multiple cytosine guanine (CpG) dinucleotides were over-represented in cis-regulatory sequences (enhancers) and were preferentially found at genes that encode relevant proteins for the

affected tissues.^{6,7} In nasal epithelial samples, differentially methylated CpG sites were over-represented at genes important for cell adhesion and the inflammatory and immune responses.⁶ In lung macrophages, the methylation change targeted genes relevant for phagocytosis.⁷ Also of interest, DNA methylation levels at multiple CpG sites correlated with lung function, namely forced expiratory volume in 1 second, in nasal epithelial samples.⁶ The causes of DNA methylation changes in CF are unknown, nor is explained the correlation between methylation levels at certain CpG sites and patient lung function. We propose that DNA methylation changes (gain and loss) in CF are the consequence of the disease and address three possible factors: (i) overproduction of reactive oxygen species (ROS), (ii) depletion of DNA methylation cofactors and (iii) susceptibility to acute and chronic bacterial infections.

3 | OVERPRODUCTION OF ROS

ROS are oxidative molecules that are released as the natural byproduct of oxygen metabolism and are produced in large amounts during the inflammatory and immune response. Overproduction of ROS has been detected in CF tissue samples. Levels of malondialdehyde, a byproduct of peroxidation, were increased in sputum and exhaled breath condensate samples from CF patients vs healthy controls.⁹ Malondialdehyde levels in sputum samples were also higher in CF patients with severe pulmonary disease than in patients with mild-to-moderate disease.⁹ High levels of ROS in CF samples can result from the massive migration of neutrophils in the airways,¹⁰ the altered glutathione transport (CFTR being a glutathione transporter),¹¹ and the intrinsic mitochondrial defect that results from CFTR impairment¹² (Figure 1).

It is now well established that under oxidative stress condition, ROS induce DNA damages (single base damage, single strand breaks and double strand breaks) and promote epigenetic changes.¹³ Two molecular mechanisms have been proposed to explain the ROS-associated epigenetic changes.

First, DNA methylation changes can be the consequence of specific single base mutations^{14,15} (Figure 2A). Oxidation of 5-methylcytosine or 8-guanine in CpG dinucleotides favors loss of DNA methylation: 5-hydroxymethylcytosines are demethylated by the ten-eleven-translocation enzymes, whereas 8-oxoguanines interfere with the recognition of the methyl-CpG dinucleotide by methyl-binding proteins.¹⁴ Conversely, incorporation of halogenated cytosines into CpG sites leads to gain of methylation: halogens at the five position of pyrimidines mimic the presence of a methyl group that, by misleading the DNA methyltransferase (DNMT), foster methylation of the complementary strand.¹⁵

An alternative and nonmutually exclusive mechanism that explains the ROS-associated epigenetic changes refers to the activation of the DNA damage repair system (Figure 2B). High levels of ROS activate the DNA repair system to correct part of the mutational burden.¹³ This activation is associated with chromatin and DNA methylation changes that temporarily stop transcription to facilitate

