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TITLE

Identification of MMP9 modulation as biomarker related to clinical responses of Cystic Fibrosis patients to CFTR Modulators therapy: new development from bench to bedside

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ABSTRACT

Cystic Fibrosis (CF) is a multi-organ hereditary disease caused by a mutation in the gene coding for Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein. Currently, no specific care is available for CF patients. Although various compounds have been developed to improve CFTR function, clinical responses are variable and sometimes even absent. Therefore, it is necessary to find biomarker to evaluate the effectiveness of CFTR modulators therapy related to the individual patient's response. For this purpose, nasal epithelial cells and intestinal organoids are usually the models used; however, the discovery of CFTR expression in non-epithelial cells and the recognition of inflammation's involvement in CF have raised interest in the role of immune cells in this disease. Additionally, immune cells are easily and quickly isolable from patients, making them a useful cellular model. In this context, we firstly identified changes in the proteomic profile linked to the restoration of CFTR channel activity in CF leukocytes after *ex vivo* treatment with the potentiator VX770. Subsequent bioinformatic analyses revealed the downregulation of proteins within the leukocyte transendothelial migration and regulation of actin cytoskeleton pathways in response to VX770 treatment. In particular, we focused our attention on matrix metalloproteinase 9 (MMP9). Since the high expression of this protease can contribute to CF-related lung damage, its downregulation could be a positive effect of CF therapies in slowing disease progression. To confirm these data also *in vivo*, we used leukocytes isolated from CF patients before and following Ivacaftor therapy. We assessed MMP9 levels by Immunoblotting and CFTR activity by GST-HS-YFP assay. These data showed that the therapy promoted a decrease in leukocyte MMP9 levels together with a recovery of CFTR activity and improved of clinical parameters. Subsequently, we amplified our study and evaluated MMP9 expression in leukocytes from CF patients before and during Trikafta® therapy, the best combination of potentiator and modulators for the most CF patients. Particularly, we could observe that MMP9 was downregulated in the clinically responsive CF patients, while levels remained elevated in non-responder. Moreover, we also assessed MMP9 activity by Zymography on plasma samples from the same CF patients undergoing Trikafta® therapy. The results obtained revealed that the MMP9 levels measured in plasma reflected the same trend observed in leukocytes.

Furthermore, we also analyzed the intracellular signaling pathway associated with MMP9 expression, in particular the modulation of the extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor-kB (NF-kB) pathways. The results obtained allowed us to demonstrate that the modulation of MMP9 following treatment with Trikafta® may be controlled by the NF-kB pathway. Finally, we can conclude that the downregulation of MMP9 expression could be considered a promising biomarker of therapy efficacy, useful to understand the molecular events underlying the variable clinical responses of CF patients to CFTR modulators. This knowledge, obtained with a simple blood withdraw, can be useful for future studies of personalized medicine.

SUMMARY

1. INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive hereditary disease caused by a defect in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein expression. This chloride channel is located in the apical membrane of various organs, including the lungs, bones, liver, pancreas, kidney, intestinal epithelium, sweat glands and deferent ducts. The underlying cause of the disease lies in a defect within the CFTR gene, which is responsible for synthesizing the CFTR protein. Positioned on the long arm of chromosome 7 (7q31.2) [1], this gene's aberration leads to malabsorption and recurrent chronic infections throughout the body, with particularly adverse effects on the lungs. Mutation of the CFTR protein alters the volume and composition of fluids secreted by epithelial cells. Maintaining the proper quantity and composition of fluids within organs is essential for their functionality: in the airways, it aids in the clearance of respiratory epithelium by ciliated cells; in the intestines, it enables proper digestion; and in the pancreas, it facilitates the transport of digestive enzymes [2,3].

In epithelial cells, CFTR plays a key role in various physiological functions. CFTR mutations, or its complete absence in severe cases, primarily results in pulmonary complications such as recurrent airway infections, bronchiectasis, and nasal polyps, as well as intestinal issues. Other notable manifestations include fat malabsorption, growth and developmental delays, reduced bone density, increased vulnerability to kidney stone formation, male infertility, portal hypertension, and effects on the immune response. The most critical aspects of the disease involve pancreatic insufficiency and chronic airway obstruction, which predispose individuals to bacterial colonization, predominantly by *Pseudomonas aeruginosa* and *Staphylococcus aureus* [4].

The CFTR mutation affects the epithelial cells responsible for mucus and sweat secretion. By regulating chloride secretion, the CFTR channel establishes an electrical gradient that draws sodium ions towards the luminal compartments of hollow organs through paracellular secretion. This creates an osmotic gradient that guides water molecules across secretory epithelia via transcellular and paracellular pathways. Under normal conditions, the presence of water leads to the production of a fine layer of mucus. However, in individuals with a CFTR channel mutation, there is a deficiency in

the continuous flow of water molecules. Consequently, this leads to the development of denser, adhesive mucus that predisposes individuals to severe bacterial infections [3].

During the 1960s, the initial approved treatments for Cystic Fibrosis adopted a holistic approach, a strategy that persists in contemporary treatment [5]. Recent advancements in scientific knowledge have significantly enhanced our comprehension of the disease, leading to a remarkable improvement in the longevity of CF patients. In its early stages, CF was regarded as a fatal disease, particularly during infancy. Present-day statistics indicate an average life expectancy of approximately 40 years for individuals with CF, and there are expectations that newborns may experience a further increase in life expectancy, potentially reaching up to 50 years of age [6].

1.1 CFTR PROTEIN

The CFTR gene, located on chromosome 7 (7q31.2), encodes a single polypeptide chain that comprises 1480 amino acids, organized into 27 exons. It possesses a molecular weight of 168 kDa. This particular protein acts as an anion channel located on the apical membrane of epithelial cells. It is responsible for the secretion of chloride, bicarbonate, glutathione, and thiocyanate upon stimulation by cAMP/PKA signaling [3]. Belonging to the ATP binding cassette (ABC) protein family, most of its equivalents employ ATPase activity to transport substrates across membranes. However, CFTR is unique among ABC family members as it forms an ion channel [7]. The CFTR protein consists of four domains: two transmembrane domains (MSD1 and MSD2), each comprised of six helices, and two nucleotide-binding domains (NBD1 and NBD2) [8-11]. Positioned between NBD1 and MSD2 is a regulatory domain (RD), which plays a crucial role in CFTR regulation by second messengers. Notably, it contains two serine residues that can be phosphorylated by Protein Kinase A (PKA). The intracellular portion of CFTR constitutes a significant 80% of the total protein mass and encompasses ATP-dependent gating and PKA phosphorylation sites [12]. Activation of CFTR is triggered by RD phosphorylation through PKA, while gating is facilitated by ATP binding to the NBD domains. Phosphorylation of the RD induces a structural alteration that enhances ATP binding to

the NBD domains [13-15]. This event initiates the formation of the NBD1-NBD2 heterodimer, which interacts with the MSD domains, ultimately leading to a conformational shift that results in channel opening [12]. CFTR is synthesized on ribosomes associated with the Endoplasmic Reticulum (ER) and undergoes folding with the assistance of chaperone proteins like calnexin, Hsp70, Hdj-2, Hsc70, and Hsp90 [16]. Misfolded CFTR is degraded via the ubiquitin/proteasome pathway. Properly folded CFTR undergoes further glycosylation in the Golgi apparatus before being transported to the plasma membrane. CFTR present at the plasma membrane is recycled or degraded by lysosomal proteases through endocytosis.

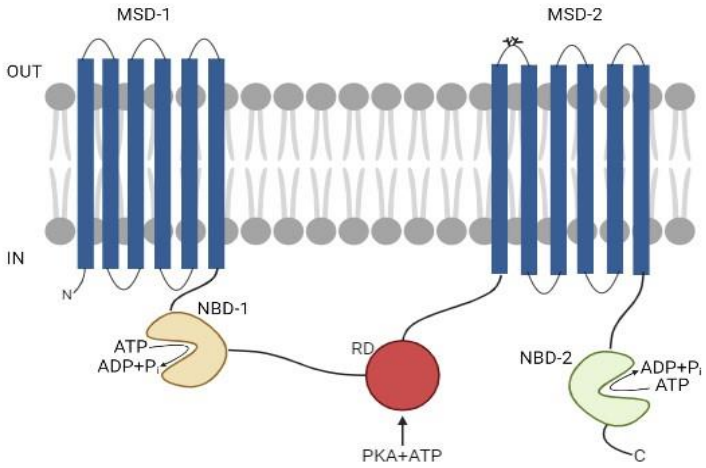

Figure 1. Structure of the CFTR channel.

1.2 ETIOLOGY AND MUTATIONS

Cystic Fibrosis, a widely prevalent hereditary disease, is considered one of the most lethal genetic disorders worldwide. Complications arising from this condition lead to the death of approximately 90% of patients. The global incidence stands at around 70,000 cases, with an additional thousand new cases documented each year (www.cff.org; Cystic Fibrosis Foundation). Remarkably, the North Caucasian population, with a carrier frequency of 1/25, exhibits an exceptionally high occurrence rate, affecting

approximately 1/2500 newborns (Cystic Fibrosis Foundation patient registry: annual data report to the Center directors, 2021), although disparities exist among different ethnic groups.

Due to advancements in medical science, significant progress has been made in improving the condition of Cystic Fibrosis patients. Once primarily perceived as a childhood disease culminating in premature mortality, it has now predominantly transitioned into an adult condition. Experts even predict that by 2025, European countries with robust healthcare systems may witness a 70% increase in the population of adults living with Cystic Fibrosis, owing to heightened life [17].

Over 2,000 mutations of the CFTR protein have been identified, which can lead to the Cystic Fibrosis phenotype. Traditionally, these mutations, causing Cystic Fibrosis, have been classified into six groups based on their specific functional alterations.

The intention behind this classification system (Figure 2) was to categorize mutations causing CF based on potential therapeutic strategies for developing mutation-specific personalized therapies [18]. Approximately one-third of the CFTR mutations associated with Cystic Fibrosis are located in the channel's gating site, situated on the intracellular side of the protein [19]. The most prevalent CFTR mutation is the deletion of three bases encoding a phenylalanine residue at position 508 (F508del). Patients with class I, II, or III mutations exhibit a more severe disease phenotype due to significantly diminished or non-existent channel activity associated with these mutations in comparison to those carrying other mutations.

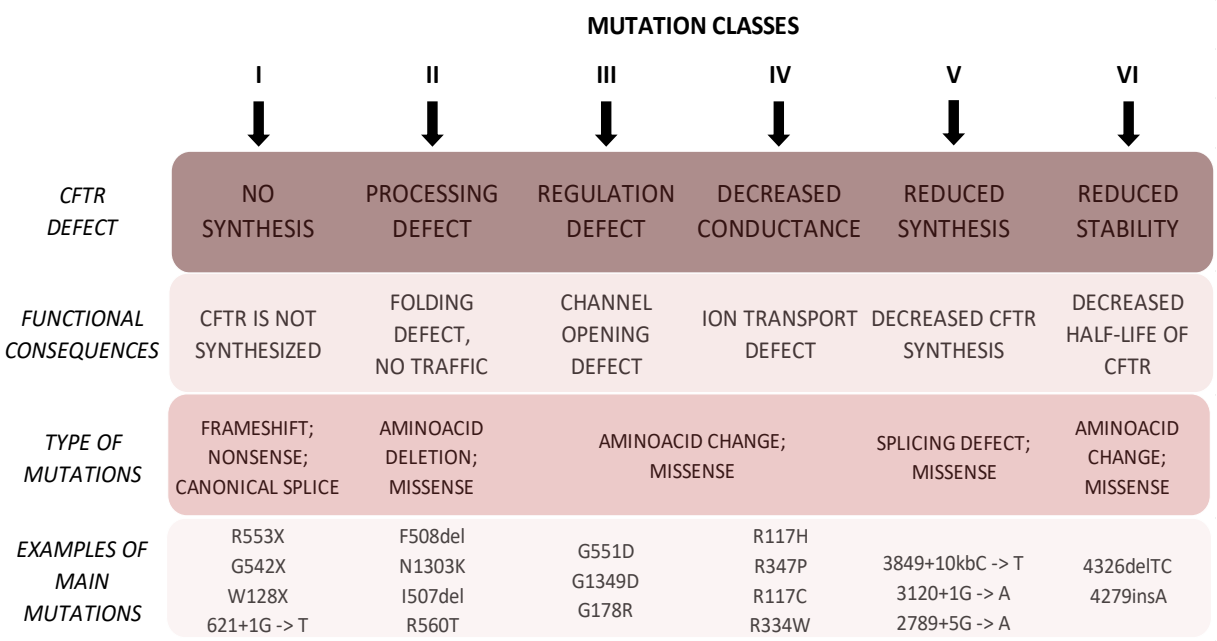

Figure 2. Classification of CFTR mutations. Data sourced from: Boyle, M. P., & De Boeck, K. (2013). A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect. The Lancet. Respiratory medicine, 1(2), 158–163. https://doi.org/10.1016/S2213-2600(12)70057-7.

Class I mutations, arising from deletions, frameshifts, and nonsense mutations, lead to premature termination of messenger RNA, resulting in truncation and instability of the CFTR peptide sequence, followed by rapid degradation. This results in a severe disease phenotype manifested on the cell membrane of affected individuals, where the protein is deficient [20,21]. Among the class I category, R553X and G542X mutations are particularly relevant.

Class II mutations induce alterations in post-translational modifications that impede proper localization of the protein within the cell. Individuals carrying these mutations exhibit a reduced functional quantity of CFTR protein on the cell membrane [20,21]. The most common example of a class II mutation is F508del (also known as ΔF508), which is found in a homozygous state in 50% of Cystic Fibrosis patients and in a heterozygous state in 90% [22]. In this scenario, the mutation affects the NBD1 domain of the protein, impeding its exit from the endoplasmic reticulum and leading to degradation through the endoplasmic reticulum quality control system (ERQC).

Class III mutations lead to a reduction in chlorine channel activity. In individuals carrying these mutations, the protein is correctly located at the cell membrane, but there is a reduction in the timing of channel opening [20,21]. Notable mutations in this class include G551D and G1349D.

Class IV mutations impact the ion permeability pathway mediated by the CFTR protein, resulting in decreased channel conductance and a reduction in the overall rate of cellular anion efflux [23,24].

Class V mutations result in an altered splicing mechanism in the messenger RNA, leading to the translation of a lesser amount of functional protein in comparison to a healthy individual.

Lastly, class VI mutations lead to increased protein turnover due to an alteration at the C-terminal end [22-25].

1.3 DIAGNOSIS AND SCREENING

Cystic Fibrosis is usually associated with a range of symptoms in adults, indeed most individuals exhibit typical symptoms that lead to a diagnosis. This diagnosis is confirmed through sweat testing, a procedure based on Darhling's observation that the sweat of CF patients becomes highly salty under specific stimuli. This test comprises four main phases: inducing sweat production through iontophoresis with pilocarpine, collecting the sweat, quantifying it by volume or weight, and measuring chloride concentration [26]. In routine tests, pilocarpine applied to the skin stimulates sweating, creating a potential gradient to the sweat glands. The collected sweat is then analyzed for Cl^- concentration and sometimes Na^+ as well. A chloride concentration above 60 mEq/L indicates a Cystic Fibrosis diagnosis, while a value between 30 and 60 mEq/L suggests a borderline situation, often related to mutations resulting in partial CFTR function. However, it is not uncommon for some individuals to reach adulthood without any suspicion of the disease due to the vague and difficult to diagnose symptomatology of CF patients [27,28].

In recent years, newborn screening for Cystic Fibrosis has been implemented in various countries with a high prevalence of the condition. The primary goal of these screenings is early detection of CFTR mutations, allowing prompt referral of affected children for appropriate interventions [29]. The screening process involves collecting blood samples from the baby's heel, which are then stored as dried blood spots on specialized filter paper. These dried blood spots are used for testing various conditions, including CF [30].

The screening employs different algorithms, typically following a two-tier approach. Initially, the dried blood spots are tested for levels of Immunoreactive Trypsinogen (IRT), a precursor of pancreatic enzymes present in the blood. IRT levels are usually elevated in CF, as abnormal secretion in the pancreatic duct leads to increased release into the circulation. Consequently, serum IRT levels rise in newborns with CF, regardless of predicted or actual pancreatic function.

The second screening level involves either a second IRT analysis about 1-2 weeks after the initial measurement (IRT/IRT) or a CFTR mutation analysis for infants with an initial IRT concentration surpassing a predefined threshold (IRT/DNA). Babies with positive results from the newborn screening are then referred for further diagnostic tests, specifically the sweat chloride test or molecular genetic testing of the CFTR gene [31].

Moreover, genetic tests using Next-Generation Sequencing (NGS) or Polymerase Chain Reaction (PCR) techniques are typically performed to identify the specific mutation responsible for the channel protein's abnormal function. This genetic information is crucial, especially in devising therapeutic strategies aimed at improving the function of the channel with a specific mutation. Research, therefore, plays a vital role in deepening our understanding of the disease, its progression, and the potential development of therapies that could ultimately reduce the mortality rate and enhance the quality of life for those affected [32].

1.4 STUDY MODELS

1.4.1 Animal models

Since the early 1990s, mice, ferrets and pigs have all served as models for the study of CF, playing a crucial role in comprehending the pathophysiology of Cystic Fibrosis and in advancing therapeutic treatments. This three species approach has collectively provided our comprehension of the disease, ranging from the mechanisms underlying to heightened infection susceptibility to the role of CFTR function.

Among these models, mice share certain similarities with humans in the upper respiratory tract, yet they differ structurally and functionally in their lower airways, rendering them less susceptible to pulmonary infections compared to humans. To overcome this limitation, alternative mouse models have been engineered to simulate the inflammatory and muco-obstructive conditions observed in CF patients [33]. Nevertheless, for a more faithful representation of the human CF lung phenotype, knockout pigs and ferrets have been developed, showing a greater similarity to the disease as it manifests in humans. Currently, ferrets stand as the most reliable preclinical model for studying lung and pancreatic failure as well as intestinal disease.

