MicroRNA global profiling in cystic fibrosis cell lines reveals dysregulated pathways related with inflammation, cancer, growth, glucose and lipid metabolism, and fertility: an exploratory study

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Abstract. Background and aim: Cystic fibrosis (CF), is due to CF transmembrane conductance regulator (CFTR) loss of function, and is associated with comorbidities. The increasing longevity of CF patients has been associated with increased cancer risk besides the other known comorbidities. The significant heterogeneity among patients, suggests potential epigenetic regulation. Little attention has been given to how CFTR influences microRNA (miRNA) expression and how this may impact on biological processes and pathways. Methods: We assessed the changes in miRNAs and subsequently identified the affected molecular pathways using CFBE41o-, and IB3 human immortalized cell lines since they reflect the most common genetic mutations in CF patients, and 16HBE14o- cells were used as controls. Results: In the CF cell lines, 41 miRNAs showed significant changes (FC (log2) ≥ +2 or FC (log2) ≤ -2 and p-value≤0.05). Gene target analysis evinced 511 validated miRNA target genes. Gene Ontology analysis evidenced cancer, inflammation, body growth, glucose, and lipid metabolism as the biological processes most impacted by these miRNAs. Protein-protein interaction and pathway analysis highlighted 50 significantly enriched pathways among which RAS, TGF beta, JAK/STAT and insulin signaling. Conclusions: CFTR loss of function is associated with changes in the miRNA network, which regulates genes involved in the major comorbidities that affect CF patients suggesting that further research is warranted. (www.actabiomedica.it)

Keywords: cystic fibrosis, miRNA, growth, inflammation, cancer, glucose metabolism, lipid metabolism, fertility

1. Introduction

Cystic Fibrosis (CF) is a recessive genetic disease with an average incidence of 1/4500 in Western Europe and 1/6000 in Northern and Central Europe (1-7). Currently over 2000 variants were listed in the CF transmembrane conductance regulator (CFTR) gene (8). Although CFTR functions mainly as a chloride channel, it provides several regulatory roles in the homeostasis of ions and other metabolites. In this chronic inflammatory disease, lung infections and disease prevail, but with time a number of comorbidities can develop. These show significant variability in terms of age at presentation and severity which can be only partially explained by the specific genotype (9,10). Due to improvement of treatment, CF patients to date
live longer (11). Based on the 2018 Cystic Fibrosis Foundation Patient Registry data, the life expectancy is predicted to be 44 years for CF patients born between 2014 and 2018. Furthermore, half of CF patients born in 2018 have a predicted life expectancy of 47 years or older (12). An increasing number of studies have described an association between CF and cancer risk and development (13). In recent years, it has been reported that CF patients have a higher risk of developing colon, bowel, biliary tract, and pancreatic cancers with respect to the general population (14). Furthermore, CFTR down-regulation has been reported in nasopharyngeal carcinoma (15), lung cancer (16,17), hepatocellular carcinoma (18,19), colorectal cancer (20,21), prostate cancer (22,23), and bladder cancer (24,25). Studies regarding CFTR mutations report that changes in CFTR are related with breast cancer (26), lung cancer (27), thyroid cancer (28) and pancreatic adenocarcinoma (29-31) although with some discrepancies (32,33). Chronic inflammation is recognized to contribute to cancer development through the effect of increased inflammatory mediators, such as chemokines and cytokines, and of inflammatory cells that can alter growth, migration and differentiation of various cell types (34). In addition, autophagy also plays a role in tumour development and progression contributing to the regulation of stemness and resistance to anticancer reagents (35). Overall, the increased cancer risk in CF is at least in part, due to the increased chronic inflammatory status that characterizes these patients (36,37), and to the increased autophagy subsequent to CFTR malfunction (38,39).

Among the most frequent comorbidities are changes in glucose metabolism related with insulin-resistance and impaired insulin secretion, which lead to Cystic fibrosis related diabetes (CFRD) which represents the major co-morbidity (40-42), delayed puberty and growth failure. Furthermore, changes in serum lipids are known to occur as well, related with CF itself but also with the hypercaloric diet recommended for these patients (43,44). Hypogonadism and infertility are often found in CF patients and issues of reproductive health have become important in the management of CF (45,46). In particular, male fertility is compromised due to spermatic duct atresia caused by an undehydrated environment with thick mucus established during the development of reproductive apparatus (47,48) and female fertility is reduced mainly due to hypothalamic suppression of hormone secretion, thick cervical mucus, reduced uterine fluid volume and smaller ovarian reserve (49).

