A network of pro-inflammatory genes repressed by clock signalling in bronchial epithelium

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The peripheral circadian clock in COPD patients is inversely coexpressed with inflammatory pathway genes https://bit.ly/3TWqkgf

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Abstract

Background Circadian rhythms are biological cycles that regulate various physiological processes, including immune responses, tissue repair and oxidative stress. Previous studies indicated a role for distorted circadian signalling in COPD.

Methods In this study, we performed an unbiased analysis of the gene network that coexpressed with circadian clock signalling in COPD using weighted gene coexpression network analysis on RNA sequencing data from bronchial brushes of COPD patients.

Results We found that a large network of pro-inflammatory genes, including *CXCL8*, *IL1B*, *IL1A*, *CSF1* and *TGFB1*, was inversely correlated with the expression of core clock genes in bronchial brushes of COPD patients. In contrast, genes that positively coexpressed with circadian clock signalling associated with ciliated cell differentiation. Furthermore, we found that both circadian clock genes and their coexpressed genes were differentially expressed in lung tissues of COPD patients compared with healthy smokers.

Conclusions Our results provide an unbiased and comprehensive analysis of the gene expression network coexpressed with circadian clock signalling in bronchial epithelium. Our findings suggest an association between circadian clock signalling and enhanced inflammatory gene expression in COPD patients.

Introduction

COPD is a chronic inflammatory lung disease characterised by airflow obstruction. It is typically caused by long-term exposure to noxious particles or gases, most often originating from cigarette smoke. Symptoms include dyspnoea, cough, mucus production and wheezing [1]. Inhaled treatment with long-acting bronchodilators remains the most important form of treatment [2].

According to a recent study, disrupted circadian rhythm is associated with a higher prevalence of emphysema, chronic bronchitis and asthma [3]. Circadian rhythms are physical, mental and behavioural changes that follow a roughly 24-h cycle [4]. These rhythms are found in most living organisms and help regulate many physiological processes, such as sleep–wake cycles, hormone secretion and metabolism [5]. In mammals, genetic influences of circadian rhythms occur at the transcriptional level, post-transcriptional level and epigenetic level, modulated by core clock genes (CCGs) [6]. CCGs include *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *REV-ERBα* and *RORα*. These represent a group of genes that interact with each other and make up an auto-regulatory feedback loop, in which the activation and repression

cycle takes about 1 day [4], and in which proteins regulate their own expression. Generally, the expression of biological clock genes is regulated by two feedback loops. First, a core feedback loop in which BMAL1 and CLOCK form a transcriptional complex that regulates the expression of *PERs* and *CRYs*. Period circadian regulators (PERs) and cryptochromes (CRYs) are important regulators that can enter the nucleus at high concentrations and inhibit further transcription by binding to the BMAL1–CLOCK transcriptional complex. Second, RAR-related orphan nuclear receptors (RORs) and reverse strand of the c-erbα genes (REV-ERBs) are part of a second feedback loop by regulating the expression of *BMAL1*. This genetic clockwork that regulates circadian rhythms is present in cells throughout the body (figure 1) [7]. Circadian rhythms have been shown to influence immune responses, tissue repair and oxidative stress in the lungs [8, 9]. For instance, we identified that PGE_2 and prostacyclin mimetics effectively restore cigarette-smoke-induced defects in alveolar epithelial progenitor cells by modulating the circadian clock and cell cycle/apoptosis signalling pathways [10]. Conversely, inflammation can also affect circadian rhythms by altering clock gene expression [11]. Moreover, REV-ERBα deletion exacerbates inflammatory responses in COPD/emphysema [8]. Recently, a distorted expression of the peripheral circadian clock was observed in COPD [12]. For example, BMAL1 abundance is reduced in the lungs, sputum cells and peripheral blood mononuclear cells (PBMCs) of COPD patients [13]. Circadian rhythms were found to control pro-inflammatory mediator release in lipopolysaccharide-treated PBMCs from nonsmokers, smokers and patients with COPD, including cytokines such as interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-α [14]. These findings underscore the critical role of circadian rhythms in maintaining immune homeostasis and suggest that circadian disruption may exacerbate inflammatory diseases such as COPD. In this study, we aimed to investigate the association between expression of CCGs and coexpressed genes in an unbiased manner in COPD. We used RNA sequencing data from bronchial brushes of 26 COPD stage Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2–3 patients as well as from lung tissues of COPD patients and healthy smokers. The expression of clock genes can be affected by many aspects, such as sampling time, disease manifestations and individual differences. Our approach was to take advantage of the variation in CCG expression among these samples. By applying weighted gene