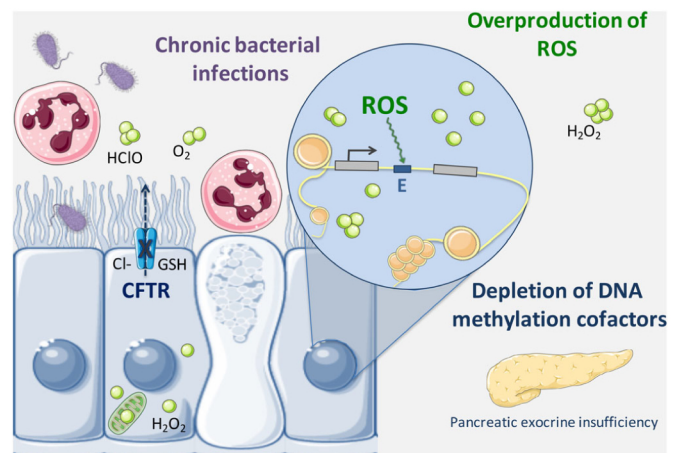


FIGURE 1 Three possible factors responsible for gain and loss of DNA methylation in cystic fibrosis. A, Overproduction of ROS (green bubbles) may result from the massive migration of neutrophils (pink) in the airways,¹⁰ altered GSH transport,¹¹ and intrinsic mitochondrial defect due to CFTR impairment.¹² B, Depletion of DNA methylation cofactors may result from the malabsorption of dietary and biliary phospholipids due to pancreatic exocrine insufficiency. C, The insufficient mucociliary clearance sensitizes the airways of CF patients to colonization by bacteria, leading to chronic infections. DNA methylation changes in CF samples were overrepresented in active genes and in cis-regulatory sequence (E, enhancer).^{5,6} This figure was created with images adapted from Servier Medical Art (Servier). Original templates are licensed under a Creative Commons Attribution 3.0 Unported License (<https://smart.servier.com/>). CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; GSH, glutathione; ROS, reactive oxygen species

recognition and repair of DNA damage.¹³ It has been suggested that, in cancer cells, these transient epigenetic changes at damage sites occasionally do not go back to normal levels and become mitotically stable epigenetic alterations.¹³ Similarly, because they are chronically exposed to inflammation, CF airway tissues may undergo transient epigenetic changes at genomic damage sites and accumulate DNA methylation changes over time (Figure 2B).

All these findings lead us to conclude that high levels of ROS can be a first factor responsible for DNA methylation changes in CF samples.

4 | DEPLETION OF DNA METHYLATION COFACTORS

Genomic DNA is methylated by DNMT and the source of methyl groups is provided by the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH)¹⁶ (Figure 3). The SAM metabolism pathway requires either choline or folate to recycle methionine, the SAM precursor, from homocysteine. Innis et al showed that CF patients have lower plasma levels of choline, betaine and a lower ratio of SAM:SAH than healthy controls.¹⁷ Depletion of these metabolites may result from the malabsorption of dietary and biliary phospholipids