Despite these significant advances, the utility of CF animal models remains limited in fully replicating the severity and systemic nature of the human disease.

1.4.2 Ex vivo models

To delve deeper into the pathogenesis of Cystic Fibrosis and closely monitor the molecular functioning of the potentially restored CFTR channel after specific therapies, the most valuable cellular models are those directly isolated from CF patients. Currently, three primary *ex vivo* models are central in this research: nasal potentials, organoids, and leukocyte cells.

Nasal potentials are acquired by collecting epithelial cells through a procedure known as brushing. This involves gently swabbing the upper interior of the nasal cavities with a specialized instrument. This minimally invasive method eliminates the need for

anaesthesia and allows for the collection of a substantial number of cells [34]. While nasal epithelial cell culture has emerged as a useful tool for investigating the molecular effects of specific CFTR mutations, primarily due to its similarity to lung cells, it does have limitations. After collection, these cells require a relatively long period to establish a suitable culture for subsequent studies. During this time, changes may occur in cellular arrangement that might no longer perfectly correspond to the phenotype isolated from the patient under investigation.

Organoids, first developed in 2009, are 3D tissues of aggregated cells that closely mimic the phenotype of the organ they were derived from. They consist of progenitor cells cultured in a solid medium (scaffold), arranged in a three-dimensional structure to replicate the cellular distribution within an organ. Organoids offer the advantage of using respiratory and intestinal progenitor cells directly extracted from patients, effectively replicating the disease condition. However, the main challenge with this system lies in the collection process, which involves invasive procedures such as intestinal or respiratory biopsies that require local anaesthesia [35,36].

Thus, current scientific research focuses on the use of leukocytes as an alternative cell model for studying Cystic Fibrosis to overcome the problem of invasive collection and long culture times. This model offers several advantages, including the ease of obtaining leukocyte cell samples through repeated blood draws. The immediate availability of the sample allows for direct analysis of isolated leukocyte cells, making it a suitable model for monitoring disease progression. Additionally, leukocytes obviate the need for protracted handling processes, thus preserving their intrinsic characteristics, facilitating the monitoring of treatment responses in clinical trials [37-39] and assessing of how new compounds affect individual patients in controlled *in vitro* environments. Despite their different characteristics from epithelial cells, leukocytes still express significant levels of the CFTR protein. Numerous studies have shown that CFTR mutations in these cells contribute to abnormalities in the immune response against inflammatory processes. Perturbations in the channel function lead to altered cytokine secretion, impaired phagocytosis of bacteria, and the accumulation of immune cells in the bronchial mucosa of individuals with the disease [40-42].

Moreover, evidence suggests that restoring channel activity through pharmacological modulators can normalize the inflammatory state and reduce susceptibility to infection in Cystic Fibrosis patients. Thus, assessing the improvement of channel function following drug therapy becomes extremely valuable, making the use of innate immune cells highly beneficial.

To validate leukocytes as a reliable *ex vivo* model for Cystic Fibrosis research, our laboratory has developed a method for assessing CFTR activity in these cells. This method utilizes a recombinant GST-HS-YFP protein, which has been specifically mutated at two sites to render it sensitive to halides [40]. Consequently, the ion flux through the CFTR channel can be quantified, and the intensity of the emitted fluorescent signal by the protein directly correlates with the change in ion flux through the channel [43].

1.5 THERAPEUTIC STRATEGIES

Since the discovery of the CFTR in 1989 [1], the pursuit of a therapy targeting the underlying defect has led to various strategies. Despite extensive research efforts, a definitive cure for Cystic Fibrosis remains elusive. The manifestation of the condition varies due to different mutations impeding CFTR's proper function. This substantial variability has made the development of a universal drug applicable to all patients unattainable. While current treatments effectively manage symptoms, they do not address the genetic anomalies affecting CFTR. These therapeutic approaches encompass antibiotics, mucolytics like DNAse, anti-inflammatory medication, hypertonic saline spray, and lung transplantation.

Currently, therapies for Cystic Fibrosis fall into three main categories:

1. **Symptom alleviation**:

   The first category focuses on measures to alleviate symptoms. It includes implementing a high-calorie, high-fat diet to counter malabsorption, alongside the proper use of pancreatic enzymes and gastro protectors for managing pancreatic insufficiency. The primary goal is to facilitate mucus clearance from

the lungs through airway clearing techniques, thus regulating and preventing bacterial infections with antibiotics and mitigating lung inflammation. While these combined therapies have shown improvements in lung function, they do have limitations. Nonetheless, they have significantly extended the lifespan of those with CF. A comprehensive and detailed treatment plan targeting all clinical symptoms is crucial. Additionally, respiratory physiotherapy plays a central role in eliminating respiratory secretions and enhancing ventilation. Osmotic compounds aid in mucus hydration, promoting better clearance of respiratory passages. Due to recurrent infections, antibiotic treatment is usually necessary. In severe cases with extensive respiratory damage, lung transplantation may become necessary. Furthermore, anti-inflammatory therapy can slow down lung function deterioration and reduce the frequency of acute lung attacks, contributing to overall disease management.

2. **Gene therapy**:

Following the cloning of the CFTR gene in 1989, gene therapy emerged as a potential treatment. Over 25 gene therapy studies have been conducted, utilizing various viral and non-viral gene transfer agents. Human studies have explored multiple vector systems, such as adenovirus vectors, adeno-associated viruses, and cationic lipids. Despite promising *in vitro* findings, the outcomes of various *in vivo* experimental projects have not consistently replicated these results [44].

3. **Targeting the genetic defect**:

Recent years have seen significant progress in therapies targeting the fundamental genetic defect, resulting in substantial advancements in the life expectancy of affected individuals. Previously seen primarily as a childhood disease, Cystic Fibrosis now predominantly affects adults. Until a few years ago, treatments focused solely on alleviating the disease's effects [29]. However, the discovery of CFTR modulators has revolutionized CF treatment. These molecules have the potential to improve or partially restore CFTR abnormalities, thereby restoring its function [45].

CFTR modulators encompass correctors and potentiators, representing drugs that can enhance the channel's functionality, with effects tailored to specific mutations. Correctors consist of small molecules that promote increased availability of the channel on the apical surface of epithelial cells, aiding in the protein's transfer to the membrane. Conversely, potentiators are compounds that boost the performance of the mutated protein found in the membrane, facilitating channel opening and ion passage.

The first CFTR modulator introduced was Ivacaftor (VX770) (Vertex. Kalydeco® Product Monograph. 2019). Subsequently, other drugs emerged, combining Ivacaftor with various correctors: Orkambi® (combining Lumacaftor (VX809) with Ivacaftor) (Vertex. Orkambi® Product Monograph. 2019); Symkevi® (resulting from the combination of Tezacaftor (VX661) and Ivacaftor) (Vertex. Symdeko® Product Monograph. 2020); Trikafta® (integrating Ivacaftor's activity with Elevacaftor (VX445) and Tezacaftor) (Vertex Trikafta® Prescribing Information. 2020).

While CFTR modulators have indeed certainly improved lung function and extended the life expectancy of patients with specific channel mutations, it is crucial to recognize that the underlying mutation responsible for the condition remains unchanged. Therefore, modulators cannot be considered a definitive cure for Cystic Fibrosis. Moreover, clinical responses to the modulators sometimes are variable due to the intricate interplay of genetic, clinical, and phenotypic factors [46,47]. Indeed, the clinical responses to CFTR modulators exhibit genotype-specific variability, making it challenging to predict outcomes consistently, even within the same genotype. Furthermore, reports indicate that also the efficacy of Trikafta® therapy, which proves beneficial for treating the majority of CF patients, requires refinement [48]. Hence, it is imperative to seek new biomarkers that can accurately anticipate an individual patient's response. Additionally, it remains important to identify patients who do not respond to treatment and investigate the underlying mechanisms of therapeutic failure. The ultimate goal will be the pursuit of personalized medicine, tailoring treatments to the unique genetic composition of each patient. This approach will

undoubtedly pave the way for even more effective interventions for individuals affected by CF.

Name	Mode of action	Clinical Stage	Mutations
Ivacaftor (VX770)	Potentiator	FDA-approved 2012	R117H, G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N e S549R
Orkambi® (Lumacaftor + Ivacaftor)	Corrector + potentiator	FDA-approved 2015	F508del homozygous
Symkevi® (Tezacaftor + Ivacaftor)	Corrector + potentiator	FDA-approved 2018	Patients heterozygous for F508del and one of the following mutations: P67L, R117C, L206W, R352Q, A455E, D579G, 711+3A→G, S945L, S977F, R1070W, D1152H, 2789+5G→A, 3272 26A→G o 3849+10kbC→T
Trikafta® (Elexacaftor + Tezacaftor + Ivacaftor)	Corrector + corrector + potentiator	FDA-approved 2019	F508del heterozygous

Table 1. CFTR Modulators for the Treatment of Cystic Fibrosis.

1.5.1 Kalydeco® (Ivacaftor, VX770)

Ivacaftor (IVA), also known as Kalydeco® or VX770, is an innovative orally administered CFTR potentiator developed by Vertex Pharmaceuticals. It gained FDA approval in 2012 for individuals over six years old with at least one G551D allele. The positive outcomes observed in patients treated with Ivacaftor led the FDA to recommend its therapeutic in individuals carrying a greater number of mutations. Currently, both the EMA and FDA have approved the drug for administration to subjects with mutations including G551D, G1244E, G1349, G178R, G551S, S1251N, S1255P, S549N, and S549R (Vertex. Kalydeco® Product Monograph. 2019).

Preclinical studies have demonstrated Ivacaftor's effective correction of ion transport through CFTR in human bronchial epithelium cell cultures (HBE), increasing the probability of channel opening on the cell surface. This leads to an enhanced passage of chloride through epithelia [49,50]. Specifically, Ivacaftor extends the channel's open

state by directly binding to the CFTR protein. The drug is particularly efficacious in class III gating mutations, such as G551D, which, however, occur in only 2.3% of patients (www.who.int).

Ivacaftor's binding to the protein necessitates phosphorylation of the CFTR regulatory region, yet ATP is not required for dimerizing the NBD1 and NBD2 domains. This mechanism can correct the G551D mutation, which does not respond to ATP and is therefore rarely activated. Double-blind placebo-controlled clinical trials conducted on patients with this specific mutation have shown that Ivacaftor leads to up to a 10% improvement in lung function. Additionally, the success of this therapy is evident in the sweat test, as a lower chloride concentration is recorded post-drug usage [51].

However, Ivacaftor therapy has shown significant efficacy only for a fraction of potential CFTR mutations. Research has also been conducted on class IV mutations, but it has not resulted in notable improvements in lung function. Nevertheless, a decrease in chloride concentration, as analyzed in the sweat test, has been evident [52]. Tests on patients with homozygosity of F508del, where the absence of the protein is manifested at the epithelial membrane level, revealed that Ivacaftor fails to provide benefits in terms of respiratory phenotype or chlorine concentration detected by the sweat test. These findings underscore that using a CFTR potentiator alone may not effectively apply to the majority of Cystic Fibrosis patients [53,54].

1.5.2 Orkambi® (Ivacaftor/Lumacaftor)

After the promising outcomes of the Ivacaftor trial in a small subset of patients, the research focus shifted towards potentially restoring CFTR function in the broader population of Cystic Fibrosis patients carrying the F508del mutation. This led to the emergence of Lumacaftor (LUM, VX809), another CFTR modulator, recognized for its corrective effects in patients with the F508del mutation. In cases where the mutated protein fails to properly reach the epithelial cell surface, Lumacaftor plays a crucial role. Due to the limited expression of CFTR with this mutation at the membrane level, Ivacaftor alone cannot improve the condition for patients homozygous for F508del [22]. Therefore, Lumacaftor, classified as a corrector-type modulator, is employed. It exerts

its effect translationally by altering the protein conformation of the MSD1 domain, leading to a more stable connection between the MSD1 and NBD1 domains, partially correcting the F508del mutation.

To augment the protein's presence on the cell surface and improve its conductance, Lumacaftor is combined with Ivacaftor (commercially known as Orkambi®). This combined therapy received approval in 2015 for Cystic Fibrosis patients over twelve years of age, homozygous for the F508del mutation. Subsequently, in 2016, the FDA extended its use to patients over six years old [55]. Double-blind clinical trials employing randomized dosages of the drug and placebo demonstrated a significant improvement in lung function among subjects treated with the drug [56,57]. Despite the evident benefits, complications related to tolerance and potential drug interactions have been observed in many cases. Respiratory complications, including infectious relapse, coughing, dyspnea, and chest oppression, have been reported [54,57]. Consequently, researchers have been driven to explore alternative pharmacological approaches to address these issues.

1.5.3 Symkevi® (Ivacaftor/Tezacaftor)

Since 2018, a third drug combining Tezacaftor and Ivacaftor (TEZ/IVA), produced by Vertex Pharmaceutics, has been employed to treat patients over twelve years old with an F508del mutation and residual channel function (Vertex. Symkevi® Product Monograph. 2020). Symkevi® has shown effectiveness across a wide spectrum of mutations with residual CFTR channel function, making it suitable for individuals who are homozygous for the F508del mutation and also for heterozygotes carrying a second mutated allele with partial protein function [58].

Tezacaftor (VX661) shares a similar structure with the previous corrector and functions as a CFTR corrector. It enhances the processing of the mutated protein and prevents degradation at the endoplasmic reticulum, thus facilitating its reach to the membrane. Notably, Tezacaftor does not induce CYP3A4 enzymes, resulting in fewer drug-drug interactions compared to Lumacaftor. Consequently, Tezacaftor exhibits a more favourable safety profile from a clinical perspective. The F508del mutation also induces

gating abnormalities in channels, which are partially corrected by the use of the potentiator Ivacaftor once the protein is located on the cell surface. *In vitro* studies have demonstrated that Tezacaftor can enhance ion transport through CFTR, with the enhancer's effect being further amplified when combined with Ivacaftor [59].

The administration of this drug has been linked to enhanced lung function, reduced incidence of infectious airway flare-ups, and an improvement in respiratory symptoms associated with the disease. Phase III studies of TEZ/IVA and LUM/IVA have indicated that both combinations yield benefits related to lung function [57,60,61]. Specifically, Symkevi® seems to hold a slight advantage in terms of lung function, although it leads to a smaller reduction in chloride concentration during the sweat test when compared to monotherapy with Ivacaftor. In contrast to Lumacaftor/Ivacaftor, Symkevi® appears to induce fewer adverse respiratory effects and demonstrate fewer interactions with other drugs [54].

1.5.4 Trikafta® (Ivacaftor/Tezacaftor/Elexacaftor)

In 2019, Vertex Pharmaceuticals introduced a second-generation modulator called Trikafta®, which combines Tezacaftor and Elexacaftor (ELX, VX445) correctors with the potentiator Ivacaftor. This triple combination recently received FDA approval for treating Cystic Fibrosis in patients over twelve years old with at least one allele carrying the F508del mutation (Vertex Trikafta® Prescribing Information. 2020).

Compared to combinations TEZ/IVA and LUM/IVA, Trikafta® demonstrates efficacy even in patients with one CFTR allele carrying the F508del mutation and the other allele carrying mutations resulting in a protein with minimal function (including nonsense, insertion/deletion, splicing, and several severe protein misfolding mutations) [62]. Thus, the primary objective was to correct CFTR function by restoring the sole F508del allele, regardless of the mutation present in the second allele. Elexacaftor and Tezacaftor play crucial roles in facilitating CFTR transfer to the epithelial membrane, each interacting with a distinct site on the protein, leading to an additive effect. Meanwhile, the presence of Ivacaftor increases channel conductance once it is properly positioned on the surface (Vertex Trikafta® Prescribing Information. 2020) [63]. The collective action of these

three molecules partially restores CFTR activity, despite the negative influence of the unresponsive allele [54,63].

Phase II and III studies indicate that this triple combination results in a significant increase in lung function, reduced instances of infectious airway exacerbations and an overall improvement in quality of life. After treatment with Trikafta®, tested subjects exhibited a 10-point increase in FEV1 (Maximum Expiratory Volume in One Second), a key parameter for assessing lung function. Overall, Trikafta® emerges as one of the most effective therapeutic approaches for adequately restoring CFTR channel function in patients with at least one mutated F508del allele [62,64,65]. While this drug represents a notable advancement in the research for CF modulators, it is important to note that it does not constitute a definitive cure for CF. Discontinuation of therapy leads patients to return to their previous treatment regimens.

Recently, the National Health System approved the free distribution of Ivacaftor, Trikafta®, Orkambi®, and Symkevi® to individuals carrying mutations compatible to treatment with these specific therapies (www.fibrosicisticaricerca.it). The use of these drugs allows appropriate treatment for a considerable number of patients, improving their clinical condition and extending their life expectancy. However, not all CF subjects are suitable candidates for these therapies. The combination of various mutations in heterozygous patients results in highly variable responses to the drugs. Additionally, individuals with class I mutations still lack a suitable therapy. Research in the field of Cystic Fibrosis is partly focused on deepening our understanding of the effects of these drugs even in subjects with mutations not previously considered fully compatible. *Ex vivo* models are of fundamental importance in monitoring individual patient responses for personalized therapy and supporting research in the pursuit of new therapeutic possibilities.

1.6 INTRACELLULAR CALCIUM HOMEOSTASIS AND INFLAMMATION IN CYSTIC FIBROSIS

The crucial role of intracellular calcium ion concentrations ($[Ca^{2+}]_i$) in orchestrating signal transduction processes is widely recognized. These processes govern fundamental cellular functions like cell proliferation, motility, secretion, and the precise regulation of gene expression [66,67]. Minor variations in $[Ca^{2+}]_i$ levels have been linked to a range of acute and chronic conditions, including but not limited to cancer and disorders of the airway epithelium.