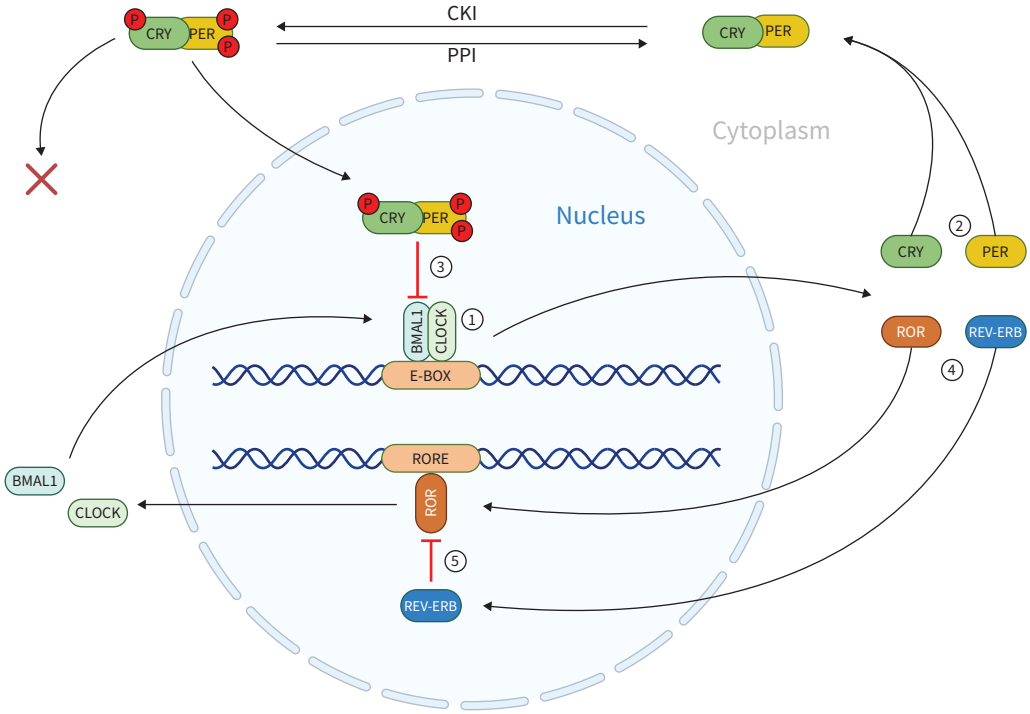

FIGURE 1 Molecular regulation of the peripheral circadian clock. In the main loop, a transcriptional complex consisting of basic helix-loop-helix ARNT-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK) (①) constitutes the positive regulatory arm, which drives expression of circadian genes, including the *CRY* and *PER* genes (②). When PER and CRY proteins reach a sufficiently large concentration, these will subsequently repress the BMAL1–CLOCK transcriptional complex (③) to form a circadian cycle. A secondary loop made up of REV-ERBα and RORα (④) consolidates the primary feedback loop and functions to repress (REV-ERBα) or enhance (RORα) *BMAL1* gene expression (⑤). CKI: casein kinase I; PPI: protein–protein interaction.

coexpression network analysis (WGCNA) on bronchial brush samples from COPD patients, we were able to identify a significant inverse correlation between the expression of CCGs and a network of pro-inflammatory genes. This study provides unbiased and comprehensive insights into the intricate relationship between circadian clock dysfunction and inflammation in COPD, suggesting potential pathways for therapeutic intervention.