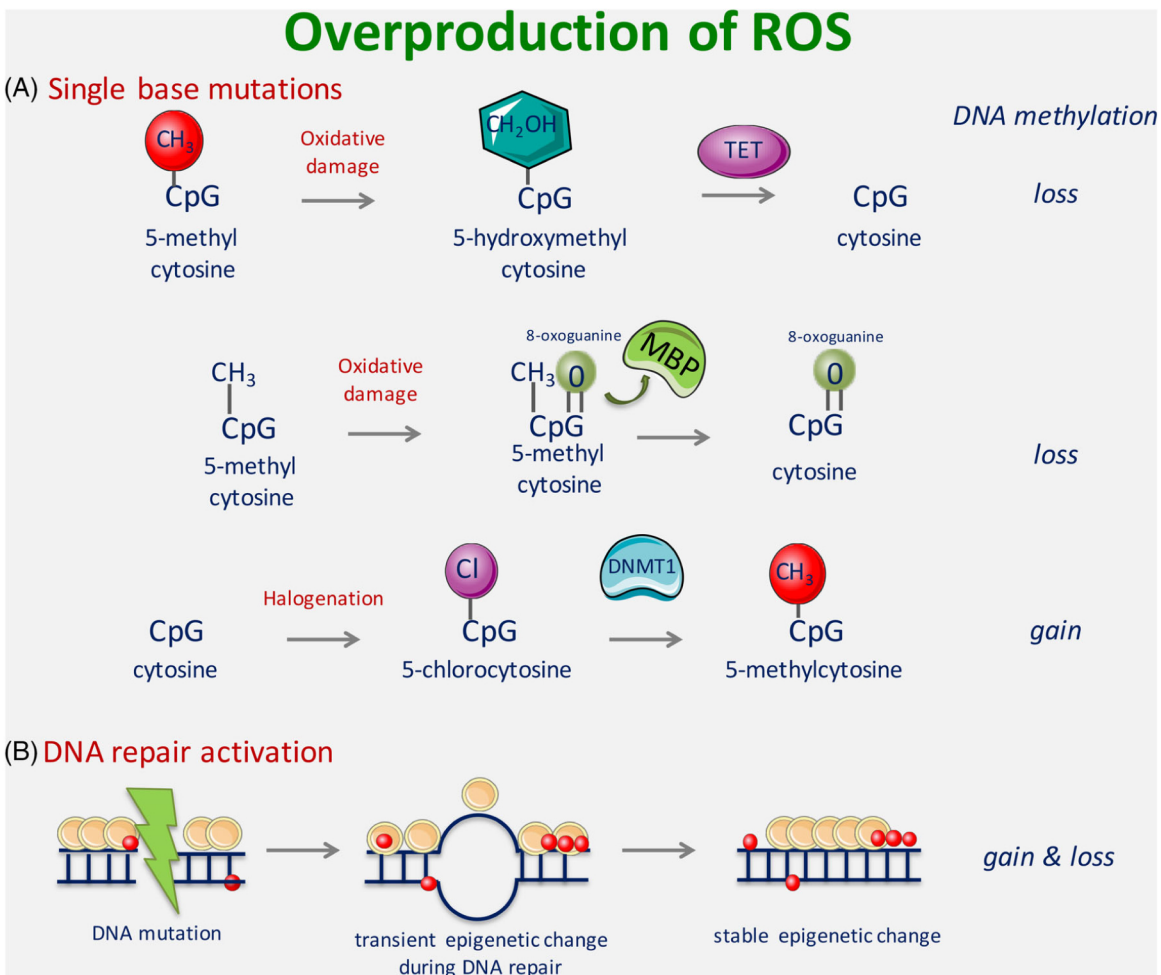


FIGURE 2 Molecular mechanisms whereby overproduction of ROS can alter DNA methylation. A, ROS generate specific single base mutations: 5-hydroxymethylcytosines are demethylated by the TET enzymes; 8-oxoguanines interfere with the recognition of the methyl-CpG dinucleotide by MBP; incorporation of halogenated cytosines into CpG sites mimics the presence of a methyl group that, by misleading the DNMT, foster methylation of the complementary strand.^{14,15} B, ROS activate the DNA repair system, which corrects part of the mutational burden; this activation is associated with transient epigenetic changes at damage sites that occasionally become mitotically stable epigenetic alterations.¹³ CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DNMT, DNA methyltransferase; GSH, glutathione; MBP, methyl-binding proteins; ROS, reactive oxygen species, TET, ten-eleven-translocation

due to pancreatic exocrine insufficiency. To conclude, depletion of methylation cofactors and primarily the low ratio of SAM:SAH is a second factor that may be critical to loss of DNA methylation in CF cells.

5 | SUSCEPTIBILITY TO ACUTE AND CHRONIC INFECTIONS

The insufficient mucociliary clearance sensitizes the airways of CF patients to colonization by bacteria, leading to chronic infections that contribute to the decline of pulmonary function. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are very common opportunistic pathogens in CF. To the best of our knowledge, whether bacterial infections directly modify the DNA methylation profile of eukaryotic

cells in CF is unknown, however, a few studies provide indirect evidence. Bestor showed that DNMT1, the enzyme that methylates hemimethylated DNA during cell replication, acquires a de novo methylation activity after cleavage between the regulatory (N-terminal) and the catalytic (C-terminal) domains by the *S. aureus* V8 protease¹⁸ (Figure 4). It is tempting to speculate that a chronic exposure to *S. aureus* and subsequent accelerated de novo activity of the DNMT1 are responsible for aberrant methylation in CF. Pérez-Novo et al found gain and loss of DNA methylation in cultured nasal polyps treated with *S. aureus* enterotoxin B, but the mechanism whereby the toxin alters the methylation profile of the eukaryotic cells has not been elucidated.¹⁹ Specific studies are required to assess whether opportunistic bacteria modify the DNA methylation profile of CF cells. A major challenge will be to distinguish between a possible direct effect of bacteria on DNA methylation of the eukaryotic host cells,