The significant heterogeneity existing between patients and time of presentation of the different comorbidities suggests, however, a potential epigenetic regulation (50). MicroRNAs (miRNAs) are endogenous noncoding RNAs, about 22 nucleotides long that act as post-transcriptional regulators by binding mRNAs determining their translational repression or degradation (51,52). MiRNAs can also determine transcriptional gene activation and transcriptional gene silencing (53). MiRNAs regulate physiological functions and metabolic pathways (54) often acting simultaneously on the same target gene with different effects (52). It has been suggested that miRNAs are also involved in pathological states such as inflammation and cancer (55) and miRNA signatures have been proposed as potential biomarkers of disease (56-61), however, this aspect needs further studies. MiRNAs contribute to regulate the physiology of body growth both controlling the hypothalamic-pituitary-IGF axis and growth plate function (62). We have previously shown that changes in specific miRNAs are associated with insulin resistance related with CFTR malfunction and reduced FOXO1 gene expression (63). This latter is a key factor in the insulin signalling cascade. MiRNAs are also functionally involved in lipid metabolism and in fertility due to their involvement in the regulation of developmental functionality of reproductive organs (64,65).

In recent years a few studies have explored the role of miRNAs in the variability of CF clinical manifestations, and the possible role they might have in affecting CFTR expression (66-68).

Although many studies have focused on the role of miRNAs in regulating CFTR gene expression (68-70), little attention has been given to how CFTR mutations influence their expression (71) and how this could affect cell growth and differentiation, and promote oncogenesis and CF related co-morbidities (72).

The aim of this exploratory study was to investigate miRNA changes in two different bronchial
epithelial cell lines bearing two different CFTR gene mutations, using a global profiling approach, and to investigate their potential impact using gene ontology analysis, pathway and protein-protein interaction analyses.

2. Materials and Methods

2.1 Cell lines

The following airway epithelial cell lines were used:

1. CFBE41o-, homozygous for the F508del mutation, derived from a bronchial isolate from a CF patient homozygous for the F508del CFTR mutation (73);
2. IB3, heterozygous F508del/W1282X derived from CF bronchial epithelium (74);
3. 16HBE14o- derived from normal bronchus as non-CF control (75).

All cell lines were immortalized with the pSVori-plasmid that contains a replication-deficient simian virus 40 (SV40) genome. CFBE41o- cells were a kind gift from Dr. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA, USA), whereas 16HBE14o- and IB3 cells were a kind gift from Prof. L. Maiuri (European Institute for Research in Cystic Fibrosis, San Raffaele Scientific Institute, Milan, Italy). CFBE41o- and 16HBE14o- cells were grown in Minimum Essential Media (MEM) (Gibco Cat. No.11095080) supplemented with 10% FCS, 100 µg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere under 5% CO2 at 37°C in coated flasks. IB3 cells were grown in LHC-8 basal medium (Gibco Cat. No. 12678017) supplemented with 5% FBS in a humidified atmosphere under 5% CO2 at 37°C in coated flasks.

2.2 Study design

The objective of this study was to identify gene pathways which expression could be altered because of abnormal epigenetic regulation in cystic fibrosis subsequent to CFTR malfunction. To achieve this goal, we proceeded with the following 3 steps:

1. miRNAs expression profiling analysis: identification of differentially expressed miRNAs in cystic fibrosis cell lines (CFBE41o-, IB3) in comparison with a wild-type cell line (16HBE14o-).
2. miRNAs target genes prediction: identification of differentially expressed miRNAs target genes.
3. From genes to pathways: identification of gene pathways of differentially expressed miRNA targets.

2.3 miRNA expression profiling

The miRNA expression profiling was previously performed by our group [76]. In the present study, we analysed the data of the miRNA profiling which was used at that time for a different purpose. Briefly, total RNA, including small RNAs, were extracted from cell lysates using mirVana isolation kit (Cat. No. AM1560 Ambion, Austin, USA) according to the manufacturer’s protocol. MiRNA expression profiling was performed using the Taq-Man® Array Human MicroRNA Card Set v3.0 (TLDA) (Cat. No. 4444913 Applied Biosystems, Foster City, USA) which is a two-card set containing a total of 384 TaqMan® MicroRNA Assays per card. The set enables accurate quantitation of 754 human microRNAs and three endogenous controls to allow data normalization and one TaqMan® MicroRNA Assay as a negative control. The workflow consisted of: Megaplex RT Reaction: RNA (350 ng) from cell line samples was reverse transcribed using Megaplex™ RT Primers (Pool A Cat. No. 4399966, Pool B Cat. No. 4399968, Applied Biosystems) that contain a pool of 758 individual miRNA-specific primers, including controls, and TaqMan microRNA Reverse Transcription Kit (Cat. No. 4366596, Applied Biosystems). Preamplification reaction: 2.5µl of RT reaction were preamplified using TaqMan PreAmp Master Mix kit (Cat. No. 4391128, Applied Biosystems) and Megaplex™ PreAmp Primers (Pool A Cat. No. 4399233, Pool B Cat. No. 4399201, Applied Biosystems). Real-time PCR reaction: the array was run on
2.4 Statistical analysis