Material and methods

RNA sequencing datasets

RNA sequencing data from the AIRFLOW-2 study was reused [15]. Airway mucosal brush samples of 26 COPD stages GOLD 2–3 patients collected at the 3-month follow-up visit in both the sham-control group and the targeted lung denervation (TLD) treatment group were used for this study. Clinical characteristics were summarised previously [15]. The inclusion criteria for the patients are 1) diagnosis of COPD with 30%⩽forced expiratory volume in 1 s ($FEV_{1)}$<60% and FEV_1/forced vital capacity (FVC)<70% (post-bronchodilator); 2) patient ⩾40 and ⩽75 years of age at the time of consent; 3) the patient has no child-bearing potential or a negative pregnancy test (serum or urine), if applicable; 4) smoking history of at least 10 pack-years; 5) nonsmoking for a minimum of 2 months before consent and agrees to continue not smoking for the duration of the study; and 6) participated in a pulmonary rehabilitation program or engaged in regular physical activity under professional supervision in the past 12 months. No significant differences were found between the TLD and sham-control groups regarding age, body mass index and smoking pack-year (supplementary table 1), nor were there significant sex differences between the groups (p=0.69). All patients were using inhaled tiotropium 18 $\mu g \cdot d^{-1}$ and could continue other inhaled medication such as short-acting bronchodilators at the discretion of their physician.

For each patient, three brushes were collected from the right lower lobe, which constituted one sample. Samples were processed using methods as described previously [15]. Data analysis was performed on a short-read dataset obtained using Illumina next-generation sequencing technology. RNA sequencing data were conducted using the Illumina NovaSeq 6000 sequencer by GenomeScan (www.genomescan.nl/). The procedure included data quality control, adaptor trimming, alignment of short reads and feature counting. Library preparation was checked by calculating ribosomal (and globin) content. Checks for possible sample and barcode contamination were performed and a set of standard quality metrics for the raw dataset was determined using quality control tools (FstQC v.0.34 and FastQA). Prior to alignment, the reads were trimmed for adaptor sequences using Trimmomatic v.0.30. To align the reads of each sample, the human reference GRCh37.75 was used. 26 samples (14 and 12 in the TLD and sham-control arms, respectively) passed quality control and were used in the analysis. All local ethics committees of the participating hospitals approved the study, and all patients provided written informed consent [16].

A second, publicly available Gene Expression Omnibus (GEO) dataset was used for differential expression analysis. This was performed using the expression microarray dataset GSE76925 (platform, GPL10558, Illumina HumanHT-12V4.0 expression beadchip) to identify differentially expressed genes (DEGs) between 40 healthy former smokers' lung tissues and 111 lung tissues from severe COPD patients. For patient characteristics, we refer to previous work [17].

Correlation analysis on clinical samples

A correlation analysis was performed to identify genes correlated to the expression of a composite clock signature consisting of five main core clock components: *PER1*, *PER2*, *PER3*, *CRY1* and *CRY2* using R package DESeq2 (v.1.12.0). The geometric means and z-scores of the normalised expression value of the five clock components mentioned were calculated into a so-called CCG score. The CCG score was used as a continuous variable to discover genes that correlated to the five CCGs. Treatment (sham/TLD) and biological sex were included as a covariable in the analyses to correct for possible differences between these two groups.

Weighted gene coexpression network analysis on clinical samples

WGCNA was performed using the R package WGCNA (v.1.72-5), with R^2=0.9 and the soft threshold β=6. Subsequently, the clinical samples were equally divided in high (n=13) and low CCG score (n=13) for *post hoc* comparisons. The adjacency matrix was transformed into a topological overlap matrix, and modules were identified with hierarchical clustering (minModuleSize=30). The eigengene was calculated, and the modules were hierarchically clustered. The module eigengene (ME) and the module membership (MM) were used to distinguish the vital modules associated with circadian clock expression. The relationship between the clinical data and the modules was calculated to obtain vital modules. ME shows the first principal component in the module and describes the expression pattern of the module. MM stands for the relationship between genes and MEs, which refers to the reliability of genes being part of modules.

Genes in the key module were studied with functional enrichment analysis (Gene Ontology (GO) pathway) using clusterProfiler (v.4.12.0). Genes in the brown module were extracted based on the cut-off criteria (|MM|>0.8 and |gene significance (GS)|>0.35) as hub genes and analysed using STRING and visualised using the software Cytoscape v.3.10.1 to visualise connectivity between key genes.