In this context, emerging evidence highlights the importance of perturbed Ca^{2+} signaling in the physiopathology of CF lung disease. Initially resulting from intrinsic defects associated with CFTR mutation, the abnormal Ca^{2+} profile observed in CF airway epithelial and immune cells is further exacerbated by recurrent pathogen infections and the overstimulation of proinflammatory mediators, culminating in injurious lung inflammation [68,69].

At the airway level, Ca^{2+} signals regulate ciliary beating and the secretion of fluids and antimicrobial agents, in addition to controlling CFTR protein expression levels and internalization [70,71]. Studies have demonstrated that there is an imbalance in Ca^{2+} regulation in primary bronchial epithelial cells from individuals with CF and in respiratory cell lines [72]. This imbalance is associated with the retention of F508del-CFTR proteins in the endoplasmic reticulum. Correcting the abnormal trafficking of F508del-CFTR through pharmacological therapy leads to a recovery of Ca^{2+} mobilization in CF cells [73].

The dysregulation of intracellular calcium homeostasis has been observed in various primary human cells derived from CF patients: airway epithelial cells [74], bronchial goblet cells [75], skin fibroblasts [76], kidney cells [77], and immune cells such as leukocytes, neutrophils, and lymphocytes [78]. In all these cells, $[Ca^{2+}]_i$ is elevated compared to non-CF cells, affirming that functional CFTR plays a key role in conditioning Ca^{2+} homeostasis.

Moreover, our research group reported compelling evidence demonstrating that in Peripheral Blood Mononuclear Cells (PBMCs), isolated from CF patients homozygous for the F508del mutation, calcium homeostasis is abnormal [40]. As a result, these cells

undergo non-regulated activation of the calcium-dependent protease known as Calpain [79]. In such scenarios, the calpain-mediated degradation of CFTR [80], leads to the removal of CFTR from the plasma membrane, followed by its internalization within endocytic vesicles [81]. The concurrent absence of functional CFTR, combined with increased $[Ca^{2+}]_i$ levels, can trigger altered immune responses in CF lymphocytes [82].

Moreover, our research has unveiled a correlation between fluctuations in $[Ca^{2+}]_i$ and the secretion of Matrix Metalloprotease 9 (MMP9) from CF PBMCs [83]. The dysregulated function of MMPs has been intricately linked to the pathogenesis of various chronic pulmonary disorders, such as asthma, emphysema, and acute lung injury. These diseases are characterized by abnormal tissue remodelling accompanied by the accumulation of extracellular matrix (ECM), wherein MMPs play a pivotal role. Notably, MMPs have recently emerged as contributors to lung remodelling, a significant determinant of lung disease severity in CF patients.

MMPs, a superfamily of metallo-endopeptidases known as metzincins, are initially synthesized as inactive proenzymes [84] by various structural and immune cell types, including macrophages, neutrophils, epithelial cells, endothelial cells, and fibroblasts. While polymorphonucleates continually produce MMPs, many other cell types release these enzymes only in response to inflammatory stimuli and related to tissue remodelling and wound repair, mediated by the modulation of transcription factors. Following their synthesis, MMPs become enzymatically active through precise cleavage events, subsequently cleaving specific substrates to execute pivotal biological functions. These functions encompass the degradation of connective tissue and alveolar epithelium, the release of cytokines and growth factors, and the regulation of cellular mobility and migration through ECM remodelling. Furthermore, MMPs actively participate in the wound repair process by catalysing the normal turnover of the ECM. While MMPs share structural similarities in their catalytic site containing zinc ions (Zn^{2+}), they exhibit distinctions in substrate specificity, cellular and tissue localization, membrane associations, and regulatory mechanisms. Several MMPs have been postulated to play a pathological role in CF [85]. Notably, gelatinase B MMP9 has been observed to be both quantitatively upregulated and to exhibit heightened enzymatic activity in the lower airway secretions of CF patients [86]. The human MMP9 gene is

situated within the genomic region 20q13.12 and encodes a protein comprising 707 amino acids. This protein is secreted into the extracellular space in the form of an inactive pro-enzyme known as pro-MMP9. Pro-MMP9 remains in an inactive state due to the presence of 80 amino acid residues at its N-terminal region, where a cysteine switch motif coordinates the zinc ion, forming the catalytic domain of the protein and thereby keeping it in an inactive state [87]. Within the extracellular space, other proteinases, such as MMP3 or MMP2, cleave the inactive pro-MMP9, converting it into the active form with a molecular weight of 84 kDa. Upon secretion, MMP9 acquires the capability to degrade collagen, potentially contributing to tissue deterioration and dysfunction, in addition to its capacity to potentiate ELR-containing chemokines that play a pivotal role in modulating inflammatory processes [88,89].

In other studies, it has been documented [90] that the presence of MMP9 in the sputum of children with CF displays an inverse correlation with FEV1. Similarly, research [91,92] has indicated that an elevated level of MMP9 in the serum of adult CF patients is associated with a decline in FEV1. These collective findings imply an association between the presence of MMP9 in either serum or airway secretions and the impairment of lung function observed in CF patients. Among the proposed mechanisms governing MMP9 secretion, several reports suggest a calcium-mediated process [93], influenced by the activity of protein kinase C (PKC) [94-96].

PKC is a member of a key family of enzymes involved in signaling pathways that specifically phosphorylates substrates at serine/threonine residues. Phosphorylation by PKC is important in regulating a variety of cellular events such as the regulation of gene expression. In this context, we previously reported that in CF PBMCs, the activation of the PKC/ERK1/2 pathway, which is promoted by the altered intracellular calcium homeostasis, induces MMP9 expression [83]. However, in the context of MMP9 regulation, there is an intricate interplay of these intracellular mechanisms. It is essential to recognize that our current understanding is only a starting point, underscoring the need for future studies that further investigate the intricate network of processes that regulate MMP9 production.

2. AIMS

The specific aim of my Ph.D. thesis was, firstly, to identify new leukocyte biomarkers related to drug recovery of CFTR by correlating the functional data obtained by GST-HS-YFP assay with proteomic leukocyte changes after VX770 cell treatment. The results demonstrated that *ex vivo* therapy downregulated MMP9, which, as reported in several previous studies, plays a crucial role in the progression of Cystic Fibrosis, as elevated levels of MMP9 are associated with declining lung function in CF patients.

The second aim of the thesis was to confirm the downregulation of MMP9 as a useful biomarker for the effectiveness of CFTR modulator therapy. Therefore, we conducted *in vivo* assessments of changes in protein expression. Initially, we examined these changes in leukocytes obtained from CF patients before and during the therapy with the potentiator Ivacaftor. This allows us to correlate alterations in intracellular MMP9 expression with some clinical parameters such as FEV1 and sweat test.

Subsequently, we extended our research to patients undergoing Trikafta®, the triple CFTR modulator therapy suitable for a large number of mutations. Since patients defined as responders and non-responders to this treatment according to clinical parameters were available, we aimed to relate the levels of MMP9 expression with the individual patient's response to therapy. Therefore, to achieve this third goal of the thesis, we analysed in these patients the expression of MMP9 not only in leukocytes but also in plasma samples before and during Trikafta® therapy.

Finally, we investigated the intracellular signaling pathway associated with MMP9 expression to comprehend the molecular events underlying the clinical response of CF patients to CFTR modulators. This knowledge could be useful to understand the variable clinical responses of patients with CF to CFTR modulators.

The research for non-invasive biomarkers of an individual patient's response is crucial in Cystic Fibrosis. Hence, the ultimate goal of this Ph.D. project is to confirm MMP9 as a biomarker of clinical response to CF therapies, useful for future studies in personalized medicine.

3. MATERIALS AND METHODS

3.1 MATERIALS

Anti-MMP9 antibody, anti-P-ERK1/2 antibody, anti-ERK1/2 antibody, horseradish peroxidase (HRP)-linked anti-rabbit and anti-mouse secondary antibody, protease inhibitor cocktail (100X) and phosphatease inhibitor cocktail (100X) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-IkBα and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). RPMI 1640, fetal bovine serum (FBS), penicillin–streptomycin solution 100X, L-glutamine 100X 200mM, Lympholyte®-H and prestained protein SHARPMASS VI MW marker were purchased from Euroclone SpA (Milan, Italy). Dibutyryl-cAMP, isopropyl β-D-1-thiogalactopyranoside (IPTG), yeast extract, tryptone, PGex6P1, 6,7-Dihydro-7,9-dimethyl-6-(5-methyl-2-furanyl)-11-phenylpyrimido (4',5',3,4) pyrrolo (1,2-a) quinoxaline –8,10 (5H,9)-dione (PPQ-102), a reversible and voltage-independent CFTR inhibitor, gelatin, Triton X-100, BRIJ®35 Detergent Calbiochem® and the potentiator Ivacaftor (VX770) were purchased from Sigma-Aldrich (Milan, Italy). Monocytes Isolation Kit II was purchased from Miltenyi Biotec Srl (Bologna, Italy). pEYFP-C1 plasmid was obtained from Clontech Laboratories (Mountain View, CA, USA). QuickChange site-directed mutagenesis kit was from Stratagene (San Diego, CA, USA). GSH-sepharose™, ECL Select™ Western Blotting Detection Reagent, ECL Western Blotting Detection Reagent, and Amersham™ Protran® Premium 0.45-µm nitrocellulose were obtained from GE Healthcare (Chicago, IL, USA). BamHI and EcoRI restriction enzymes by Fermentas were purchased from Life Technologies Italia (Monza, Italy). Brillant Blue R-250 and Acrylamide/Bis Solution were obtained from Bio-Rad Laboratories Srl (Segrate, MI, Italy).

3.2 ETHICS STATEMENT

All participants provided their written informed consent before being enrolled in the study, including permission for the storage and exclusive research use of the samples.

The study protocol conformed to the guidelines outlined in the Declaration of Helsinki and those of the G. Gaslini Children Hospital in Genoa, Italy. The Ethic Committee of Genoa approved the study under protocol A-CF2014 460REG2014.

3.3 DONOR SUBJECTS AND SAMPLE COLLECTION

At the Cystic Fibrosis Center of the Giannina Gaslini Institute of Genoa, blood samples were obtained from patients during their routine clinical examinations. For the initial study (Figure 3), we enrolled twenty-six CF patients, all with the F508del$^{+/+}$ mutation (14 females, 8 males; mean age: 38), along with twenty-six non-CF donors. Additionally, the CFTR assay was conducted on PBMCs isolated from two other CF patients, who carried G1349D and F508del mutations (2 males; mean age: 16), both before and during Ivacaftor therapy (Figures 4 and 5). PBMCs for *ex vivo* treatment with VX770 were isolated from sixteen CF patients (Figure 6) carrying class III gating mutations and non-gating mutations with residual functioning CFTR, all eligible for Ivacaftor therapy (8 females, 8 males; mean age: 39). In the final part of the study, we recruited another sixteen CF patients (9 females, 7 males; mean age: 27) undergoing Trikafta® therapy (Tables 3 and 4) and four non-CF donors. The patients' clinical condition were further evaluated using ppFEV1 (%) and sweat chloride tests. For each patient and non-CF donor, approximately 8 mL of blood was collected in three vacuette® PREMIUM tubes, each containing 3 mL of 5mM EDTA.

3.4 PBMCs AND MONOCYTES ISOLATION AND PLASMA COLLECTION

The blood samples were mixed with an equal volume of RPMI 1640 in a 50 mL Falcon™ tube, and then carefully layered over an equivalent volume of Lympholyte®-H Cell Separation Media. Subsequently, the samples were centrifuged at 800×g for 20 min at 22 °C without brake. Following centrifugation, the central layer consisting of

lymphocytes, monocytes, and platelets was collected from the interface between the upper layer containing the plasma fraction and the lower layer containing the Lympholyte®-H. To remove the platelets, the central layer of leukocytes was washed twice with RPMI 1640 and then resuspended in PBS (Phosphate Buffered Saline) or CFTR buffer for the relevant subsequent analysis. For monocytes purification, we employed the Monocytes Isolation Kit II (MiltenyiBiotec), following the manufacturer's instruction. The magnetically labelled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabelled monocytes passed through the column. For plasma collection, 0.5 mL of blood sample was centrifuged at 800xg for 10 min. The supernatant was centrifuged at 16,000xg for 10 min. The obtained plasma was divided into aliquots of approximately 80 µL and stored at -80 °C to be used for subsequent analysis.

3.5 CELL TREATMENT

For *ex vivo* treatment with VX770, PBMCs were resuspended in complete culture medium (RPMI 1640, containing 10% (*v*/*v*) FBS, 10 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine) at a concentration of 10^6/mL. The resulting cells were then equally divided into two tubes. The first tube underwent incubation with potentiator VX770 (5µM) diluted in DMSO, while the second tube received an equal amount of DMSO, ensuring identical conditions in both samples except for the drug addition. Both were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Following incubation, the PBMCs were washed twice with CFTR buffer, counted, and subsequently subjected to the CFTR activity assay or processed for Western blot or proteomic analysis.

As for the monocytes, after purification, they were plated in a 96-well plate without FCS. After 30 min, the complete medium was added. The following day, the monocytes were washed twice with CFTR buffer (20mM sodium borate (pH 7.5), 0.25M sucrose, 5mM glucose, and 0.2mM $CaCl_2$) before proceeding with the assay.

3.6 RECOMBINANT GST-HS-YFP PURIFICATION

The YFP nucleotide sequence was amplified through PCR using the Sn YFP-BamHI: 5′ AA-GGATCC-ATGGTGAGCAAGGGC and Asn YFP-EcoRI: 5′ A-GAATTC-TTACTTGTACAGCTCGTCCATGC primers derived from the pEYFP-C1 plasmid. The resulting double filament underwent denaturation at 94 °C for 1 min, followed by 35 cycles of reaction at 94 °C for 30 s. Subsequently, the temperature was raised to 58 °C for 30 s to facilitate primer binding to the template, then increased to 68 °C for 1 min. Finally, after the last PCR cycle, a final extension cycle of 5 min at 68 °C was performed. The PCR product (amplicon) was cloned into the pGex6P1 expression vector, which contains Glutathione S-transferase (GST) as tag protein. To create an YFP protein sensitive to halides [97], the YFP-H148Q mutation was introduced. Additionally, the I152L mutation, which results in an YFP with very high affinity for I-ions [43], was incorporated. Mutagenesis was carried out using the QuickChange site-directed mutagenesis kit based on the polymerase chain reaction. The nucleotide sequence of the mutated YFP was confirmed through sequencing with CEQ 2000XL DNA analysis system (Beckman Coulter).

The mutated YFP, expressed as a GST-HS-YFP fusion protein, was produced in E. Coli DH5α and purified to homogeneity via affinity chromatography with GSH-Sepharose. Briefly, transformed DH5α cells were cultured in Super Broth medium (3.5% tryptone, 2.0% yeast extract, 0.5% NaCl, pH 7.0) containing 100 μg/mL ampicillin at 37 °C for 16 h. The cells were then ten-fold diluted and grown at 37 °C until the optical density at 600nm was ≥ 0.6. Induction of recombinant protein expression under the control of the LAC operon was achieved with 1.2mM IPTG for 16 h at 25 °C. The cells were washed once with H_2O and lysed in the following lysis buffer: 0.1M Tris/HCl pH 8.3, 0.15M NaCl, 1% Triton-X100, 10mM EDTA, 2 mg/mL lysozyme, 1X Protease Inhibitor Cocktail. After 20 min at 0 °C, 10mM $MgCl_2$ and 10μg/mL DNase were added, and the lysate was incubated for an additional 20 min at 0 °C. The lysate was cleared by centrifugation (100,000×g for 20 min at 4 °C) and the resulting supernatant was loaded onto 2 mL GSH-sepharose column, pre-equilibrated with 50mM sodium borate pH 7.5, containing 0.15M NaCl and 5mM DTT (buffer A). The resin was washed with 20 column volumes of buffer A, and GST-HS-YFP was eluted with 50mM sodium borate pH 9.0, containing

0.15M NaCl, 5mM DTT, and 10mM GSH. The eluate was subjected to desalting procedure using a PD10 column (GE Healthcare) pre-equilibrated with 50mM sodium borate pH 8.0. Purity of GST-HS-YFP was evaluated by SDS–10% PAGE followed by blue Coomassie staining.

The fusion protein has a K_I = 2.34 ± 0.17 (mean ± SEM) whereas HS-YFP has K_I = 2.05 ± 0.19 (mean ± SEM). According to *t* test (p = 0.282, for GST-HS-YFP n = 9 and for HS-YFP n = 6), the two K_I values are not statistically different.

3.7 CFTR ASSAY

For evaluating CFTR activity, the cells (PBMCs: 1.5 x 10^6; monocytes: 2 x 10^5) were subjected to a 20 min incubation at 37 °C with 100µM Dibutyryl-cAMP and 5µM VX770 in 200 µL of CFTR buffer (stimulated cells). The unstimulated sample is maintained for 20 min at 37 °C in 200 µL of CFTR buffer. Subsequently, 5mM NaI was introduced to both the stimulated and unstimulated cells. After 30 s, the supernatants were clarified by centrifugation (13,000×g for 20 s at room temperature). Finally, the supernatants were transferred to a black 96-well plate, and 1µg of highly purified GST-HS-YFP was added. Following 5 min of shaking, fluorescence was measured with a Mithras LB940 plate reader (Berthold Technologies); the excitation/emission wavelengths were λ_{ex} = 485 ± 15 nm and λ_{em} = 535 ± 10 nm, respectively. Additionally, a GST-HS-YFP NaI-quenching curve was established. Unknown NaI concentrations were derived from the GST-HS-YFP NaI-quenching curve, and CFTR activity was quantified as NaI exchange, expressed as pmol/min/10^3 cells.