The relative quantification analysis of miRNA expression was performed using Expression Suite v1.1 software (Applied Biosystems). The small non-coding U6 RNA and RNU 48 were selected as endogenous genes, and RNA extracted from 16HBE14o- cell line was used as calibrator sample. Ct values >34 were considered as non-expressed. Contamination was excluded by the analysis of a negative control. The procedure of fold change (FC) calculation included the evaluation of the statistical significance using the Student’s t-test for sample group comparisons followed by the Benjamini-Hochberg false discovery rate for multiple testing correction and results were filtered by p-value. The adjusted p-values of ≤ 0.05 reflect the statistical significance, while the FC (log2) is an evaluation of the biological meaning. We chose this threshold (FC(log2) ≥ +2 or FC ≤ -2) to narrow down the search to those miRNAs that were effectively changing and reflected differences between the two cell lines bearing two different CFTR class 1 gene mutations. The combination of these criteria allowed to find the most biologically meaningful sets of miRNAs with respect to using p-values alone. Differentially expressed miRNAs were those with a FC (log2) ≥ +2 or FC (log2) ≤ -2 in CFBE41o- or IB3 cells with respect to 16HBE14o- cells, and with a p-value ≤ 0.05. These were the miRNAs considered for the following bioinformatics analyses.

2.5 miRNA target prediction

In silico analysis was performed to identify the validated target genes for each differentially expressed miRNA using the database miRWalk (http://mirwalk.umm.uni-heidelberg.de/; last accessed 10 January 2021) [77]. MiRWalk v.3 stores predicted data obtained with a machine learning algorithm including experimentally verified miRNA-target interactions. We selected those targets that were both predicted in miRDB and validated in miRTarbase simultaneously.  

2.6 From genes to pathways, Gene ontology and Protein–protein interaction analysis

GO and pathway analyses were performed using the web-server DIANA-mirPath v3.0 (http://snf-515788.vm.okeanos.grnet.gr/; last accessed 13 January 2021) [78] an online software suite for the assessment of miRNA regulatory roles, that starting from a list of miRNAs identifies Gene Ontology (GO) terms and pathways associated with each of their target genes by using standard, unbiased empirical distributions and/or meta-analysis statistics [78]. This tool enables to identify those pathways and GO categories controlled by a group of miRNAs based on experimental data (TarBase v.7.0) [79]. The pathway and GO terms with a p-value ≤ 0.05 were considered significant.

Networks are built based on both direct (physical) and indirect (genetic) interactions between gene products (proteins). For network analysis, we utilized the STRING 11.0b web server (https://string-db.org/; last access 12 February 2021) [80]. The resulting network provides information of the degree of overall connectivity across imputed gene products (as quantified by the ratio between observed and expected interactions [a.k.a. “edges”] between proteins [a.k.a. “nodes”], and formally tested by means of a PPI enrichment test). Moreover, it suggests cluster of interacting proteins, which can help identify specific cell pathways. All associations are provided with a probabilistic confidence score, which is derived by separately benchmarking groups of associations against the manually curated functional classification scheme of the KEGG database. Each score represents a rough estimate of how likely a given association describes a functional linkage between two proteins that is at least as specific as that between an average pair of proteins annotated on the same ‘map’ or ‘pathway’ in KEGG. To functionally classify the proteins in the interaction network, we performed pathway enrichment analysis using KEGG database [81]. The count number larger than 2 and FDR less than 0.0074 were chosen as cut-off criterion.
3. Results

3.1 miRNA expression profiling

The expression of 754 miRNAs was investigated by qPCR in three different bronchial epithelial cell lines: CFBE41o-, IB3 with CFTR mutations and 16HBE14o- cells used as the normal counterpart. The analysis of raw Cts pointed out that 243 microRNAs were not detected in any cell line (Ct>34). Among the expressed miRNAs, 423 were present in all cell lines, whereas 23 were present only in 16HBE14o-, 23 were present only in IB3, and 8 were present only in CFBE41o-. Furthermore, some miRNAs were commonly expressed in two cell lines and, in particular, 7 were expressed both in 16HBE14o- and IB3, 19 were expressed both in 16HBE14o- and CFBE41o-, and 10 were expressed both in IB3 and CFBE41o-.