Data analysis and statistics

The RNA sequencing data was normalised using the DESeq2 package in R v.4.4.1. Annotation process was performed by the R package AnnotationDbi (v.1.66.0). Volcano plots were made using the R package EnhancedVolcano (v.1.22.0). The heatmap was made using the R package pheatmap (v.1.0.12). The DEGs obtained from microarray data were identified using the R package limma (v.1.12.0). Pathway enrichment was done using the R package clusterProfiler. Protein–protein interaction (PPI) network analyses were performed using STRING (https://string-db.org) and visualised in Cytoscape. Gene signatures were further analysed using gene set enrichment analysis and MSigDB (https://www.gsea-msigdb.org).

Results

CCG score inversely correlates with pro-inflammatory gene expression in COPD

We first calculated a composite gene expression score, which we named the CCG score, per patient for a gene set consisting of the core clock modulators *PER1*, *PER2*, *PER3*, *CRY1* and *CRY2*. These genes are part of the repressive arm of the molecular clock and are expressed in response to activation of the BMAL1–CLOCK complex [18]. When analysing the expression of this CCG score, we observed that within patient samples, the expression of individual CCGs (both positive and negative regulators) correlated quite well (supplementary figure 1). Thus, high CCG score expressers tended to have high expression of both positive and negative regulators and *vice versa*, which was lower across the board for low-CCG expressers. No differences were observed in the expression of CCGs between sham and TLD-treated patients (supplementary figure 2).

We analysed RNA sequencing data of bronchial brushes from 26 patients with clinical stages GOLD 2–3 COPD. A total of 1796 genes were found correlated with the CCG score (figure 2a), the full gene list can be found in supplementary table 2. As a confirmatory approach, we performed an additional analysis to explore genes correlated with *CRY2* gene expression only and obtained 2833 genes (figure 2b). Correlation analysis of the genes associated with CCG score and with *CRY2* only showed clear similarities (supplementary figure 3) in the direction and magnitude of the association, suggesting that these analyses report similar gene associations.