3.8 IMMUNOBLOTTING ANALYSIS

Freshly isolated CF and non-CF PBMCs were sonicated in Laemmli sample buffer at a concentration of 10^7/mL and then heated for 5 min at 95 °C. Subsequently, 30 µL

aliquots from each sample were subjected to SDS/PAGE (6% or 10%) followed by Western blot analysis. The nitrocellulose membranes were initially blocked with 5% (*w/v*) skim milk powder in PBS containing 0.05% (*v/v*) Tween-20 for 1 h at room temperature. Afterward, the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-MMP9 (1:1000), anti-β-actin (1:1000), anti-P-ERK1/2 (1:2000), anti-ERK1/2 (1:2000), and anti-IkBα (1:1000). The peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse) was applied for 1 h at 22 °C (diluted 1:5000). A stripping and re-probing procedure was performed to assess the membranes with all the primary antibodies.

Immunoreactive signals were developed using ECL Select™ Western Blotting Detection Reagent, acquired and quantified using ChemiDoc™ XRS equipped with Quantity One Image Software 4.6.1 (Bio-Rad Laboratories Srl, Segrate (MI), Italy). Alternatively, ECL Western Blotting Detection Reagent was used.

3.9 PROTEOMIC ANALYSIS

The methodology for identifying and quantifying proteome modulation associated with CFTR activity was conducted as previously reported [98]. Initially, PBMCs were isolated, washed and lysed in 1X PBS with protease inhibitor cocktail (Roche) and 0.1% SDS. Protein precipitation and denaturation were achieved through the use of cold acetone, followed by resuspension in 100mM NH_4HCO_3. Subsequently, protein concentration was determined by Bradford Protein assay (Sigma-Aldrich, St. Louis, MO). 30 µg of proteins underwent reduction (employing DTT from Sigma-Aldrich), alkylation (utilizing iodoacetamide from Sigma-Aldrich) and trypsin digestion at 37 °C overnight. The resulting peptides were then subjected to label-free LC–MS/MS analysis, employing a micro-LC system (Eksigent Technologies, Dublin, USA) interfaced with a 5600+ TripleTOF mass spectrometer (AB Sciex, Concord, Canada). The samples initially underwent data-dependent acquisition (DDA) analysis to construct the SWATH–MS spectral library. Subsequently, cyclic data independent analysis (DIA), utilizing a 25-Da window, and each sample was analyzed in triplicate for robustness. MS data were acquired using Analyst

TF v.1.7 (AB SCIEX), while PeakView v.1.2.0.3, Protein Pilot v.4.2 (AB SCIEX) and Mascot v. 2.4 (Matrix Science) programs were utilized for peak-list generation. The UniProt/Swissprot (v.2015.07.07, 42131 sequences entries) was employed for database searches. The Protein Pilot software v. 4.2 (AB SCIEX, Concord, Canada) was utilized for inputting samples, with specific parameters including cysteine alkylation, trypsin digestion, no special factors and a false discovery rate at 1%. For Mascot search, the following parameters were used: trypsin as digestion enzyme, allowance of two missed cleavages, peptide mass tolerance of 50 ppm and MS/MS tolerance of 0.1 Da. Peptide charges were set to 2+, 3+ and 4+, and the search was set on monoisotopic mass. The instrument was configured to ESI-QUAD-TOF, and specific modifications such as carbamidomethyl cysteines as fixed and oxidized methionine as variable were specified. False discovery rate was controlled at 1%.

The files obtained from the DDA acquisitions were employed in generating the library, adhering to an FDR threshold of 1%. Protein quantification was conducted using PeakView v.2.0 and MarkerView v.1.2. (ABSCIEX) programs, extracting ten peptides per protein with the highest MS1 intensity from SWATH files, along ten transitions per peptide. Peptides with FDR lower than 1.0% were exported.

3.10 ZYMOGRAPHY ANALYSIS

Zymography was used to assess plasmatic MMP9 activity. Initially, 1 µL of CF and non-CF plasma samples were diluted in a modified Laemmli sample buffer. Subsequently, the samples underwent electrophoresis at 4 °C for 1.5 h, without boiling or reduction, utilizing an 8% (*v/v*) polyacrylamide gel copolymerized with 1 mg/mL gelatin. The gel was incubated for 1 h at 25 °C in 0.05M Tris (pH 7.4) containing 2.5% (*v/v*) Triton X-100, followed by two washes with 0.05M Tris (pH 7.4) and an overnight incubation at 37 °C in 0.05M Tris (pH 7.4), containing 10mM $CaCl_2$, 0.15M NaCl and 0.05% (*v/v*) BRIJ®35 Detergent. Subsequently, the gel was fixed and stained for 2 h with a pre-warmed solution containing 45% (*v/v*) methanol, 10% (*v/v*) acetic acid, and 0.25% Coomassie Blue R-250. The zymograms were then de-stained using 30% (*v/v*) methanol-10% (*v/v*)

acetic acid. The relative levels of MMP9 activity were calculated via computer-assisted planimetry, and the intensity of the MMP9-dependent lytic areas was determined using Quantity One Image Software 4.6.1 (Bio-Rad Laboratories Srl, Segrate (MI), Italy).

3.11 STATISTICAL ANALYSIS

Where applicable, the data were presented in the form of mean $\pm$ SD and subjected to a Kolmogorov–Smirnov test (test of normality) to assess their distribution. The significance of the differences was analyzed using either non-parametric or parametric tests, as specified in the corresponding figure legend. The Prism 4.02 software package (GraphPad Software, San Diego, CA, USA) was utilized for this analysis, whit a minimum threshold of $p<0.05$ considered as statistically significant.

For the statistical evaluation of proteomics data, MarkerView software (Sciex) and Metaboanalyst were employed. Proteins were considered up and downregulated used p-value < 0.05 and fold change > 1.3 or < 0.769. The bioinformatic analyses of proteomic data was performed using STRING V.11.0 software.

3.12 REGRESSION ANALYSIS

A data analysis methodology has been implemented in the R language to transform fluorescence intervals obtained from the CFTR channel into iodide fluxes. Additionally, it aims to ascertain the most suitable mathematical model for these data. The regression method is non-iterative and totally autonomous, negating the need for an initial input of parameters value. The method implements three categories of functions: polynomial (P), exponential (E), and hyperbolic functions (H). Each of these families encompasses a wide array of models.

Given the (n x m) matrix of the experimental data, where the first column comprises measurements of the independent variable $x = (x_1, x_2, x_n)$, and the other columns

represent arrays of samples of the dependent variables Y = (Y_1, Y_2, Y_m), where Y_i = (Y_{1i}, Y_{2i}, Y_{ni}), the method fits the three family of functions to the data in the following way:

$$\bar{Y} = P(x) \leq p = \sum_{j=1}^{p} a_j \, x^j$$

$$\bar{Y} = E(X) = a \cdot \exp(b \cdot x) + c, a, b, c \in \mathbb{R}$$

$$\bar{Y} = H(X) = \frac{1}{a+bx} + c, a, b, c \in \mathbb{R}$$

where $\bar{Y} = \sum_i^m = 1^{Y_i}$

Subsequently, model discrimination was carried out based on the following parameters:

- Mean sum of residuals
- Total sum of squares
- R-squared (where possible and reasonable to use this parameter).

The final output is the model with the lowest sum of squares, and the R-squared greater than 95%.

4. RESULTS

In our laboratory, we have developed an innovative approach to evaluate CFTR activity in PBMCs isolated from peripheral blood samples [40]. This method is based on a recombinant protein derived from Yellow Fluorescent Protein (GST-HS-YFP), incorporating two specific mutations that significantly amplify its sensitivity to halides. This enhanced sensitivity renders it a useful instrument for precisely quantifying ion flux through the CFTR channel. Once CFTR is properly functioning, the conformation of the protein allows the chloride ions (Cl^-) efflux that contributes to the maintenance of cellular homeostasis. However, individuals with Cystic Fibrosis carry CFTR gene mutations that disrupt this ion flux, giving rise to symptomatic manifestation of the disease.

To evaluate CFTR function, we induce cAMP stimulation, triggering phosphorylation events that facilitate channel opening and subsequent ion flux into the cell [40]. In our assay, we introduce the potentiator VX770 to optimize this process. CFTR activity is measured by monitoring the increased fluorescence of the GST-HS-YFP protein, which occurs as a direct consequence of reduced iodine ion (I^-) levels in the extracellular medium, resulting from its internalization in cells following CFTR channel activation.

Although the primary function of the CFTR channel *in vivo* is to expel Cl^- ions from the cell, it also exhibits the property of allowing the transit of all halides, including I^-. We took advantage of this property and used I^- ions in our CFTR assay, as the fluorescence response of the GST-HS-YFP protein is particularly sensitive to changes in its concentration [40].

4.1 APPLICATION OF CFTR ASSAY *EX VIVO*

In the initial phase of our investigation, we demonstrated the effectiveness of our assay method based on the GST-HS-YFP protein in discerning variations in channel activity between non-CF subjects and individuals carrying a CFTR mutation [40].

To evaluate the accuracy of our assay, we also introduced PPQ-102 (pyrimido-pyrrolo-quinoxalinedione-102), a synthetic inhibitor of the CFTR channel (Figure 3). PPQ-102, indeed, exerts a direct influence on the cytoplasmic nucleotide-binding domain, thereby preserving the channel's closed conformation [99].

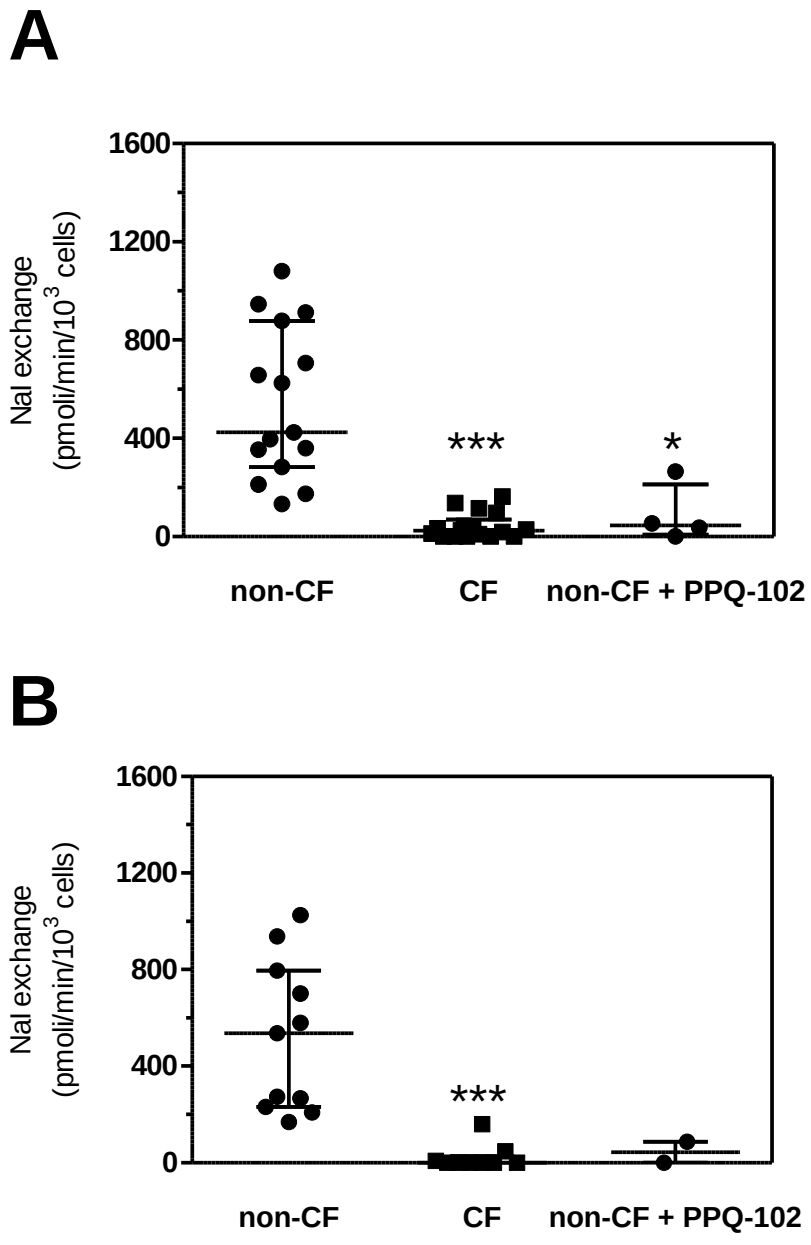

Figure 3. Assessment of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) activity in peripheral blood mononuclear cells (PBMCs). (**A**) CFTR activity was evaluated in PBMCs freshly isolated from non-CF donors, either in the absence (n = 15) or in the

presence of 10μM CFTR inhibitor PPQ-102 (n = 4), and Cystic fibrosis (CF) patients F508del$^{+/+}$ (n = 17). (**B**) CFTR activity was measured in monocytes obtained from non-CF donors, in the absence (n = 11) or in the presence of 10μM CFTR inhibitor PPQ-102 (n = 2), and CF patients F508del$^{+/+}$ (n = 9). The data is presented as a median along with the interquartile range. Statistical analysis was conducted by means of Kruskal–Wallis test followed by Dunn's multiple comparison test. *** p<0.001; * p<0.05.

In particular, as reported in Figure 3A, we performed the CFTR assay using PBMCs of non-CF subjects, of individuals with homozygous F508del mutations and of non-CF subjects exposed to the inhibitor PPQ-102 during stimulation. Notably, PBMCs with fully functional channels exhibited a significantly higher ion flux than the other two conditions. When PPQ-102 was introduced into PBMC samples from non-CF donors, effectively mimics the state of a non-functioning CFTR channel. Consequently, the ion flux data observed in these samples closely aligns with the results obtained from individuals carrying mutated CFTR channels.

Similar results were obtained from experiments conducted on isolated monocytes (Figure 3B), which excluded the amount of lymphocytes present in PBMCs. The rationale behind this approach is based on previous research, which consistently demonstrated that the most substantial presence of active CFTR within the leukocyte population is localized on the surface of monocytes [100].

The data acquired from our experiment has provided compelling evidence of the assay's capability to discern CFTR activity in PBMCs from individuals with the disease, where the channel exhibits reduced efficiency compared to cells from non-CF subjects. However, the insights obtained from this assay are not able to define a threshold indicative of the cellular function recovery associated with physiological chloride efflux facilitated by CFTR.

Indeed, as indicated in Figures 3A and 3B, channel activity shows a heterogeneous pattern, with values ranging from approximately 150 to a maximum of 1000 picomoles of iodide exchanged per minute per 10^3 cells. While the CFTR assay allows us to discern differences between patients and controls, further investigation is imperative to

determine its suitability for monitoring the recovery of CFTR function following treatment with modulators and thus their efficacy.

4.2 APPLICATION OF CFTR ASSAY *IN VIVO*

In support of the hypothesis that GST-HS-YFP testing on PBMCs or monocytes from CF patients may be useful in assessing treatment efficacy, we monitored CFTR activity *in vivo* before and during Ivacaftor therapy. The subject in our study exhibited a heterozygous genotype, with the G1349D mutation on one allele and the F508del mutation on the other CFTR allele. Individuals with at least one gating mutation like G1349D are eligible for treatment with the potentiator Ivacaftor (VX770), which could lead to a restoration of channel activity.

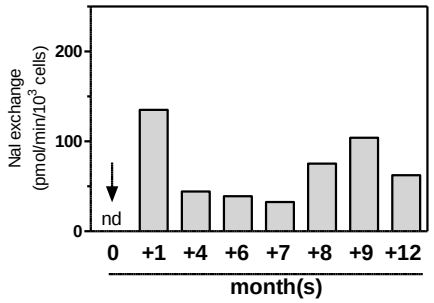

Figure 4. Monitoring of CFTR function was conducted in freshly isolated PBMCs from a CF patient carrying both G1349D and F508del mutations of the CFTR gene. Assessments were made at various time points: before (0 months) and subsequent to oral Ivacaftor therapy (+1, +4, +6, +7, +8, +9, and +12 months). "nd" denotes non-detectable activity. The arrows indicate the initiation of the therapy.

As reported in Figure 4, the beginning of the therapy (0 months) reveals an absence of I^- exchange through CFTR in PBMCs. However, after just one month of treatment, a significant increase in I^- exchange values became evident, a trend consistently observed

during the subsequent monitoring times. This data indicates that our assay could discern CFTR function differences in CF PBMCs pre- and post-therapy.

To confirm this outcome, we examined CFTR activity in PBMCs of a second patient with identical genotypic characteristics of the first one before and after *ex vivo* VX770 treatment (Figure 5A). As reported, the cell treatment promoted a significant recovery of CFTR activity. Then, we evaluated CFTR activity also *in vivo* when the patient underwent Ivacaftor therapy (Figure 5B). Again, only after the treatment, the iodine exchange values significantly increased.

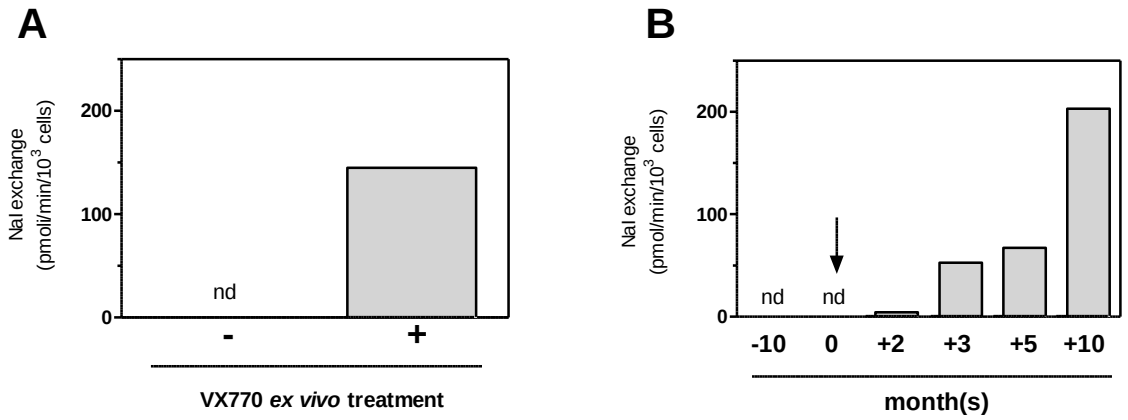

Figure 5. Examining CFTR activity in a second patient with G1349D and F508del mutations, both *in vivo* and *ex vivo* treatments with VX770 were administered. (**A**) Freshly isolated PBMCs from the CF patient with CFTR G1349D and F508del mutations were cultured either without (–) or with (+) 5µM VX770. After a 24-hour incubation period, CFTR activity was measured. "nd" denotes non-detectable activity. (**B**) CFTR activity was evaluated in freshly isolated PBMCs from the CF patient, both before (–10 and 0 months) and following Ivacaftor oral therapy (+2, +3, +5, and +10 months). "nd" signifies undetectable levels. The arrow signifies the initiation of the therapy.