Subsequently, miRNA relative expression in CFBE41o- and IB3 cell lines was determined. Overall, 41 miRNAs were differentially expressed in the cystic fibrosis cell lines with respect to the 16HBE14o- control cell line, considering significant miRNAs with a FC (log2) ≥ +2 or FC (log2) ≤ -2 with a p-value ≤ 0.05 (Figure 1 and Table S1).

![Figure 1](image-url)  
**Figure 1.** Hierarchical clustering of differentially expressed miRNAs. The Log-transformed values of the relative expression levels based on RT-qPCR assays were used to perform heatmaps. The colour scale represents relative expression levels with respect to the 16HBE14o- control cell line with red and green colours as high and low values, respectively. Each row represents a miRNA, each column represents CFBE41o- and IB3 cell lines.
In particular, five miRNAs were up-regulated (miR-155-5p, miR-370-3p, miR-886-5p, miR-10b-3p, miR-577-5p) and one miRNA (miR-1257) was down-regulated in both CFBE41o- and IB3 cells. MiR-200b-3p showed an opposite trend in the two CF cell lines being up-regulated in CFBE41o- and down-regulated in IB3 cells (Figure 2).

Furthermore, four miRNAs (miR-493-3p, miR-337-3p, miR-432-5p, miR-154-3p) were up-regulated only in the CFBE41o-; eight miRNAs (miR-140-3p, miR-452-5p, miR-486-5p, miR-509-5p, miR-675-5p, miR-767-5p, miR-27b-5p, miR-505-5p) were up-regulated only in the IB3 cells.

Twelve miRNAs (miR-193b-3p, miR-197-3p, miR-483-5p, miR-501-5p, miR-511-5p, miR-574-3p, miR-616-3p, miR-30c-1-3p, miR-378a-3p, miR-616-5p, miR-942-5p, miR-1255B-5p) were down-regulated only in the CFBE41o-; ten miRNAs (miR-141-3p, miR-146a-5p, miR-183-5p, miR-200c-3p, miR-372-3p, miR-124-3p, miR-183-3p, miR-193b-5p, miR-335-3p, miR-9-3p) were down-regulated in the IB3 cell lines.

### 3.2 Target genes identification and GO analysis

Through an in silico analysis, the validated target genes of the 41 differentially expressed miRNAs were determined. In total, 511 human genes were identified, and some of them were targeted by more than one miRNA (Figure 3).

In detail, about 66% of the selected miRNAs resulted to have in common at least one target gene (27/41 = 65,8%). Some genes were targeted by more than one miRNA, highlighting their possible relevant role. Intriguingly, an isolated group of 4 miRNAs (miR-767, miR-140, miR-675 and miR-505) sharing 3 target genes (Deleted In Azoospermia-Associated Protein 2; OTU Deubiquitinase 4; Transducin Beta Like 1 X-Linked Receptor 1) was also identified (Figure 3 panel 4E).

The whole set of target genes was then analysed by a functional enrichment analysis to identify the biological processes in which they were mainly involved. Interestingly, this analysis revealed that the miRNAs could mainly affect biological processes related with cancer, inflammation, body growth, glucose metabolism, lipid...
metabolism (Figure 4) and fertility. For this latter only androgen receptor signalling emerged.

All the biological processes, molecular functions and cellular components are reported in Tables S2, S3, S4, respectively.

3.3 Protein-protein interaction network analysis

The list of the 511 human genes, which represent the validated targets of the previously selected up- and down-regulated microRNAs, was analysed to obtain the corresponding proteins and subsequently the representation of the protein–protein interaction network (Figure 5).

The network obtained is significantly enriched (p < 1.0e-16), indicating that a high level of interactions is present. Twenty-five proteins (PIK3R1, GRB2, SHC1, VEGFA, RHOA, RPS6KB1, PTPN11, ITGB1, KDR, SOD1, SMAD2, MAP8, IL6R, PPP2CB, IGF1R, NRAS, MAPK3, STAT3, ESR1, MAPK8, CDKN1A, SP1, EP300, CREBBP, NOTCH1) are hub nodes in the network, with a connectivity degree >10.
Figure 3 (Continued)
Figure 4. Gene ontology analysis of the biological processes involving the target genes of those miRNAs dysregulated in the CF cell lines. The pie charts highlight the biological processes involved in cancer (blue), inflammation (red), body growth (green), glucose metabolism (yellow), and lipid metabolism (grey). The area of each sector is proportional to the -Log10 (p-value) of each GO term.