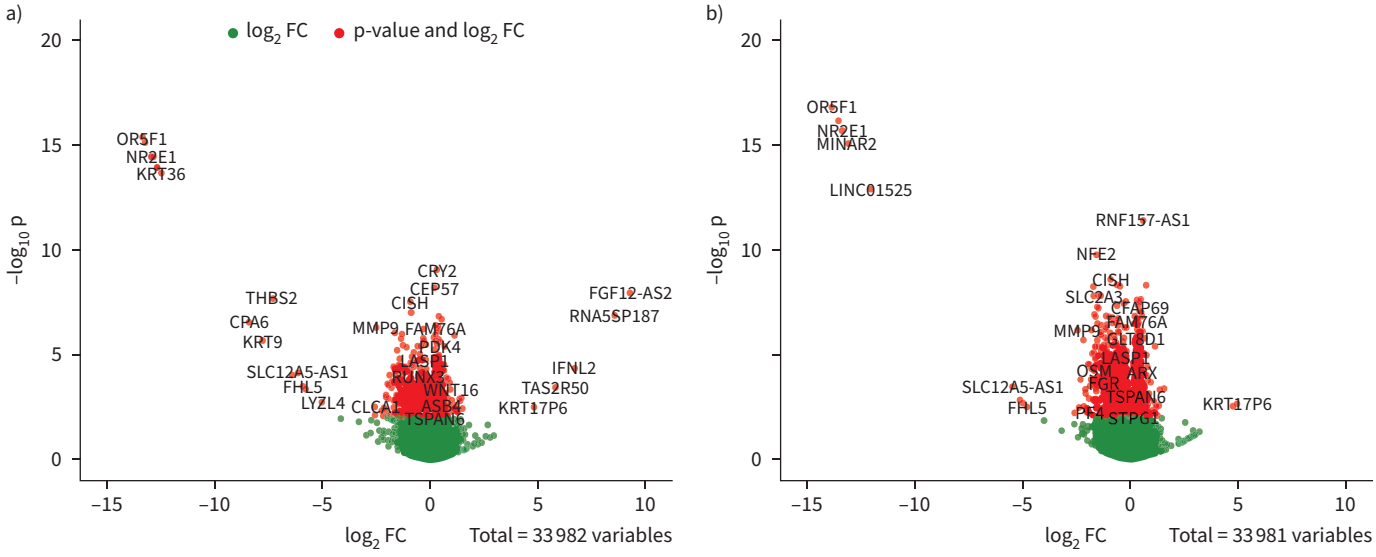

FIGURE 2 Coexpression analyses of the core clock genes (CCG) score and of CRY2 with gene expression in bronchial brushes of COPD patients. a) Volcano plot of genes found to be associated with the CCG score consisting of the core clock components *PER1*, *PER2*, *PER3*, *CRY1* and *CRY2*. 1796 genes were found to be correlated. b) Volcano plot of 2833 genes found to be associated with *CRY2* expression. The cut-off values we used are a p-value threshold of 0.01 and a fold change (FC) cut-off of 0. In the volcano plot, the genes coloured in green have p-values >0.01 and genes coloured in red have p-values <0.01.

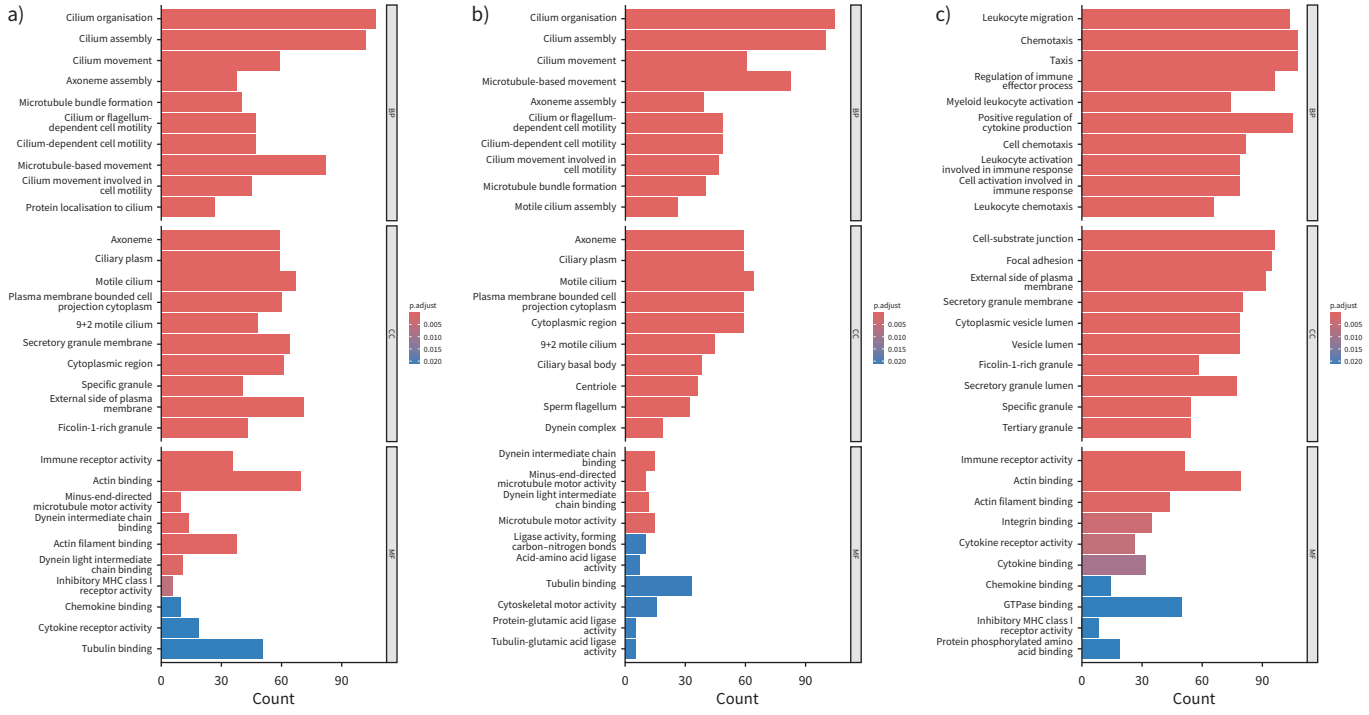

FIGURE 3 Pathway enrichment analysis in bronchial brushes samples for a) all genes associated with core clock genes (CCG) score; b) positively associated genes with CCG score; c) negatively associated genes with CCG score. Pathway associations are shown for Gene Ontology (GO) Biological Process; GO Cellular Component; and GO Molecular Function pathway definitions. All pathways shown meet the adjusted p<0.05 threshold value and are colour-coded according to p-value as specified in the legend. The x axis indicates the number of genes in the pathway definition that was found in the associated gene list. MHC: major histocompatibility complex.