All these results affirm the feasibility of evaluating CFTR activity in leukocytes after *ex vivo* treatment and its ability to predict *in vivo* effects.

Notably, the outcomes of the GST-HS-YFP assay in both patients correlated with certain clinical parameters. Specifically, we compared the functional CFTR data from the two patients with FEV1 (maximum expiratory volume in the first second) and Cl^- concentration values in sweat (Table 2). FEV1 is a crucial parameter in evaluating CF patients, typically expressed as a percentage of expected values for individuals with similar characteristics, such as ethnicity, age, sex, and height. Statistically, a healthy adult typically has an FEV1% value above 80% [101]. As for the Cl^- concentration in sweat, abnormal levels are indicative of Cystic Fibrosis. CF individuals have CFTR-related disruptions that obstruct normal ionic flow, leading to an accumulation of salts in sweat. Values in a healthy individual can vary widely but generally are below 30 mmol/L. Values above 60 mmol/L suggest a pathological condition, while values between 30 and 60 mmol/L are considered borderline [32].

			Sweat chloride (mEq/l)	
Month(s)	**CFTR activity[§]**	**FEV1 (%)**	**NaCl**	**Cl**
0	0	54	93	115
+1	135	89	66	38
+8	75	105	51	28
+12	62	105	75	47
0	0	75	123	106
+3	53	122	45	22

Table 2. In the table, we present the CFTR activity and clinical parameters following VX770 treatment in two patients with G1349D and F508del mutations in the CFTR gene. Patient 1's data is featured in the upper section, while patient 2's data is presented in the lower section. The clinical assessments include FEV1, represented as a percentage value, along with the levels of NaCl and chloride ions (Cl^-), expressed in mEq/L. [§](I^- pmoli/min/10^3 cells).

Table 2 reveals that in the first months of treatment for both patients, the CFTR activity was restored paralleled with a gradual increase in FEV1. Notably, in both cases, there was a rapid reduction in sweat salt levels following the initiation of Ivacaftor therapy.

These data indicate that the recovery of CFTR activity in both patients correlated with the restoration of clinical parameters, confirming the potentiality of our assay, useful not only in monitoring CFTR functionality but also in predicting the effects of therapies.

4.3 PROTEOMIC PROFILE ASSOCIATED WITH RECOVERY OF CFTR ACTIVITY

To gain insights into the biological functions of PBMCs associated with CFTR rescue, we selected a cohort of sixteen CF patients carrying residual function, all eligible for Ivacaftor therapy. We conducted CFTR activity evaluations in PBMCs before and after *ex vivo* VX770 treatment. The *ex vivo* approach allows us to reduce the variability related to the possible outcomes of concomitant therapies that occurred *in vivo*.

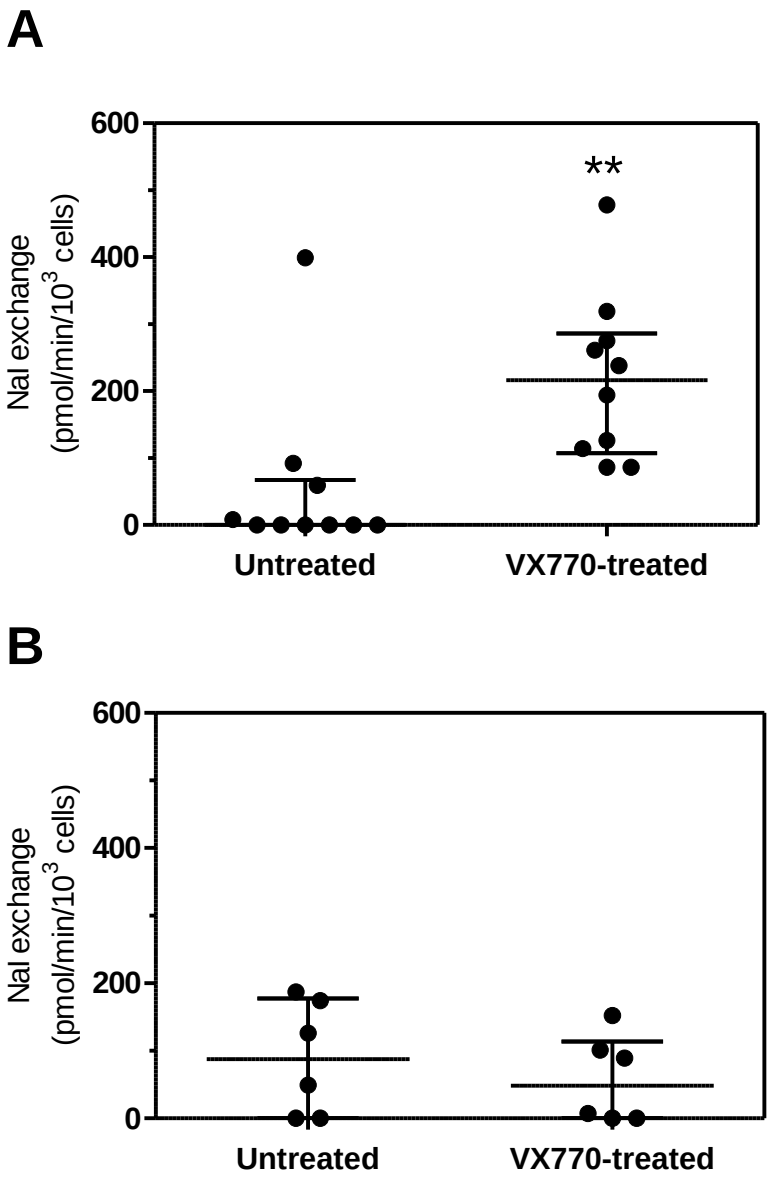

Figure 6. Evaluating CFTR functionality in PBMCs following *ex vivo* VX770 treatment. We conducted assessments on PBMCs derived from 16 CF patients, where half of each cell sample underwent a 24-hour exposure to 5µM VX770, while the other half remained untreated as control PBMCs (control). (**A**) CF patients exhibiting a positive response to the drug (n = 10). (**B**) CF patients showing no response to the drug (n = 6).

As reported in Figure 6A, among the sixteen PBMC samples treated with VX770, ten exhibited a positive response to the drug, resulting in the restoration of CFTR activity. In contrast, the other six PBMC samples maintained minimal or no CFTR activity after treatment (Figure 6B).

In parallel with the GST-HS-YFP assay for CFTR function evaluation, we conducted shotgun proteomics on PBMCs isolated from four CF patients who responded positively to VX770 treatment (Figure 7). We aimed to obtain a quantitative proteomic signature directly linked to the therapy's effects. We employed data-independent acquisition (DIA), a method that integrates discovery proteomics with selected reaction monitoring to identify and quantify thousands of proteins [102].

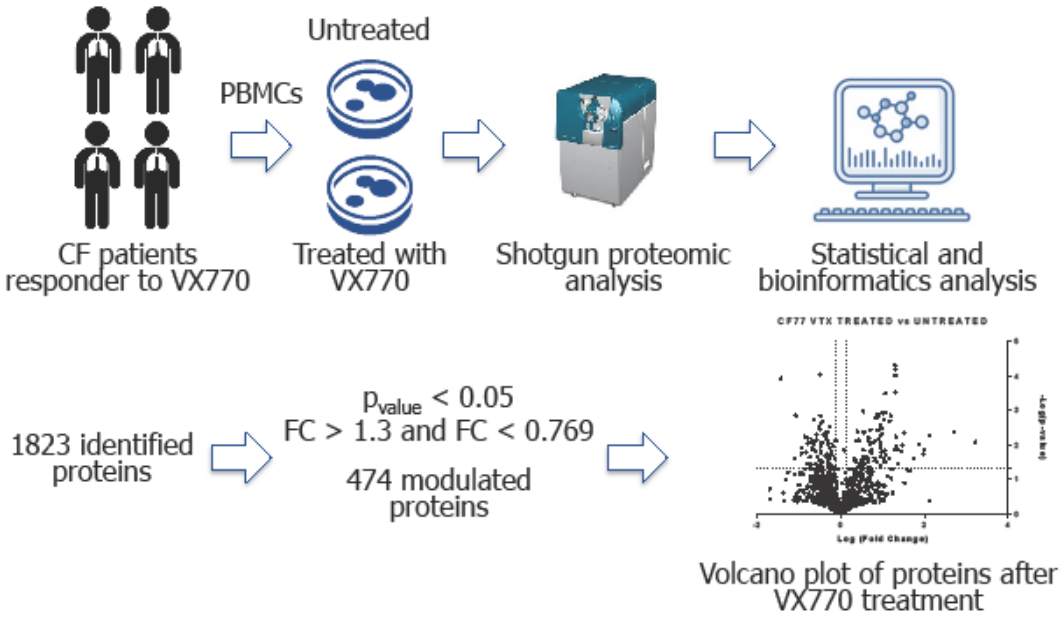

Figure 7. The proteomic analysis experimental design involved the examination of both VX770-treated and untreated PBMCs. Subsequently, the identified proteins underwent bioinformatics analysis to identify the pathways linked to the restoration of CFTR activity.

The proteomics analysis of PBMCs led to the identification of a distinct leukocyte profile including over 1800 proteins. Through statistical analysis of protein abundances, 474 proteins were found to be modulated and correlated with the restored CFTR activity.

Following this, a combined statistical and bioinformatic analysis was conducted to discern intracellular protein pathways that included these modified proteins, which demonstrated a correlation with the recovery of CFTR activity.

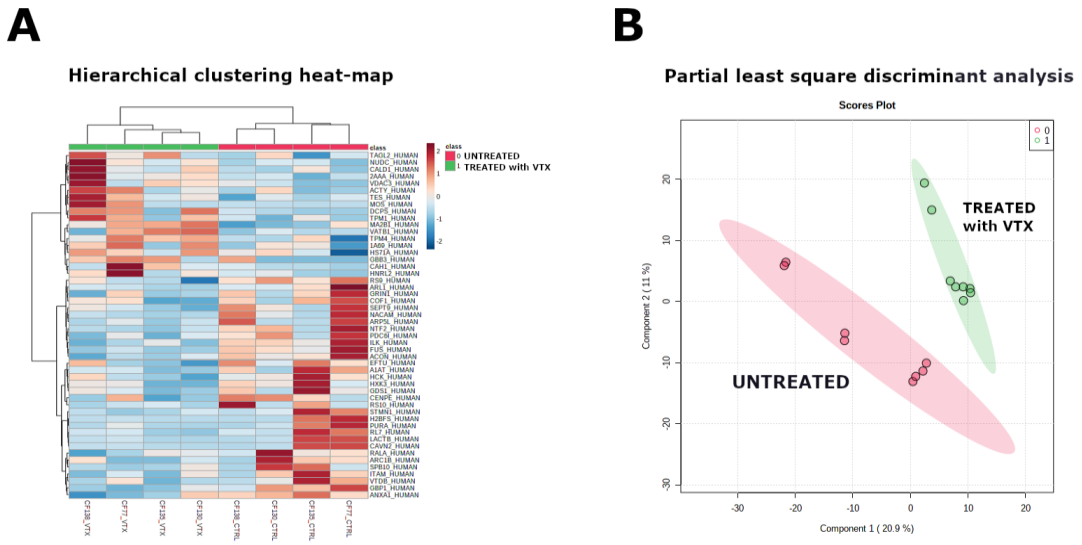

Figure 8. Comparative analysis of the leukocyte proteome was conducted through statistical and bioinformatic approaches on PBMCs from CF patients, distinguishing between those treated with VX770 and those left untreated. The findings are illustrated as follows: (**A**) Hierarchical clustering of proteins depicted in the heatmap. (**B**) Utilization of partial least squares discriminant analysis (PLS-DA) to differentiate VX-770 treated PBMCs (in green) from untreated counterparts (in red).

In Figure 8A, a heatmap is presented as a graphical tool for quantitatively evaluating protein changes. It employs a colour scale to emphasize alterations in protein expression levels between PBMC samples treated with VX770 (the first four columns, marked in green at the top) and the untreated ones (the last four columns, marked in red). Notably, the treated cells exhibited a more pronounced variation in their proteomic profile compared to the untreated cells.

Subsequently, the results underwent reprocessing through a graph known as PLS-DA (partial least squares discriminant analysis). This representation highlights maximal differentiation between distinct groups, in this case, the treated and untreated PBMCs (Figure 8B). PLS-DA confirms the presence of structurally significant proteomic information associated with CFTR recovery.

In contrast, for elucidating intracellular pathways involved in the restoration of CFTR channel function, protein modification data were subjected to analysis using the STRING

software. This approach facilitated the correlation of bioinformatic insights derived from the analyzed samples.

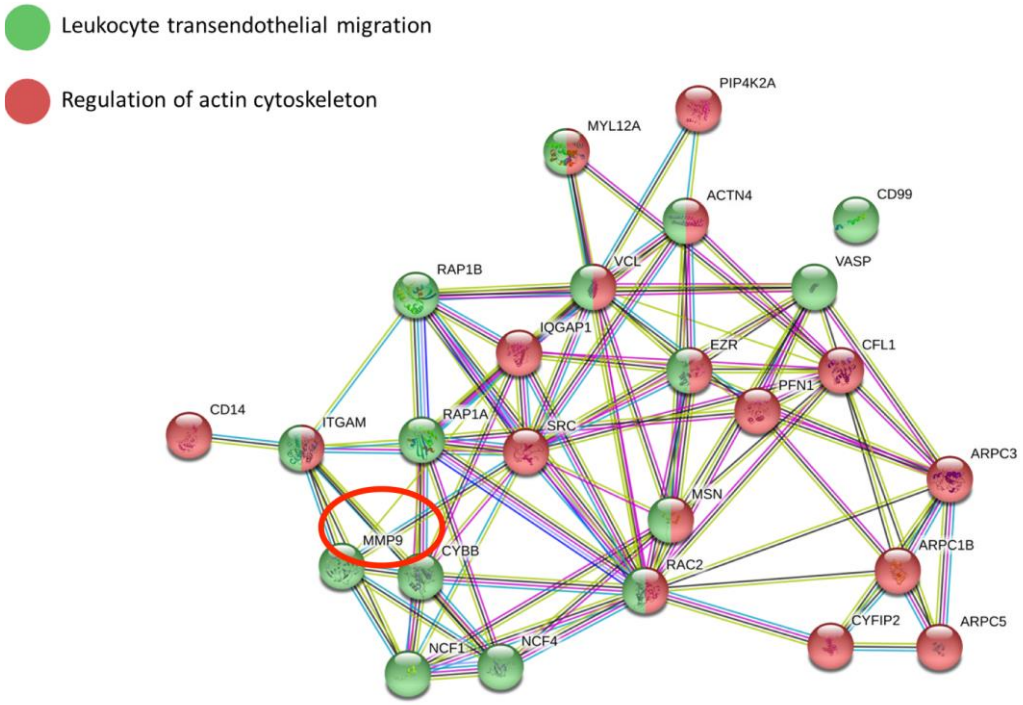

Figure 9. In the STRING graph, we observe a network analysis of proteins that have been downregulated in leukocytes following VX770 treatment. Notably, pathways associated with the transendothelial migration of leukocytes are highlighted in green, while those related to the regulation of cytoskeletal actin are denoted in red.

In particular, we identified two specific intracellular pathways in leukocytes crucially linked to the restoration of CFTR activity. One pathway is associated with the transendothelial migration process (RAP18, RAP1A, MMP9, CYBB, NCF1, NCF4, CD99, VASP, ITGAM, RAC2, MSN, EZR, VCL, MYL12A, ACTN4), while the other involves the regulation of cytoskeletal actin (CD14, IQGAP1, SRC, PFN1, CFL1, ARPC3, ARPC1B, ARPC5, CYFIP2, PIP4K2A, RAC2, MSN, EZR, VCL, ITGAM, ACTN4, MYL12A). The examination of the data revealed that a significant proportion of the analyzed proteins in these pathways were downregulated after VX770 treatment and the concomitant rescue of the CFTR activity.

Matrix metalloprotease 9 (MMP9) emerged as a notable protein from our bioinformatic analysis. This calcium-dependent endopeptidase, integral to the transendothelial migration pathway, has been extensively studied for its pivotal role in Cystic Fibrosis progression [92,103,104]. Importantly, our previous research demonstrated that PBMCs from CF patients, homozygous for the phenylalanine deletion at position 508, consistently expressed and released elevated levels of MMP9 due to alteration in intracellular calcium homeostasis [83]. This event is of great significance, as the secreted MMP9 not only possesses the capacity to degrade collagen, potentially contributing to lung parenchymal destruction and dysfunction but also can enhance chemokines involved in modulating inflammatory processes. Hence, it is plausible that the overexpression of MMP9 in CF leukocytes plays a substantial role in CF pathogenesis, potentially exacerbating the inflammatory state and inflicting damage to the lung parenchyma [85,105-107]. Therefore, the observed downregulation of MMP9 in CF PBMCs treated with VX770 could represent one of the potential positive effects of this drug in mitigating the progression of lung damage.