KEGG pathway analysis was performed using the list of proteins contained in the Network. This highlighted 50 significantly enriched pathways (Table S5). In particular, the top nine pathways (most highly significant (p< 6.93e-05) were: microRNAs in cancer (hsa05206), endocrine resistance in cancer (hsa01522), EGFR tyrosine kinase inhibitor resistance (hsa01521), FoxO signalling pathway (hsa04068), hepatitis B (hsa05161), pathways in cancer (hsa05200), proteoglycans in cancer (hsa05205), breast cancer (hsa05224) and human papillomavirus infection (hsa05165). Interestingly, the network’s hub proteins were involved in at least four of these pathways, as highlighted in Figure 5.

Among the significant regulated pathways by the 41 differentially expressed miRNAs, the following were involved in more than one single aspect referring to inflammation, cancer, growth, glucose and lipid metabolism, and fertility.

In detail, we reported regulated pathways which are related with clinical features reported in CF patients (Figure 6): the RAS signalling pathway, the TGF beta signalling pathway, the JAK-STAT signalling pathway, the insulin signalling pathway.

The RAS signalling pathway (Figure 6A) is involved in a variety of cancers and in RASopathies, a group of genetic syndromes caused by germline mutations in genes encoding proteins belonging to the RAS-MAPK pathway and having postnatal growth failure as a persistent feature. The TGF beta signalling pathway (Figure 6B), is a key pathway involved in inflammation and cancer, in glucose metabolism, in promoting lipid accumulation in the liver and is related with female fertility. Furthermore, it is involved in osteoblastogenesis and in chondrogenesis. JAK-STAT signalling pathway (Figure 6C) is involved in inflammation and its dysregulation has been evidenced in many types of cancer. JAK-STAT signalling is a key pathway for growth hormone (GH) action and it is involved in lipid metabolism also. The insulin signalling pathway (Figure 6D) regulates blood glucose levels
Figure 5. Protein-Protein interaction network. The proteins encoded by the validated target genes of the selected up- and down-regulated microRNAs were used as input in STRING software (https://string-db.org). Proteins are represented with nodes and the physical direct interactions with continuous lines. Line thickness indicates the strength of association amongst individual partners. The colour present in some nodes indicates the pathway in which the protein is involved. Endocrine resistance refers uniquely to endocrine resistance within specific treatments for cancer based on the data contained in the database.
Figure 6. Ras signalling pathway (A). TGF beta signalling pathway (B). JAK-STAT signalling pathway (C). Insulin signalling pathway (D). MiRNAs and their regulated genes (in yellow boxes) involved in these signalling cascades are connected by dashed lines. The up/down regulation of each miRNA is shown with arrows (orange or blue for CFBE41o- or IB3 respectively). Continuous violet lines specify the regulated gene. Graphical pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/).
Figure 4 (Continued)
Figure 4 (Continued)
and glucose uptake by cells and its dysregulation induces insulin resistance. Furthermore, the dysregulation of insulin signalling has been related with several types of cancer and lipid metabolism.

4. Discussion

This exploratory study suggests an association between CFTR malfunction and the dysregulation in miRNA expression. These changes could be partly related with the specific CFTR genotype, and one miRNA (miR-200-3p) showed an opposite trend in the two different affected cell lines. However, further studies are warranted to demonstrate whether the genotype is the only cause for this variability. Overall, we described changes in 41 miRNAs in the CF cell lines compared with the wild-type cells, targeting 511 validated genes. The result is a network, as expected, where a single miRNA targets several genes, and these genes are regulated by multiple miRNAs (51,52).
These 41 miRNAs were involved in the regulation of biological processes such as inflammation and immunity, cancer, body growth, glucose and lipid metabolism, and fertility. Previous miRNA profiling studies in CF reported slightly different dysregulated miRNAs possibly due to the different in vitro models used and/or different cut-offs applied (66-68,82). It has to be underlined that the biological processes evidenced could be biased by the data currently available in the databases. As a matter of fact, most of the data uploaded to date are within the field of oncology, and this could contribute to explain why this was most represented process. The inevitable gaps in the databases could contribute to explain also the poor representation of some biological processes linked with other co-morbidities in CF patients, such as fertility, glucose and lipid metabolism. However, the changes in miRNAs and the biological processes involved and described in this study match co-morbidities that can present at different times in life in CF patients. Furthermore, interestingly, our in vitro models showed differences between the two main genotypes studied, suggesting that this might account at least in part for the variability observed in the patients having these same genotypes (46). The biological processes regulated by the dysregulated miRNAs were related with chronic inflammation, delayed growth, insulin resistance and insulin deficiency, impaired glucose tolerance and diabetes, changes in serum lipids, fertility, and some forms of cancer, which are all described in CF patients (83).