To investigate the functions of the CCG score-correlated genes, we performed pathway enrichment analysis and found that many pathways related to inflammation and cytokines were enriched (figure 3a). We then divided these genes into positively and negatively associated genes and repeated the analysis. Interestingly, the positively associated genes were mostly related to cilium assembly and cell maintenance (figure 3b), while the negatively associated genes were highly related to cytokine and inflammatory processes (figure 3c). Pathway enrichment analysis was also done on *CRY2* correlated genes in the same way and very similar results were obtained (supplementary figure 4).

As the pathway enrichment analysis indicated an inverse relationship between clock genes and inflammation, we further assessed the identity of the inflammatory factors. We analysed the obtained 1796 genes using the MSigDB online database (https://www.gsea-msigdb.org/) and found 39 inflammation and cytokine-related genes, including *CXCL8*, *IL1A*, *IL1B*, *IL7* and *TGFB1*. To assess whether these genes can interact with typical clock genes, including *CLOCK*, *BMAL1*(*ARNTL*), *PER1*, *PER2*, *CRY2*, *REV-ERBα*, *REV-ERBβ*, *DBP* and *TEF*, a PPI network was constructed using the STRING database and visualised using Cytoscape software (figure 4). The Cytoscape plugin cytoHubba was used to analyse the topological structure of the whole PPI network and scored based on the importance of each node with the algorithm of maximal clique centrality (MCC). Next, we identified the top five genes interacting with *BMAL1*, *CRY2*, *CRY1*, *PER2* and *CLOCK*. The top five inflammatory genes were found to be *CXCL8*, *IL1B*, *IL1A*, *CSF1* and *TGFB1*.

As we found pro-inflammatory gene expression to inversely correlate with clock genes, a heatmap of the relationship between expression of CCGs and cytokine signature genes was made. The 39 cytokine signature genes from the correlation analysis were selected and sorted by increasing expression of CCG score from left to right, and the top 5 genes are positively associated with CCG score; the rest are negatively associated. Both sections are sorted by the p-values rising from top to bottom (figure 5). From the heatmap, we can see that most of the cytokine signature genes showed a significant trend of expression with the CCG score, and most genes showed an overall tendency of decreasing expression, which indicates that most pro-inflammatory genes are reduced in expression as the CCG score increases.