To confirm this proteomic finding, we conducted Western blot analyses on leukocytes of the patient in Ivacaftor therapy, whose CFTR activity was reported in Figure 4, and of one responsive and two unresponsive to *ex vivo* VX770 treatment. The data reported in Figure 10 unequivocally demonstrate a strong inverse correlation between MMP9 expression and the restoration of CFTR function.

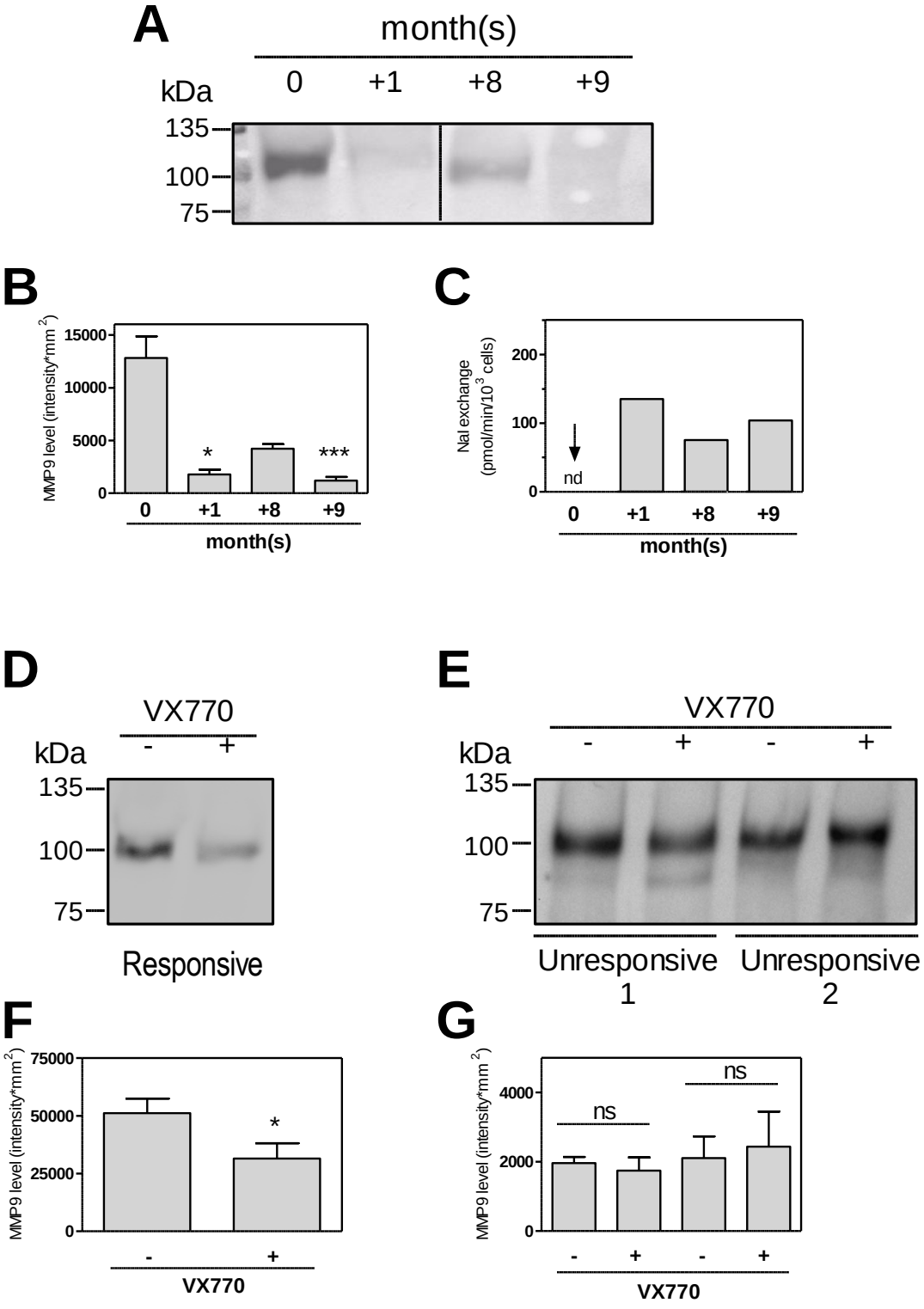

Figure 10. MMP9 Levels in PBMCs Following *in vivo* and *ex vivo* VX770 Treatment: (**A**) PBMCs were collected from a CF patient carrying CFTR G1349D and F508del mutations at specified time points from the start of Ivacaftor oral therapy. A portion of the cells underwent Western blot analysis for MMP9. Molecular weight (MW) protein markers

(kDa) are indicated. (**B**) Quantification of immunoreactive signals was performed as detailed in the Methods section. The data represents the means ± standard deviation from five quantifications. Statistical analysis was conducted using a Kruskal–Wallis test followed by Dunn's multiple comparison test. *** $p<0.001$; * $p<0.05$. (**C**) Selected CFTR activity values from Figure 4 and 5 were chosen for comparison with the MMP9 levels. (**D**) and (**E**) PBMCs from one patient responsive and two unresponsive to *ex vivo* VX770 treatment were subjected to Western blot analysis for MMP9. MW protein markers (kDa) are provided. (**F**) Quantification of immunoreactive signals was carried out as specified in the Methods section. The data represents the means ± standard deviation from six quantifications. Statistical analysis was performed using a Wilcoxon signed-rank test. * $p<0.05$. (**G**) The immunoreactive signals were quantified as detailed in the Methods section. The data represents the means ± standard deviation from six quantifications. Statistical analysis was conducted using a Wilcoxon signed-rank test. ns, not statistically significant.

4.4 CFTR ACTIVITY AND MMP9 EXPRESSION IN PBMCs OF CF PATIENTS DURING TRIKAFTA® THERAPY

To investigate whether other CF therapies with modulators could similarly influence the relationship between MMP9 expression and the restoration of CFTR function, we enrolled a large cohort of CF patients with mutations eligible for Trikafta® therapy. This triple CFTR modulator, suitable for a broad spectrum of mutations, has significantly improved the clinical condition of many CF patients. However, responses to CFTR modulators are variable, and also Trikafta®, which, while providing benefits to a majority of CF patients, may still present limitations.

To can consider MMP9 as a biomarker of therapy efficacy, we conducted an *in vivo* analysis of MMP9 expression in correlation with CFTR channel activity in CF patients undergoing Trikafta® therapy. In Table 3 are reported the clinical data of five CF patients before and after five and ten months of therapy. Among these five patients, four displayed improvements in specific clinical parameters, while one patient's condition remained unchanged.

CF patient Sample timing	Age	Gender	CFTR mutation	FEV1 (%)	Sweat Chloride test*	CFTR activity§
76-PRE	48	M	F508del/F508del	86	113	ND
76-POST I				83	67	106
76-POST II				78	NA	36
85-PRE	23	F	F508del/E585X	34	104	85
85-POST I				35	96	24
85-POST II				35	97	NA
108-PRE	29	F	F508del/L1065P	69	NA	ND
108-POST I				72	13	189
108-POST II				72	NA	166
157-PRE	14	F	F508del/I1005R	89	NA	13
157-POST I				135	NA	229
157-POST II				122	70	157
161-PRE	16	F	F508del/F508del	67	132	32
161-POST I				113	35	109
161-POST II				113	NA	136

Table 3. Details of clinical outcomes of CF patients undergoing Trikafta® therapy, categorized into responders and non-responders (n = 5). PRE: Data collected prior to initiating therapy. POST I: Information gathered at the five-month mark from the commencement of therapy. POST II: Records obtained at the ten-month point from the onset of therapy. NA: Data not available. ND: Indicating non-detectable levels. *(Cl^- mEq/L): Denotes chloride ion levels in milliequivalents per liter. §(I^- pmoli/min/10^3 cells): Represents iodide ion measurements in picomoles per minute per 10^3 cells.

As illustrated, the CFTR activity assessed by the GST-HS-YFP assay corresponds to the values of the clinical parameters, FEV1 and sweat test. Notably, the only patient who did not exhibit clinical improvement also showed no increase in CFTR channel activity after therapy. Consequently, this patient was classified as a non-responder (CF85), while the remaining four were classified as responders (CF108, CF161, CF76, CF157).

Then, we initially evaluated MMP9 levels in PBMCs before Trikafta® therapy, not only for the five CF patients mentioned above, but also for additional eleven patients who entered therapy later (Figure 11).

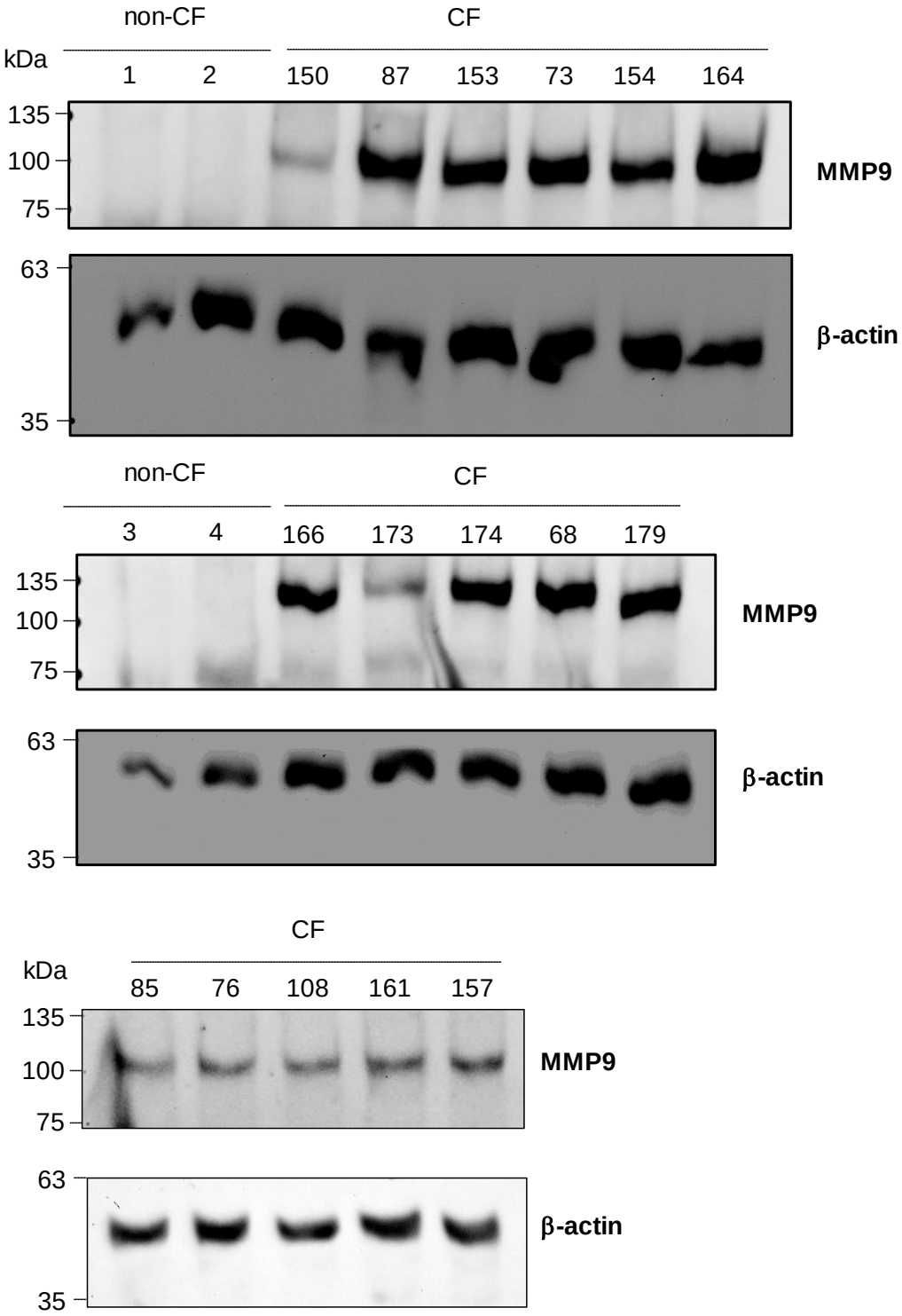

Figure 11. Analysis of intracellular MMP9 expression was conducted in PBMCs obtained from a cohort of CF patients (n = 16) and non-CF donors (n = 4) utilizing Western blot analysis. To begin, PBMCs were lysed using Laemmli sample buffer. Subsequently, an aliquot from each sample, equivalent to 3×10^5 cells, underwent SDS-PAGE (6%) followed by Western blot analysis targeting MMP9 and β-actin.

As shown in Figure 11, MMP9 expression was evident in the PBMCs of all CF patients, while no MMP9 was detectable in the PBMCs from four non-CF subjects. These findings corroborate our earlier data, in which PBMCs isolated from CF patients homozygous for F508del constitutively expressed and released MMP9 at a high rate; in contrast, in PBMCs isolated from non-CF donors, MMP9 expression and secretion were undetectable [83]. Given our previous observation that MMP9 reduction could be a potential leukocyte biomarker associated with Ivacaftor effectiveness, we proceeded to evaluate MMP9 levels in PBMCs of both non-responder and responders before and after specified durations of Trikafta® therapy.

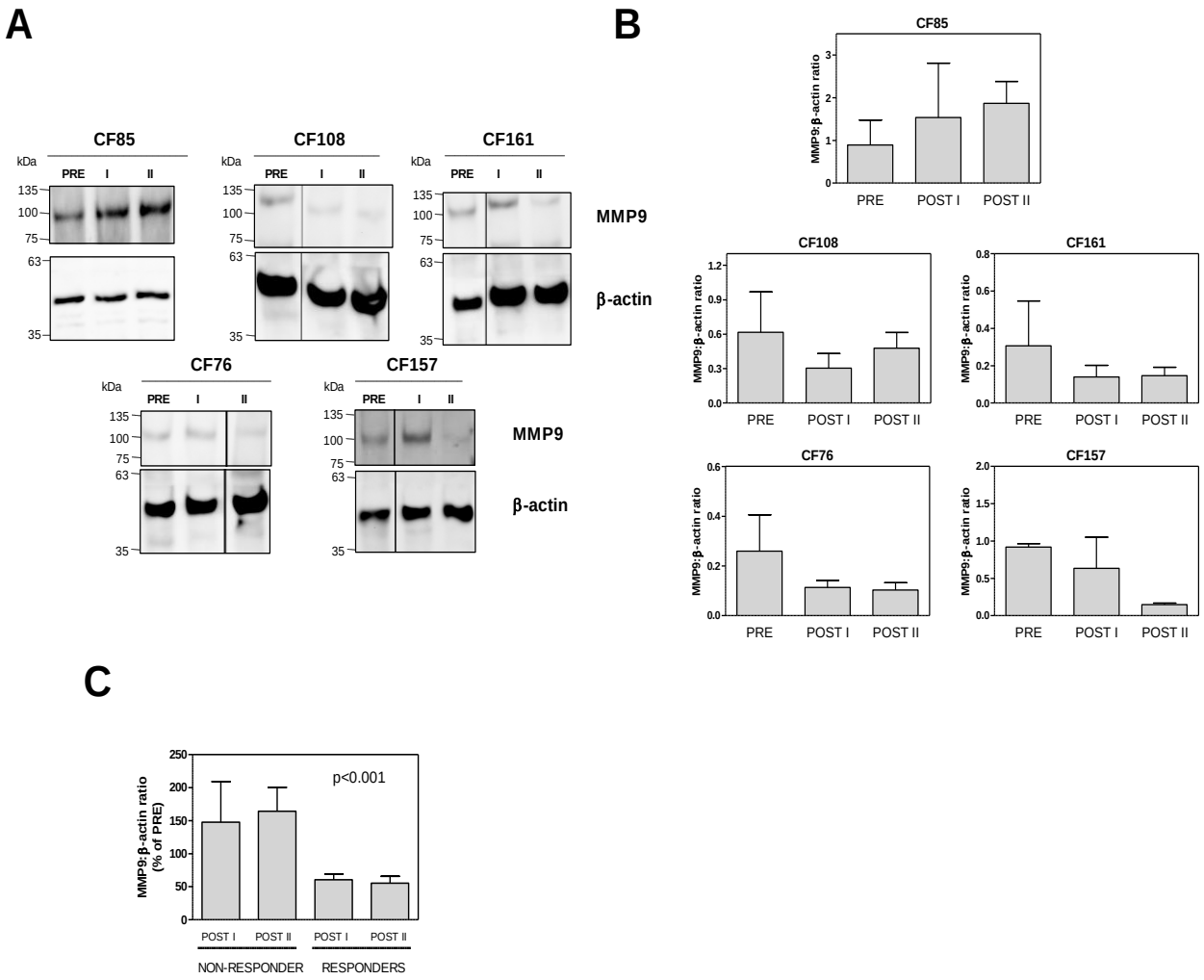

Figure 12. Intracellular MMP9 Expression in Individuals with Cystic Fibrosis post Trikafta® Therapy. MMP9 expression was assessed in PBMCs isolated from one non-responder patient and four responder patients, both prior to (PRE) and following the therapy (POST I and POST II), using Western blot analysis. An aliquot of each sample, equivalent to 3×10^5 cells, underwent SDS-PAGE (6%). Subsequently, a WB analysis for MMP9 and β-actin was conducted as a loading control. A representative blot of three is presented. (**A**) For each sample, the MMP9 levels detected in (**B**) were quantified, and the MMP9: β-actin ratios were averaged and reported with standard deviation. (**C**) The immunoreactive signals were quantified, and the MMP9: β-actin ratios obtained after therapy (POST I and POST II) were expressed as percentages of the ratios before therapy (PRE) for both non-responder and responders. The data represents means with standard deviation from three quantifications for each sample. According to a Mann–Whitney test, $p<0.001$.