Referring to inflammation, among the 511 target genes of the 41 dysregulated miRNAs, we evidenced IL-11, IL6R, IL-18, TGFBR3, TNFRSF11B, and TNFRSF21 genes. The first three are tightly related with lung and airway inflammation. In detail, IL-11 which is a member of the IL-6 family and signals mainly through the ERK and JNK pathways, has been reported to be overexpressed in the airways from patients with asthma [84] and pulmonary fibrosis (85). IL6R exists both in a soluble and transmembrane form, which respectively mediate the pro-inflammatory and anti-inflammatory activities of IL-6, through the JAK-STAT signalling pathway (86). IL6R is also important for growth, and the soluble form of IL6R is critical for bone homeostasis and, in particular, for osteoclast formation (86). Furthermore, IL6R is linked with glucose metabolism and the deletion of IL-6R in murine hepatocytes determines an impairment both in insulin sensitivity and in glucose tolerance (87). IL-18 is a member of the IL-1 cytokine superfamily and it was described as overexpressed in inflamed lungs (88). As to the latter three genes, TGFBR3 is a member of the TGF-beta receptor superfamily. As previously described, TGF-beta signalling is involved in many biological processes such as inflammation, cancer (89) and growth (90,91). TNFRSF11B and TNFRSF21 are both members of the tumour necrosis factor receptor superfamily of proteins. The first one encodes for osteoprotegerin and is directly involved in inflammatory processes especially in inflammatory bowel diseases and in several gastrointestinal carcinomas (92); furthermore, it is involved in the regulation of osteoclast development and bone turnover (92). The second, TNFRSF21 also known as DR6, has been reported to be involved in the regulation of airway inflammation in a mice model of asthma (93). Moreover, increases in IL-1, IL-6, IL-8, IL-17, IL-33, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), HMGB1, and TNF alpha have been reported in the lungs and airways of patients with CF (94). Furthermore, we previously reported an increase in circulating IL-1 beta, IL-6, TNF alpha also in serum from young adult CF patients (36). Chronic inflammatory conditions in general are characterized by changes in miRNA profiles, each with specific signatures (95,96).

Chronic inflammation is also a cause of cancer (34,97), and the onset of some of the cancers reported in CF patients is related with the state of chronic inflammation (98).

As to cancer, many biological processes involved with the onset and progression of cancer appear to be regulated by the miRNAs which are dysregulated in the CF cell lines. As detailed in Figure 4, the six biological processes mostly impacted by miRNA dysregulation were the EGFR and FGFR signaling pathways, UPR activation, G1/S and G2/M transitions of mitotic cell cycle and the cell junction assembly process. Among these in particular the EGFR signaling, the cell junction assembly process, and the UPR are an interesting link between cancer and CF. Indeed, EGFR...
alterations are frequently observed in cancer (99) and determine a number of modifications at the molecular level which include the downregulation of a number of non-coding RNAs, including miRNAs (99). Interestingly, the EGFR pathway activation is increased also in CF airway epithelium due to extracellular oxidation and this could determine tissue remodeling and inflammation in lung epithelium (100). Cell junction assembly is altered in many types of cancer (101) and junctional abnormalities were also reported in cells carrying F508del-CFTR (102). In cancer, the UPR is a pro-survival mechanism which is involved in the establishment and progression of many types of cancer (103). The F508del-CFTR activates also the UPR (104) which in turn reprograms macrophages and is associated with an increased inflammatory response in CF (105). Interestingly, 11 miRNAs among the 41 dysregulated in the CF cells show the same trend as reported in lung cancer. In particular, miR-155-5p, miR-452-5p, miR-486-5p, miR-675-5p were upregulated in CF cells and miR-141-3p, miR-146a-5p, miR-183-5p, miR-193b-3p, miR-511-5p, and miR-335-3p were downregulated as previously reported in lung cancer (106). Furthermore, miR-200b-3p which was upregulated in CFBE41o- and downregulated in IB3 cells was reported to be upregulated in lung adenocarcinoma (106). Furthermore, an increase in miR-155 and miR-200b was reported in endometrial cancer (107), a type of cancer with increased CFTR mRNA expression (108). Moreover, both CF patients have been reported also to be at higher risk of developing digestive tract cancer and especially colorectal cancer (14,106,109) with specific recommendations having been developed (110). Here we described 7 miRNAs having the same trend as reported in colorectal cancer. In detail, miR-155-5p (111), miR-886-5p (112) were up-regulated in colorectal cancer as well as in CF cells and miR-141-3p (113), miR-200b-3p (114), miR-511-5p (115), miR-574-3p (116), and miR-378a-3p (117) were down-regulated in colorectal cancer as well as in CF cells.