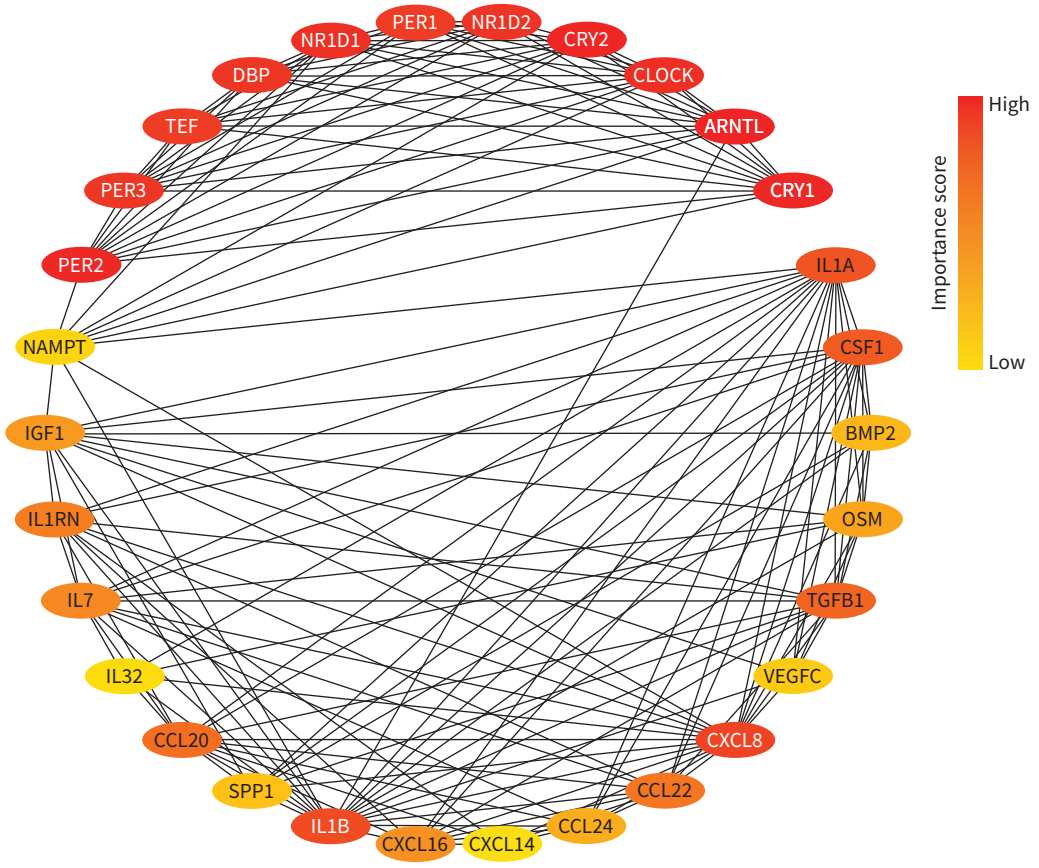

FIGURE 4 Protein–protein interaction (PPI) network of the 30 hub genes with the highest importance scores based on the maximal clique centrality (MCC) algorithm in bronchial brush samples. The hub genes are identified from 39 inflammatory/cytokine-related genes, along with typical clock genes. Clock genes were found related to the expression of inflammatory genes, including *CXCL8*, *IL1B*, *IL1A*, *CSF1* and *TGFB1*. Genes are coded by importance score ranging from 0 (low; yellow) to 1 (high; red).

Higher expression of inflammatory genes and lower expression of several CCGs in severe COPD patients compared with healthy smokers

To verify whether these cytokine genes are also differentially expressed in lung tissues, we performed a DEG analysis using GEO microarray dataset GSE76925. Although this is a dataset of lung tissues and not specifically of bronchial epithelial cells only, we chose to use this dataset to validate the expression of genes associated with the CCG score more broadly to lung tissue. In addition, using this same dataset, we previously demonstrated differential expression of CCGs [10]. Significant changes in the expression of 1107 genes were identified using the R package limma, with a substantial difference in expression between healthy smokers and COPD tissues (adjusted p-value< 0.05; $\log_2$fold change (FC)>0.695 or <−0.695), including 185 upregulated and 922 downregulated genes (supplementary figure 5a). Next, GO and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis were performed on the DEGs. Pro-inflammatory pathways were enriched in the DEGs, which is similar to the pathway enrichment results of the CCG-score-associated genes (supplementary figure 5b,c). We searched the identified 39 inflammation and cytokine-related genes together with CCGs in the DEG list; eight cytokine-related genes and four clock genes were found differentially expressed in COPD patients (supplementary table 3).

WGCNA reveals CCG score is related to inflammation

Both correlation analysis and DEG analysis showed an inverse relationship between clock genes and inflammatory genes. Next, to more accurately verify CCG score coexpressed genes and the relationship with inflammation, we conducted WGCNA. This analysis was based on the gene expression signature of CCGs in the dataset of the bronchial brushes.