Depletion of DNA methylation cofactors

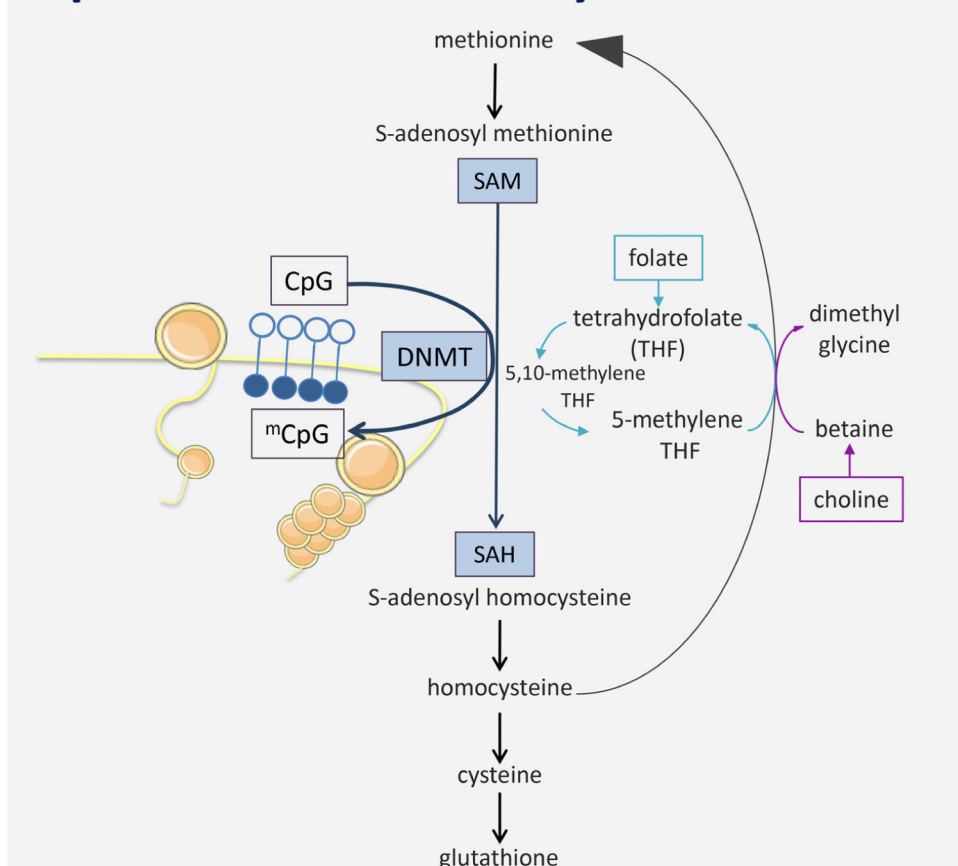


FIGURE 3 Depletion of methylation cofactors can be critical to loss of DNA methylation in CF cells. DNMTs methylate nuclear DNA with methyl groups provided by the conversion of SAM to SAH. A deficiency of either choline or folate may lead to a low SAM:SAH ratio and loss of methylation. ^mCpG is a methylated and CpG is an unmethylated dinucleotide. CF, cystic fibrosis; DNMT, DNA methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine

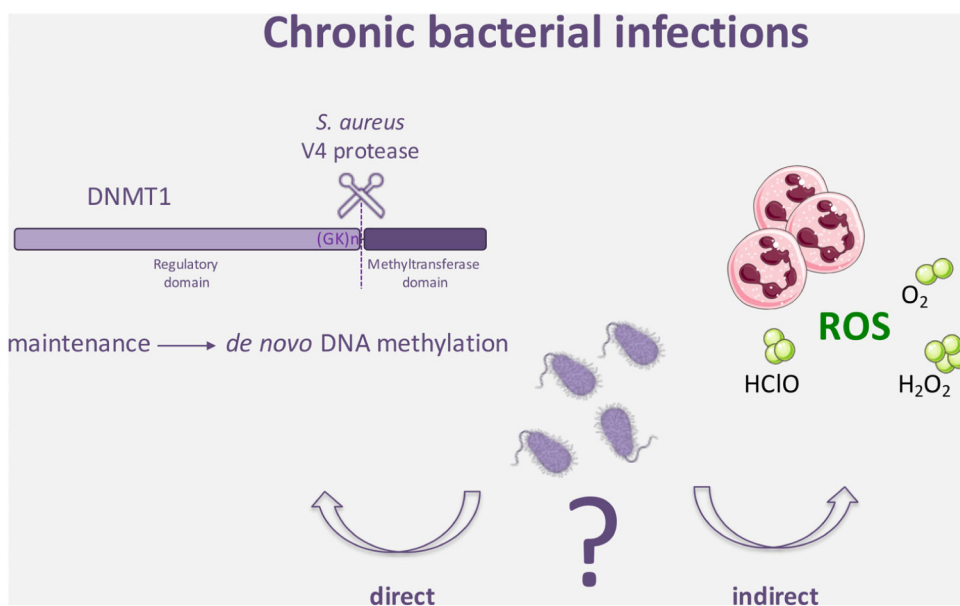


FIGURE 4 “Tentative” mechanisms whereby chronic infections can alter DNA methylation in CF samples. Direct mechanism: DNMT1, the enzyme that methylates hemimethylated DNA during cell replication, acquires a de novo methylation activity after cleavage between the N-terminal regulatory region and the C-terminal catalytic domain by the *S. aureus* V8 protease.¹⁸ (GK)_n are alternating Gly and Lys residues. Indirect mechanism: chronic infections stimulate the innate and immune response; then, the immune system generates ROS that alter DNA methylation as shown in Figure 2. This figure was created with images adapted from Servier Medical Art (Servier). Original templates are licensed under a Creative Commons Attribution 3.0 Unported License (<https://smart.servier.com/>). CF, cystic fibrosis; DNMT, DNA methyltransferase, ROS, reactive oxygen species [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.com)]

and an indirect mechanism whereby bacteria stimulate the innate and acquired immunity and the activated immune cells generate ROS (Figure 4).

6 | DNA METHYLATION CHANGES IN CF PATIENTS ARE THE CONSEQUENCE OF THE DISEASE

Of the three afore-mentioned factors, overproduction of ROS best explains the DNA methylation variations that we and other groups observed in CF samples.

- Overproduction of ROS is consistent with gain and loss of DNA methylation at multiple genomic sites in CF samples (Figure 2). It accounts for the over-representation of differentially methylated CpG sites in active genes and enhancers that are associated with an accessible chromatin thereby are prone to be impacted by ROS (Figure 1). Also, high levels of ROS explain DNA methylation variations in airway tissues, the inflammation sites, and in blood samples since peripheral circulating neutrophils from CF are already primed.²⁰
- Depletion of methylation cofactors (ie, low SAM:SAH ratio) in CF patients can explain loss of DNA methylation, but does not account for gain of methylation, nor does it explain the over-representation of DNA methylation changes in active genes and enhancers (Figure 3).
- Finally, several studies have shown an association between DNA methylation changes and viral or bacterial infections, but the specific molecular mechanism has not been identified.^{19,20} The acquired de novo methylation activity of DNMT1 in the presence of *S. aureus* was merely observed in vitro¹⁸ (Figure 4). It can explain gain but not loss of DNA methylation in CF samples, and does not account for methylation changes in blood cells that are not exposed to bacterial infections.

Overall, these findings provide ground for concluding that DNA methylation variations in CF cells are the consequence of the disease, with chronic inflammation and high level of ROS best explaining the CF-specific methylation pattern.

7 | OTHER FACTORS CONTRIBUTING TO THE DNA METHYLATION PROFILE OF CF PATIENTS

Various factors contribute to the DNA methylation profile in the general population, namely methylation quantitative trait loci (meQTL), lifestyle environmental factors, and stochastic events. meQTL are genetic loci that correlate with DNA methylation levels at specific CpG sites. A comprehensive meQTL analysis in blood samples from >700 participants at five different time points across the life course showed that single nucleotide polymorphic variants accounts for approximately 20% of variance in DNA methylation.²¹ Studies in

human cohorts and animal models have shown an association between DNA methylation marks and lifestyle environmental factors, including smoking, physical activity and pollution.²² Finally, stochastic events can contribute to the methylation variance because of occasional failure of the otherwise faithful maintenance of DNA methylation.²³ meQTL, lifestyle environmental factors and stochastic events do not necessarily explain the DNA methylation differences between CF patients and healthy controls, however, they are expected to contribute to the individual methylation pattern of each patient.