Delayed growth and puberty have been reported in CF patients (36,118-120). We previously reported that miRNAs in serum, which showed changes in CF patients and were specifically related with inflammation, were also regulators of the GH-IGF-I axis and of the IGF system contributing to explain stunted growth (72). Besides miR-155-5p which has been already identified to be dysregulated in CF patients (72,121), this in vitro study identified additional dysregulated miRNAs possibly involved in the regulation of growth. In particular, some of the 41 selected miRNAs are regulators of key pathways such as JAK/STAT, TGF beta and Ras, signalling which are known to be important for longitudinal growth in humans, as subjects having genetic defects in those genes have also short stature (93,121-123).

Insulin resistance has been well described in CF patients (46,63,124,125). The identified miRNAs regulate genes encoding for proteins along the insulin cascade (Figure 6D) which have been described in CF (63). Glucose metabolism and insulin sensitivity are tightly related. We previously reported that dysregulated miRNAs in CF, related with inflammation targeted validated genes within the insulin signalling pathway (72,76). The miRNA profiling analysis in these cells suggested that besides those miRNAs, others are implicated in that regulatory network (76).

Lipid metabolism has been studied less in patients but dysregulations are reported (43,44,126). Our study highlights that miRNAs involved in cholesterol, triglyceride and long-chain fatty acid biosynthesis besides other processes show changes in CF (Figure 4). Some of these metabolic processes are also related with insulin sensitivity (127).

Finally, as reported in the introduction, fertility can be a problem in CF patients (47,48). The data of this study showed that the miRNAs that change because of CFTR malfunction are involved with androgen receptor signalling. To our knowledge, this aspect has not been previously explored in CF, and suggests the need for further studies.

The protein-protein interaction network output helps visualize how the processes regulated by the miRNAs tightly interact, and match the complex aspects observed in human disease. This concept is further highlighted by the KEGG pathway analysis as discussed below.

The RAS pathway is related, as explained above with growth (121,122) but also with carcinogenesis as it is dysregulated in a variety of cancers (e.g. hematopoietic malignancies, pancreatic ductal adenocarcinoma,
5. Conclusions

Concluding, from a practical point of view, a dysregulated miRNA network underpins cystic fibrosis owing to CFTR malfunction showing some differences which could be dependent on the specific CFTR genotype. This contributes to explain some of the clinical variability and differences in timing and severity of presentation of the different co-morbidities.

Moreover, as improved treatment has led to ageing in these patients, the data suggest that an increased cancer risk may be present, and proper surveillance is indicated.

Finally, the results suggest that future research should consider using cell models from different organs/tissues directly from patients.

We are aware that this study has an exploratory purpose and further studies are needed to validate these findings. Studies in patients and in primary cell cultures from patients would be warranted (141). However, we think that the data from this study could offer new interesting cues and starting points for further research in this field.