As depicted in Figures 12A and 12B, a strong decrease in intracellular MMP9 expression was observed among the responders after only five months of therapy, and this downregulation persisted even after ten months of treatment (POST I and POST II). However, this trend was not observed in the non-responder (CF85), where the level of MMP9 expression remained elevated at all points of analysis. These initial findings, while promising, do not reveal a specific temporal pattern for treatments influencing MMP9 expression. Indeed, there was no statistically significant difference between the results at POST I and POST II obtained from all samples considered (Figure 12B). Nevertheless, the intracellular MMP9 expression levels in all responders, at both POST I and POST II, exhibited a significant decrease following Trikafta® treatment, a change not observed in non-responder (Figure 12C).

These results highlight MMP9 downregulation in leukocytes as an indicative biomarker of CFTR modulator effectiveness. Furthermore, they suggest its suitability in assessing the individual patient's response to Trikafta® therapy.

4.5 MMP9 ACTIVITY IN PLASMA SAMPLES OF CF PATIENTS DURING TRIKAFTA® THERAPY

In order to establish an immediate and non-invasive method to evaluate MMP9 as a potential biomarker, before the initiation of Trikafta® therapy, we assessed MMP9 expression in plasma samples collected simultaneously with the isolation of PBMCs from the same cohort of sixteen CF patients presented in Figure 11. This effort aimed not only to confirm MMP9 modulation as a potential biomarker for assessing the efficacy of CFTR modulators but also to gain deeper insights into the underlying molecular mechanism leading to the modulation of MMP9 synthesis and secretion.

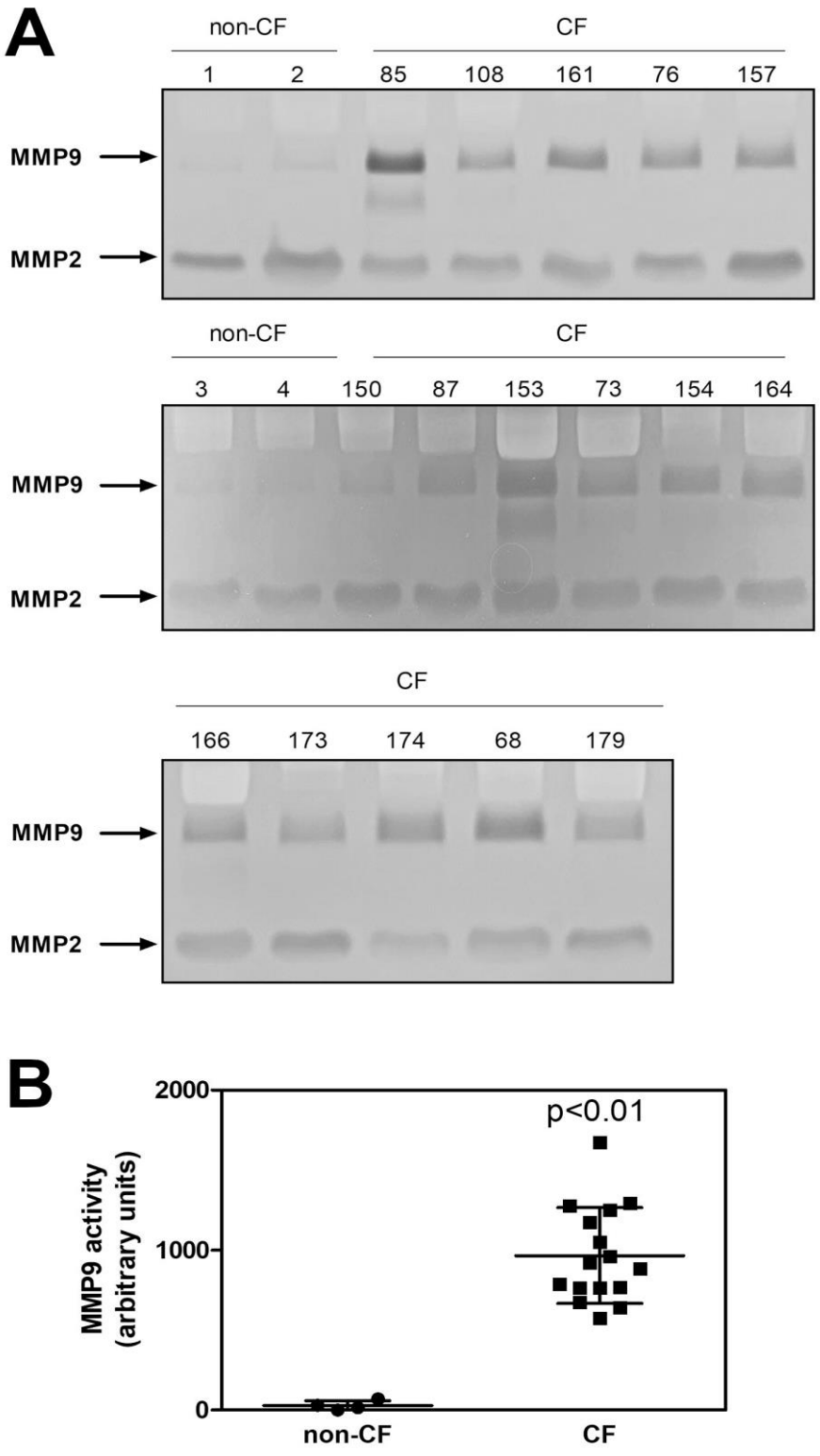

Figure 13. Assessment of MMP9 Activity in Plasma Samples from CF Patients and Non-CF Donors. (**A**) Plasma samples (1 µL) obtained from CF patients (n = 16) and non-CF

donors (n = 4) were subjected to zymography on SDS-PAGE (8%). Lytic bands indicative of MMP9 and MMP2 activity are marked by arrows. The original images were transformed into grayscale and inverted for enhanced visualization. (**B**) Quantification of MMP9-dependent lytic bands presented as means ± SD; $p<0.01$, as determined by the Mann-Whitney test.

As illustrated in Figures 13A and 13B the MMP9 activity in plasma samples from all patients before the therapy was significantly higher compared with the activity of four non-CF subjects, consistent with the results obtained from the analyses performed in PBMCs (see Figure 11).

Additionally, to determine if the MMP9 activity during therapy followed the same trend observed intracellularly, we quantified MMP9 activity in plasma samples from both non-responder and responders before and after therapy.

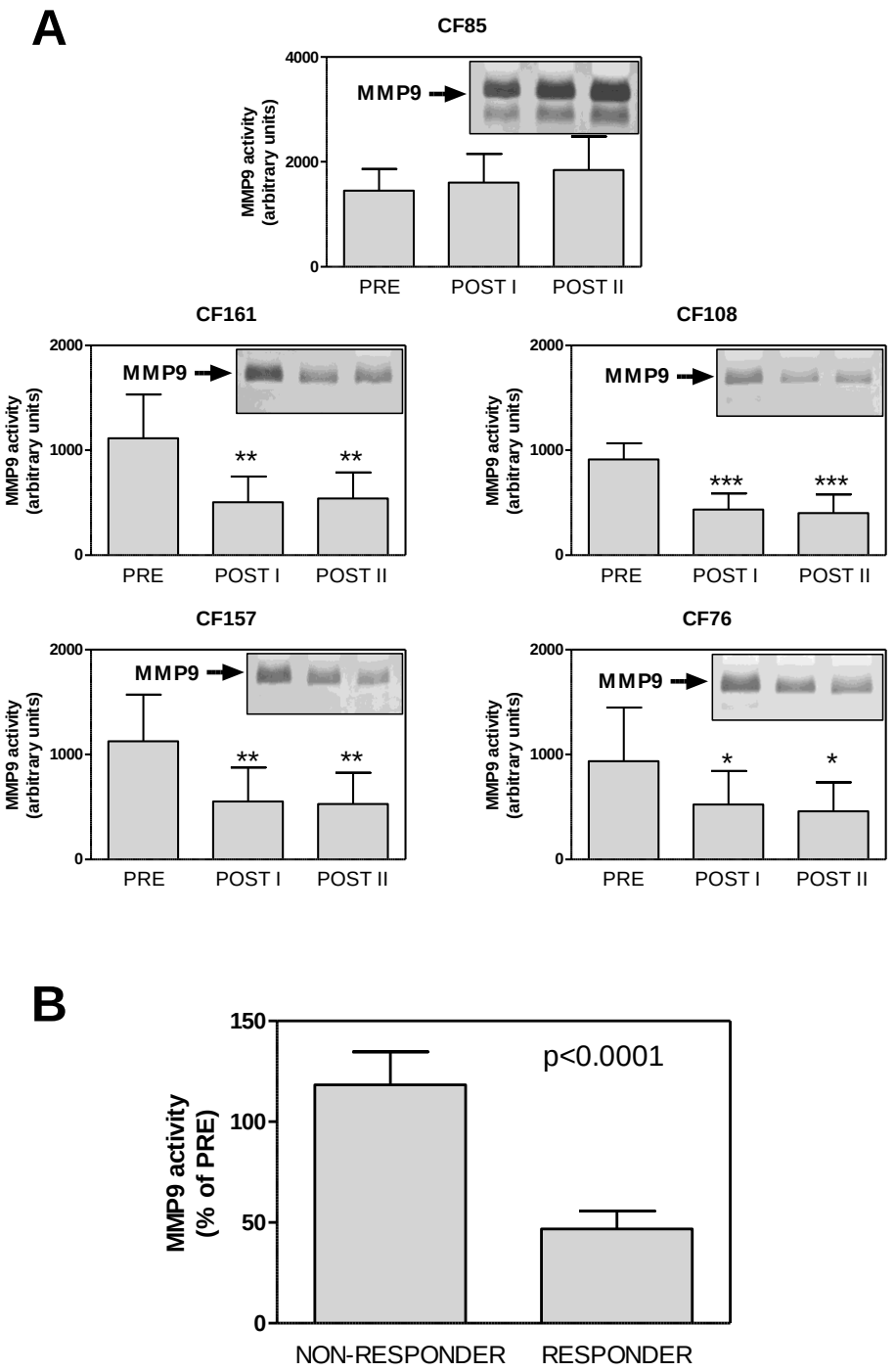

Figure 14. Plasmatic MMP9 activity in CF patients following Trikafta® therapy. (**A**) MMP9 activity has been measured in 1 µL of plasma obtained from one non-responder patient and from four responder patients, before (PRE) and following the therapy (POST I and POST II), by zymography. One representative zymogram of three is shown for each patient. The MMP9-dependent lytic bands were quantified and reported as means ± SD

from three quantification for each CF patient; *p<0.05, **p<0.01, ***p<0.001, according to ANOVA followed by Tukey's post hoc test (**B**) MMP9 activity detected following the therapy (POST I+POST II) was reported as percentage of the metalloprotease activity before the therapy (PRE), for non-responder and responders. Data are means ± SD from three quantification for each CF patient. p<0.001, according to unpaired t-test test.

As reported in Figure 14A plasmatic MMP9 activity remained unchanged in non-responder after therapy. Conversely, in responders, there was a notable reduction of the plasmatic protease of approximately 50% (Figures 14A and 14B). These findings, in line with those outlined in Figure 12, suggest that the MMP9 levels measured in plasma could correspond to those secreted by PBMCs.

Furthermore, to further support the hypothesis that plasmatic MMP9 modulation could be a useful biomarker of CFTR modulator efficacy, we also preliminary evaluated the changes in plasmatic MMP9 activity in eight of the eleven CF patients who had started Trikafta® therapy, but whose plasmatic samples and clinical outcomes were already available (see Table 4).

CF patient	Age	Gender	CFTR mutation	FEV1 (%)	Sweat Chloride test*
68	34	M	F508del/N1303K	PRE: 62 POST I: 83	PRE: 120 POST I: 94
73	35	M	F508del/G542X	PRE: 32 POST I: 44	PRE: 109 POST I: 109
87	55	F	F508del/R334W	PRE: 77	PRE: 115
150	31	M	F508del/2184insA	PRE: 83	PRE: 70
153	17	F	F508del/R553X	PRE: 43 POST I: 75	PRE: NA POST I: 47
154	25	F	F508del/W1282X	PRE: 106	PRE: NA
164	17	F	F508del/W1282X	PRE: 113 POST I: 152	PRE: 110 POST I: 50
166	22	F	F508del/F508del	PRE: 77 POST I: 99	PRE: 107 POST I: NA
173	14	M	F508del/S13R	PRE: 111 POST I: 101	PRE: NA POST I: NA
174	14	M	F508del/2183AA>G	PRE: 81 POST I: 89	PRE: NA POST I: 52
179	31	M	F508del/D110H	PRE: 92 POST I: 107	PRE: NA POST I: NA

Table 4. Clinical information for additional CF patients, including eight individuals who had initiated Trikafta® therapy at an early stage (n = 11). PRE, prior to therapy initiation;

POST I, five months after the commencement of therapy; NA, data not available. *(Cl^- mEq/L).

As shown in Figures 15A and 15B, we observed a decrease in plasmatic MMP9 at the first follow-up during Trikafta® therapy in seven CF patients identified as responders, in which there was an improvement in clinical parameters. However, in CF73, identified as a non-responder based on clinical assessment, MMP9 activity remained unchanged.

Although these findings are preliminary, together with those previously obtained, further strengthen the hypothesis that plasmatic MMP9 can serve as a promising biomarker of the efficacy of CFTR modulators. Furthermore, the modulation of plasmatic MMP9 could be an indicator of the leukocytes' molecular changes related to the response to the therapy.

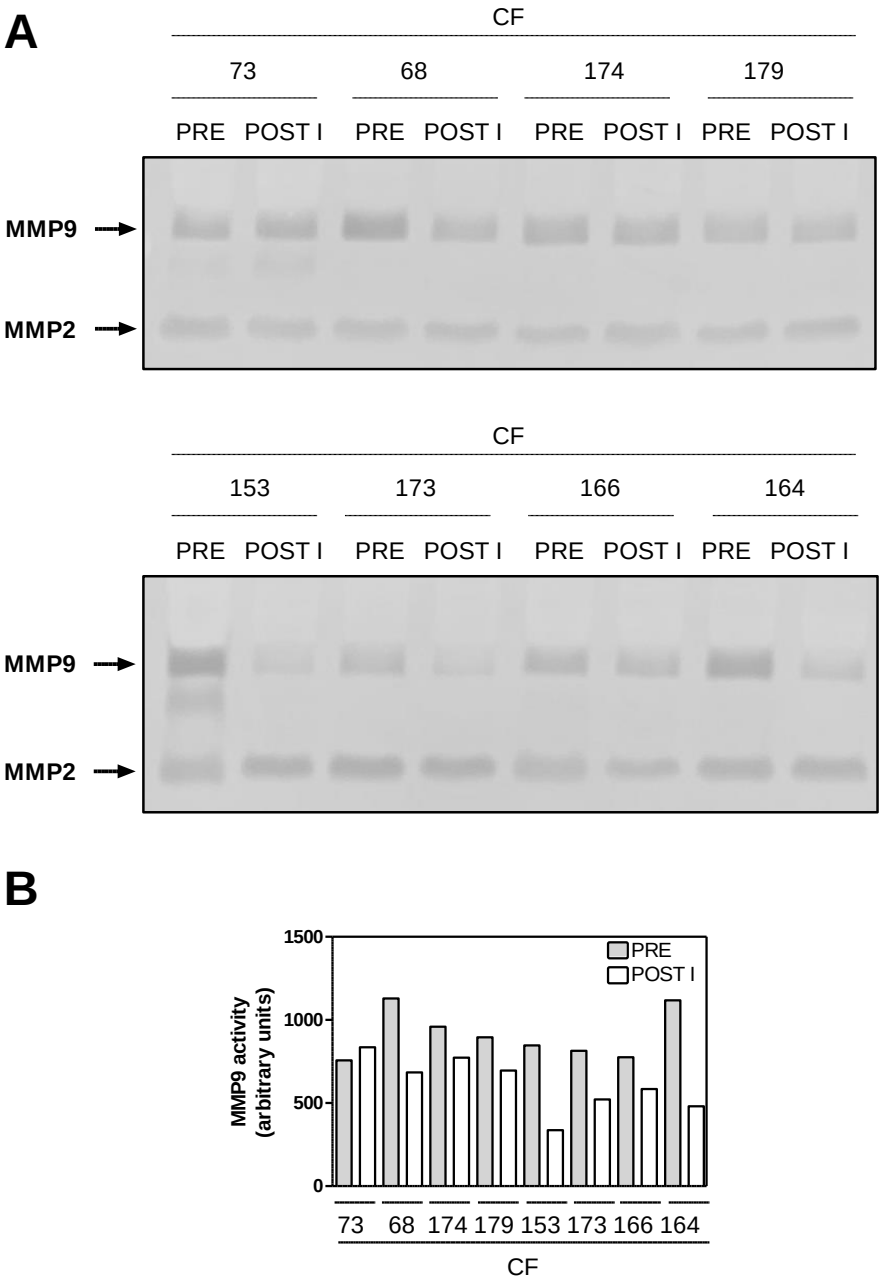

Figure 15. Plasmatic MMP9 Activity in Additional CF Patients Post Trikafta® Treatment. (**A**) Analysis of MMP9 activity was conducted on 1 µL of plasma collected from eight additional CF patients, both before (PRE) and after therapy initiation (POST I), using zymography. (**B**) Quantification of MMP9-dependent lytic bands as illustrated in (A).

4.6 INVESTIGATION OF INTRACELLULAR SIGNALING PATHWAYS ASSOCIATED WITH MMP9 EXPRESSION IN PBMCs OF CF PATIENTS DURING TRIKAFTA® THERAPY

It has been confirmed that MMP9 is the predominant MMP present in bronchopulmonary secretions derived from CF patients [86]. Bronchial wall destruction in CF patients may be influenced by MMP9 through a variety of mechanisms. MMP9 exhibits the capability to degrade elastin, fibronectin, and different types of collagen present in the basement membrane of airways [108]. This degradation of basement membrane components by MMP9 could potentially enhance inflammation by allowing the movement of inflammatory cells into both the airway lumen and bronchial walls. Additionally, MMP9 has been demonstrated to directly damage airway cartilage, as evidenced by its ability to degrade articular cartilage [109]. While neutrophils are recognized as a primary source of MMP9, other cell types, such as macrophages and epithelial cells, have also been identified as potential producers [90]. However, the specific signaling pathway responsible for the upregulation of MMP9 in Cystic Fibrosis remains not completely understood. Some studies propose that both the extracellular signal-regulated kinase 1/2 (ERK1/2) and the nuclear factor-κB (NF-κB) pathways can promote MMP9 expression [87,110]. Therefore, we investigated the possible involvement of these signaling mechanisms in the elevated MMP9 expression observed in PBMCs of CF patients as well as in the subsequent modulation of the protease after Trikafta® therapy.