8 | DNA METHYLATION AND PHENOTYPIC VARIABILITY IN CYSTIC FIBROSIS

In the first part of this review, we have provided evidence that DNA methylation changes in CF can be the consequence of the disease. We now posit that the unique DNA methylation profile of each patient can modulate the phenotype, thus contributing to the clinical diversity of CF patients and to the low concordance observed between CF monozygous twins and siblings.¹⁻⁴

Differentially methylated CpG sites between CF patients and healthy controls do not map at random in the genome; rather they are over-represented at active genes and therefore are probably to impact genes of biological relevance for the affected tissue.⁶ In addition, DNA methylation changes in CF samples are over-represented in enhancers, but also present in promoters.^{6,7} Enhancers are long-distance regulatory sequences that modulate gene transcription by interacting with the promoter region via chromatin loops. The interaction between enhancers and promoters is regulated by transcription factors (TF) and chromatin modifier proteins that act together within protein complexes.²⁴ A systematic analysis of 542 TF has shown that the effect of DNA methylation on TF specificity is more widespread and complex than previously established: 60% of the classified TF were affected by DNA methylation.²⁵ Several major classes of TF are inhibited by the presence of methylated CpG dinucleotides in their binding site. The inhibition is mainly due to steric hindrance. Other TF bind preferentially to methylated CpG sites because of a direct hydrophobic interaction with the 5-methyl group of methylcytosines. These findings lead us to hypothesize that the individual methylation profile of CF patients, particularly in regulatory regions, while affecting the affinity of TF, may change the transcription level of genes and, ultimately, modulate the clinical phenotype. We suggest therefore that not only does CF alter DNA methylation, but, very likely, the individual DNA methylation profile of each patient modulates the progression of CF.

9 | CONCLUDING REMARKS AND FUTURE DIRECTIONS

Genetic factors (*CFTR* mutations and modifier genes) do not totally account for the clinical variability of CF patients. Epigenetic

modifications may represent the missing link between noninherited factors and phenotypes. In the future, to better understand the wide variability of CF patients, genome-wide association studies, epigenome-wide association studies, and gene expression analyses should be combined.

Current DNA methylation investigations have been carried out in adult CF patients⁵⁻⁸; in the future, different patient ages should be analyzed. Of interest, the profile of young children with CF may elucidate whether the excessive inflammatory response, which is present from birth, alters DNA methylation prior to chronic infections. Also, it would be of great interest to establish if, similar to asthma, exposure to environmental factors during early periods of postnatal development has long-term effects in adult life.²⁶ Specifically, future studies should address whether exposure to bacterial/viral infections or peaks of hyperglycemia at specific age windows are critical for the phenotype of adult CF patients and the development of comorbidities, such as CF-related diabetes.²⁷ If this occurs, epigenetic modifications may provide the cellular memory of these early events.

In mammals, DNA methylation is predominantly found at cytosines of the dinucleotide sequence CpG (CpG methylation). However, high levels of non-CpG (CpA, CpT and CpC) methylation are present in pluripotent (embryonic stem and induced-pluripotent stem) cell types and neurons, whereas low levels are generally detected in other somatic cells.²⁸ Increasing evidence shows that non-CpG DNA methylation is of functional relevance, namely in neurons during fetal to young adult development²⁹ and in primary human myocytes after exposure to TNF- α or free fatty acids.³⁰ Thus, future efforts should assess non-CpG methylation in CF, focusing on cell types of biological relevance for the disease and with special attention to the fetal to adult developmental period.

Finally, to have a comprehensive epigenomic view in CF, histone modifications should be also profiled. However, histone profiling is still relatively uncommon in clinical studies because of the wide variety of existing histone modifications and the high cost of sequencing samples from many patients.

To conclude, we are at the very beginning of epigenomic studies in CF and there are more questions than answers. Yet, to understand the molecular mechanisms whereby nonheritable factors contribute to the phenotypic variability of CF patients and to find new therapeutic solutions, future studies should address not only genetic but also epigenetic factors.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

N/A Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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