Abbreviations: CDKN1A, Cyclin Dependent Kinase Inhibitor 1A; CF, Cystic fibrosis; CFD, Cystic fibrosis related diabetes; CFTR, CF transmembrane conductance regulator; CREBBP, CREB binding protein; EGFR, Epidermal growth factor receptor; EP300, E1A Binding Protein P300; ERK, Extracellular signal-regulated kinase; ESR1, Estrogen receptor 1; FC, Fold change; FDR, False Discovery Rate; FOXO1, Forkhead Box O1; G-CSF, Granulocyte colony-stimulating factor; GH, Growth Hormone; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GO, Gene ontology; GRB2, Growth Factor Receptor Bound Protein 2; HMGB1, High mobility group protein Box 1; IGF1R, Insulin Like Growth Factor 1 Receptor; IGF-I, Insulin Like Growth Factor 1; IL-1, Interleukin-1; IL-1 beta, Interleukin-1beta; IL-11, Interleukin-11; IL-17, Interleukin-17; IL-18, Interleukin-18; IL-33, Interleukin-33; IL-6, Interleukin-6; IL6R, Interleukin-6 receptor; ITGB1, Integrin Subunit Beta 1; JAK, Janus Kinase; JNK, c-Jun N-terminal kinase; KDR, Kinase Insert Domain Receptor; MAP8, Microtubule-associated protein 8; MAPK, Mitogen-Activated Protein Kinase; MAPK3, Mitogen-Activated Protein Kinase 3; MAPK8, Mitogen-Activated Protein Kinase 8; miRNA, microRNA; NOTCH1, Notch receptor 1; NRAS, NRAS Proto-Oncogene, GTPase; PIK3R1, Phosphoinositide-3-Kinase Regulatory Subunit 1; PPI, Protein–protein interaction; PPP2CB, Protein Phosphatase 2 Catalytic Subunit Beta; PTPN11, Protein Tyrosine Phosphatase Non-Receptor Type 11; RAS, rat sarcoma protein; RHOA, Ras Homolog Family Member A; RPS6KB1, Ribosomal Protein S6 Kinase B1; SHC1, Src Homology 2 Domain-Containing

colorectal cancer, non-small cell lung cancer, malignant melanoma, bladder and thyroid carcinomas, myelodysplastic/myeloproliferative neoplasms) (128). Patients having RASopathies are prone to develop cancer, such as gastrointestinal stromal tumours (129).

Interestingly, digestive tract cancers are also the most represented in patients with CF (14,109).

The TGF beta pathway modulates insulin transcription and pancreatic beta cell activity (130), and promotes lipid accumulation in the liver (131). Moreover, the TGF beta pathway is a regulator of body growth, being involved in both osteoblastogenesis, necessary for growth plate development and maintenance, and chondrogenesis (90). It promotes mesenchymal cell commitment to chondrogenic lineage, proliferation and deposition of extra cellular matrix and prevents terminal chondrocyte differentiation (90). Finally, it is involved in female fertility and especially in folliculogenesis, oocyte maturation and ovulation, embryo development and reproductive tract development (132). Furthermore, the TGF beta pathway is a key regulator of both inflammation and cancer promoting or inhibiting tumorigenesis depending on the tissue microenvironment and concomitant gene mutations (133).

The JAK-STAT signalling pathway is involved in lipid metabolism regulating adipocyte development and physiology (134). Moreover, it is pivotal for GH action; it is activated by GH binding to its receptor and mediates GH biological functions (135). The JAK-STAT signalling comprises SOCS proteins which are key regulators of inflammation (136) and is dysregulated in many types of cancer (137).

The insulin pathway is pivotal for the tight regulation of blood glucose levels and glucose uptake and its dysregulation induces insulin resistance. Interestingly, a clear association between insulin resistance and RAS signalling has been reported; indeed, the inhibition of the RAS pathway improves insulin sensitivity, glucose uptake and reduces inflammation (138). Furthermore, insulin has been related with several types of cancer both for insulin action on proliferative and anti-apoptotic signalling in cancer cells, and for the role of insulin in maintaining whole body homeostasis (139). Insulin signalling also promotes lipid synthesis, and inhibits lipolysis in adipocytes (140).
protein 1; SMAD2, SMAD Family Member 2; SOCS, Suppressor of Cytokine Signalling; SOD1, Superoxide dismutase 1; SP1, Sp1 Transcription Factor; STAT, Signal Transducer And Activator Of Transcription; STAT3, Signal Transducer And Activator Of Transcription 3; TGF beta, Transforming Growth Factor Beta; TGFBR3, Transforming Growth Factor Beta Receptor 3; TNF alpha, Tumor necrosis factor alpha; TNFRSF11B, TNF Receptor Superfamily Member 11b; TNFRSF21/DR6, TNF Receptor Superfamily Member 21; UPR, Unfolded protein response; VEGFA, Vascular Endothelial Growth Factor A.

Supplementary Material: Table S1. Biological processes obtained from DIANA-miPath V3.0 involving the 41 miRNAs deregulated in CF cell lines. Table S2. Cellular components obtained from DIANA-miPath V3.0 involving the 41 miRNAs deregulated in CF cell lines. Table S3. Molecular functions obtained from DIANA-miPath V3.0 involving the 41 miRNAs deregulated in CF cell lines. Table S4. KEGG pathway analysis of the genes deriving from the protein–protein interaction analysis reported in Figure 5.

Data Availability Statement: The datasets generated for this study can be found in the GEO repository [GSE167100].

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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