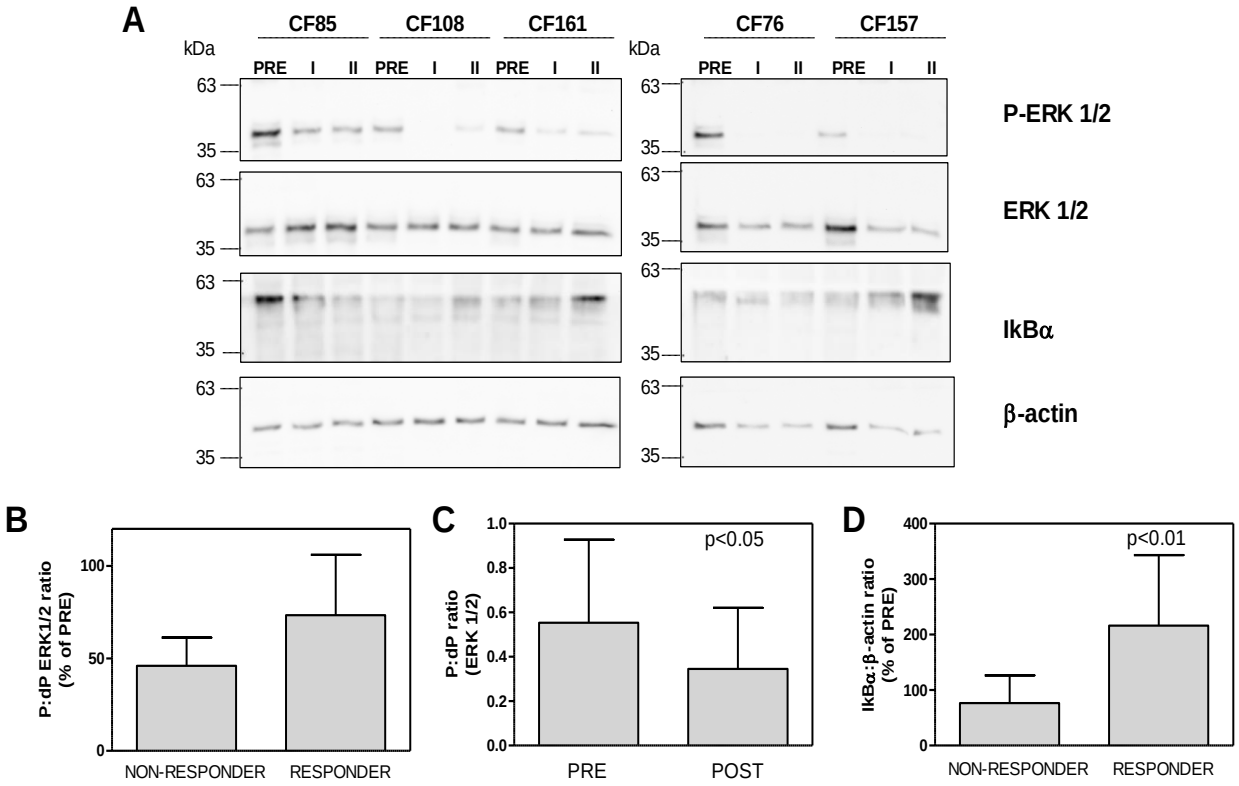

Figure 16. Intracellular signaling pathway associated with MMP9 expression in CF patients after Trikafta® therapy. (**A**) Analysis of ERK1/2 phosphorylation and IkBα modulation was conducted on PBMCs isolated from one non-responder patient and four responder patients, both prior to (PRE) and following the therapy (I and II), using Western blot analysis. An aliquot from each CF patient's sample, equivalent to 3×10^5 cells, underwent SDS-PAGE (10%) followed by WB analysis targeting the specified antigens. A representative blot of three is presented. (**B**) The immunoreactive signals were quantified, and the ratios between phosphorylated (P) and dephosphorylated (dP) forms of ERK1/2 obtained post-therapy (I+II) were expressed as a percentage of the ratios pre-therapy (PRE), for both non-responders and responders. Alternatively, (**C**) the P:dP ERK1/2 ratio was compared between the pre-therapy (PRE) and post-therapy (POST) ratios. (**D**) The IkBα:β-actin ratios obtained post-therapy (I+II) were reported as a percentage of the ratios pre-therapy (PRE), for both non-responders and responders. The data represents means with standard deviation from three quantifications for each CF patient. $p<0.05$ and $p<0.01$, according to a Mann Whitney test.

As Figure 16A shows, the levels of p-ERK1/2 in PBMCs of all five CF patients collected before Trikafta® therapy were higher than those obtained at five and ten months post-therapy initiation (POST I and POST II). The dephosphorylation of these kinases showed no significant difference between non-responder and responders (Figure 16B), suggesting that Trikafta® may induce p-ERK1/2 dephosphorylation, regardless of the therapeutic efficacy of the triple CFTR modulator therapy.

However, considering that p-ERK1/2 induces MMP9 expression in leukocytes, as we have previously reported [83], the dephosphorylation of p-ERK1/2 is in line with the MMP9 modulation data we obtained from PBMCs of the responders, though not with those obtained from the PBMCs of the non-responder. To clarify this discrepancy, we studied the downstream events particularly related to the NF-kB pathway, since it has been reported [110] that this nuclear transcription factor is involved in promoting MMP9 expression. Specifically, the nuclear factor-κB inhibitor (IkBα) phosphorylation and subsequent degradation facilitate NF-kB translocation into the nucleus binding to the sequence-specific promoter of MMP9. Therefore, we examined the modulation of IkBα in PBMCs of CF patients responding and non-responding to Trikafta® therapy.

From this analysis, we noted that PBMCs from responder patients showed a significant increase in IkBα levels after Trikafta® therapy, while those from non-responder demonstrated a decrease (Figures 16A and 16D). This modulation of IkBα corresponds with the modulation of MMP9 expression, indicating that the therapy, which is useful to restore CFTR activity, facilitates MMP9 downregulation through the NF-kB pathway.

In conclusion, the altered intracellular calcium concentration observed in cells of Cystic Fibrosis patients due to reduced CFTR activity [83], together with pro-inflammatory signals, can trigger ERK1/2 activation and the consequent IkBα phosphorylation and degradation. These events cause the dissociation of NF-kB which translocate into the nucleus, promoting MMP9 mRNA transcription, and thus increasing MMP9 protein expression. Consequently, MMP9 becomes detectable both in the extracellular environment and in the plasma of CF patients. Our findings suggest that Trikafta® therapy prevents the degradation of IkBα, and as a result, the transcription of MMP9 mRNA cannot occur. Ultimately, the influence of therapy on this molecular pathway leads to a significant reduction in MMP9 levels in patients identified as responders.

Conversely, in PBMCs of non-responder patients, while ERK1/2 dephosphorylation still occurs, IkBα continues to be degraded, inhibiting the downregulation of MMP9 expression. Thus, by elucidating the NF-kB signaling pathway associated with MMP9 expression, it may be possible to understand the molecular events underlying the variability in the clinical responses to the therapies observed in CF patients.

5. DISCUSSION

The data obtained in my Ph.D. thesis initially allowed us to identify specific proteomic profiles associated with the recovery of CFTR activity in CF leukocytes before and after *ex vivo* treatment with the potentiator VX770. Specifically, we conducted CFTR activity assay and shotgun proteomic analysis on PBMCs isolated from CF patients with residual function mutations (classes IV and V), potentially eligible for Ivacaftor therapy, both before and after treatment with VX770. The decision to perform *ex vivo* treatments with VX770 was motivated by the aim to reduce the experimental variability related to the effect of concomitant therapies often administrated to CF patients. Initially, this analysis aimed to identify leukocyte biomarkers associated with restoration of CFTR function that would allow us not only to monitor but also to predict the efficacy of new CF pharmacological therapies. Indeed, we demonstrated that restoration of CFTR function in PBMCs of CF patients, after *ex vivo* treatment with VX770, anticipated the patient's positive response once beginning therapy. Thus, our analysis could be useful for the identification of predictive biomarkers for treatment effectiveness.

Through our experimental approach and subsequent data analysis using some bioinformatics software such as Ingenuity Pathway Analysis, Cytoscape, STRING, as well as multivariate analysis software such as The Unscrambler, Statistica, MatLab, we successfully identified two leukocyte pathways, both containing downregulated proteins after VX770 treatment: one associated with leukocyte transendothelial migration and one associated with cytoskeletal actin regulation. These data align with those reported in the literature highlighting the central role of actin in CFTR activation mediated by cyclic AMP. Indeed, therapeutic treatments capable of correcting channel function have been shown to restore cytoskeletal actin disorganization in Cystic Fibrosis subjects [111]. Moreover, altered monocyte adhesion has already been shown in CF patients, which promotes leukocyte accumulation at the level of the bronchial mucosa and plays a key role in immune responses and inflammation [38]. Indeed, it is reported that a malfunctioning CFTR disrupts chemoattractant-induced integrin activation and chemotaxis in primary monocytes, thereby influencing their migration in the lung parenchyma. In addition, it was also demonstrated that CFTR's C terminus helps shape the actin cytoskeleton [112].

Among the proteins of the transendothelial migration pathway, we identified several metalloproteases usually involved in Cystic Fibrosis [85], such as matrix metalloproteinase 8 (MMP8), matrix metalloprotease 9 (MMP9), and matrix metalloproteinase 16 (MMP16). However, only MMP9 exhibited a reduction following the treatment. This is a matrix metalloprotease that belongs to the family of calcium-dependent endopeptidases and is expressed by several immune cells, such as neutrophils and monocytes/macrophages [113,114]. As previously mentioned, it is a protein that plays a crucial role in the progression of Cystic Fibrosis, as reported in several studies [92,103,104]. Indeed, MMP9 is upregulated in both quantity and activity in the lower airway secretions of CF patients [90]. Furthermore, in a previous study conducted in our laboratory, it was revealed that PBMCs from CF patients homozygous for phenylalanine deletion 508 constitutively express and release high amounts of MMP9 at elevated rates [83], due to alterations in their intracellular Ca^{2+} homeostasis. Importantly, as a result of its secretion, MMP9 acquires not only the ability to degrade collagen, potentially contributing to the destruction and dysfunction of the lung parenchyma, but also to potentiate chemokines involved in the modulation of inflammatory processes [88,89]. In addition, *in vitro* studies demonstrated that CFTR modulators altered monocyte calcium homeostasis, a critical factor in initiating aberrant MMP9 secretion in CF immune cells [115]. All this evidence indicates a pathogenetic role for MMP9 overexpression observed in CF leukocytes, potentially exacerbating airway inflammation and parenchyma lung destruction. This is due to the recruitment of a large number of monocytes in the site of inflammation and damage [116]. In this context, as recently reported [38], the altered leukocyte adhesion caused by the failure of CFTR function could result in the recruitment of many of these cells at the lung level, consequently triggering a persistent and sustained secretion of MMP9 at this site. Therefore, we can suggest that the downregulation of MMP9, observed in CF PBMCs treated with VX770, could represent one of the possible positive effects of CFTR modulators in decreasing the progression of lung damage. In support of this hypothesis, studies have documented that an RNA-Seq analysis of whole blood gene expression alterations following Lumacaftor/Ivacaftor treatment revealed a decrease in MMP9 expression among responsive patients [115].

These positive results set the basis for the possible confirmation of MMP9 as a leukocyte biomarker associated with the restoration of CFTR function. After this initial, mainly *ex vivo* work, we also conducted *in vivo* studies. In particular, we applied the same experimental approach on leukocytes from patients undergoing Ivacaftor therapy, aiming to correlate the new results with the *ex vivo* data and with some clinical parameters, such as FEV1 and sweat test, concurrently monitored in the same patients. These results *in vivo* confirmed the previously *ex vivo* data, demonstrating an inverse correlation between MMP9 levels and the improvement of the clinical condition and CFTR activity.

Subsequently, it was interesting to analyse whether other CFTR modulators could influence the relationship between MMP9 expression and restoration of CFTR function. In particular, we addressed our studies on PBMCs of CF patients with mutations eligible for the triple combination Trikafta®, recruited by the Giannina Gaslini Institute. Indeed, this therapy was recently approved by EMA and AIFA for treating CF patients with at least one mutation containing the phenylalanine 508 deletion.

However, among these patients on Trikafta® therapy, there were responders and non-responders to the therapy based on their clinical parameters. Indeed, as previously reported, clinical responses to modulators are sometimes variable [46,47] and it has been reported that the efficacy of Trikafta® therapy, which is useful in treating the majority of CF patients, also needs refinement [48]. Therefore, we studied the relationship between the level of MMP9 and the individual patient's response to the therapy to define the modulation of this protease as an indicative biomarker of CFTR modulator effectiveness.

We evaluated MMP9 levels in PBMCs and plasma samples collected from five CF patients before and after Trikafta® therapy. Assessment of MMP9 levels in plasma may be useful to determine an immediate and non-invasive method for evaluation of this protease as a potential protein biomarker. In the biological samples of clinically positive responders, MMP9 showed a downregulation after the start of therapy. In contrast, the non-responder patient exhibited elevated levels of this protease even after the start of therapy. The observed downregulation of MMP9 during therapy correlated with the

recovery of CFTR activity assayed in the same PBMC samples, along with enhanced clinical outcomes.

To strengthen the significance of our findings and to confirm these data in a higher number of patients, we extended our MMP9 analysis to PBMCs and plasma samples from eleven other patients who had not yet undergone Trikafta® therapy. Among them, we selected eight patients who subsequently had started Trikafta® therapy, and assessed their plasma levels of MMP9 after the initial phases of treatment. Our results and patient clinical data revealed a similar modulation of MMP9 in both PBMCs and plasma samples from responder and non-responder patients. This suggests that the plasmatic downregulation of MMP9 is a promising biomarker of therapy efficacy.

Moreover, since we have reported that p-ERK1/2 induces MMP9 expression in leukocytes of CF patients [83], we investigated the intracellular signaling pathway associated with MMP9 modulation in PBMCs of both responsive and non-responsive CF patients undergoing Trikafta® therapy. While a significant reduction in ERK1/2 phosphorylation levels was observed in PBMCs of responder patients, unexpectedly, the decrease in p-ERK1/2 also occurred in non-responder patient who maintained high levels of MMP9. To explain this discrepancy we analyzed the downstream events related to the NF-kB pathway [110]. Specifically, responsive patients exhibited a significant increase in IkBα levels following the initiation of therapy, coinciding with reductions in both intracellular and plasma MMP9 levels. Conversely, non-responsive patient showed a decrease in intracellular IkBα levels despite MMP9 remaining overexpressed after therapy. These data demonstrated that the activation or inhibition of NF-kB, through the degradation or synthesis of its cytosolic inhibitor, IkBα, could regulate the modulation in intracellular MMP9 expression and subsequent secretion of MMP9 in the plasma. This result aligns with the potential involvement of p-ERK1/2 in the phosphorylation process of IkBα before its degradation. Therefore, the translocation of NF-κB into the nucleus, a process facilitated by the phosphorylation, polyubiquitination, and subsequent proteasomal degradation of IkBα, could promote MMP9 expression [117]. Hence, we suggest that the downregulation of MMP9 expression in the PBMCs and plasma of responders to Trikafta® therapy may be attributed to a decrease in p-ERK1/2, sufficient to promote a consequent increase in IkBα and inhibition of NF-kB translocation into the

nucleus. In contrast, in PBMCs from the non-responsive patient, the decrease in p-ERK1/2, observed after therapy, failed to increase IkBα levels, thus failing to downregulate MMP9 expression. This event implies the possible involvement of other kinases in the phosphorylation process of IkBα, as previously documented in the literature [87,117]. Although several studies have demonstrated a relationship between increased NF-kB activation [118-120] and CFTR dysfunction in various CF cell lines, potentially leading to the expression of proinflammatory mediators, less attention has been paid to investigating the connection of this pathway with MMP9 expression in CF PBMCs. Therefore, further investigations, ideally employing a proteomic approach, are recommended to support our hypothesis and better clarify the p-ERK1/2/NF-kB signaling pathway discrepancy and MMP9 expression between responsive and non-responsive CF patients.

In conclusion, the findings reported in my Ph.D. thesis involve the direct monitoring of MMP9 levels in both PBMCs and plasma of CF patients undergoing therapy with CFTR modulators. These results provide additional information, useful to confirm MMP9 modulation as a biomarker to monitor and predict the effectiveness of therapy and to anticipate the individual patient's response to CF therapy. In future analyses, it would be interesting to further evaluate the proteomic results and explore other proteins that might be up- or down-modulated, with particular attention to those associated with MMP9, to identify additional biomarkers that might improve our understanding of an individual patient's response to CF therapy. Moreover, we investigated the relationship between the p-ERK1/2/NF-kB signaling pathways and MMP9 expression, highlighting a potential alteration in the modulation of ERK1/2 phosphorylation in the PBMCs of the non-responder patient. To elucidate the biochemical mechanisms related to the NF-kB signaling pathway and MMP9 expression, we will conduct future studies on more suitable cell models (e.g., monocytes/macrophages) directly isolated from responders and non-responders to the therapy. The resulting data will be helpful in better understanding the molecular events underlying the variable clinical responses of CF patients to CFTR modulators. This knowledge, obtained with a simple blood draw, can be useful for future studies of personalized medicine.

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