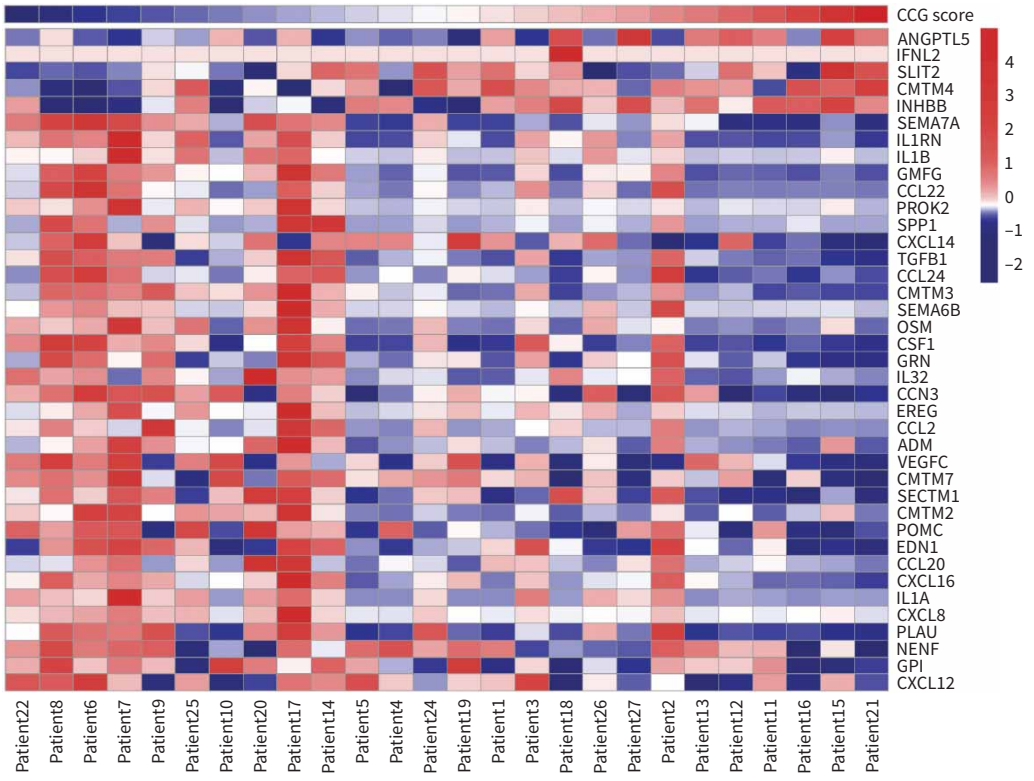

FIGURE 5 Heatmap of the expression of the core clock gene (CCG) score-correlated cytokine genes in bronchial brush samples. Gene expression was normalised to z-score and colour-coded ranging from −2 (blue; low expression) to +4 (red; high expression). The samples were sorted by the CCG score. The top five genes are positively associated with CCG score and the rest are negatively associated. Both sections are sorted by the p-values rising from top to bottom.

First, samples were divided into two equally large groups based on CCG score: a high expression group (CCG score ⩾0.05) and a low expression group (CCG score <0.05). Next, based on scale independence of >0.9, the soft thresholding power β was selected as 6 to ensure a biologically significant scale-free network (figure 6a and supplementary figure 6a). Genes were clustered into 12 modules through hierarchical clustering analysis and dynamic branch cut methods for gene dendrograms (supplementary figure 6b).

To identify CCG-score-related key modules, gene significance (GS) and MM were calculated to relate modules to CCG score expression. The MM was defined as the correlation between gene expression values and the ME. The GS was defined as the correlation between genes and samples. Module–trait relationships indicated that the turquoise and brown modules were significantly correlated with CCG score expression (supplementary figure 6c, d).

To reveal the underlying molecular biological processes, we conducted GO pathway enrichment analyses. GO enrichment was analysed with the clusterProfiler package in R (figure 6a). The results showed that regarding the Biological Process, the turquoise module contains genes related to cilia and ion channels, whereas the genes in the brown module are mainly related to leukocytes and inflammation processes, which indicates further investigation should be done on the brown module. Based on the cut-off criteria (|MM|>0.8 and |GS|>0.35), 75 genes with high connectivity in the brown module were identified as hub genes; the gene list can be found in supplementary table 4. These genes were analysed using the STRING database and visualised using Cytoscape software to determine the key genes (figure 6b). Many inflammatory genes were obtained, such as *ITGAM*, *TGFB1*, *IL17RA*, *TNFRSF1B*, *FCN1*, *FCGR2A*, *FCGR2B* and *FPR1*, which supports our findings on the association between CCG score and pro-inflammatory genes. The full gene lists of turquoise and brown modules can be found in supplementary table 5.

Discussion

In this study, we investigated the relationship between the core clock and gene expression in an unbiased manner in COPD patients using RNA sequencing data from bronchial brushes. Our main finding is that

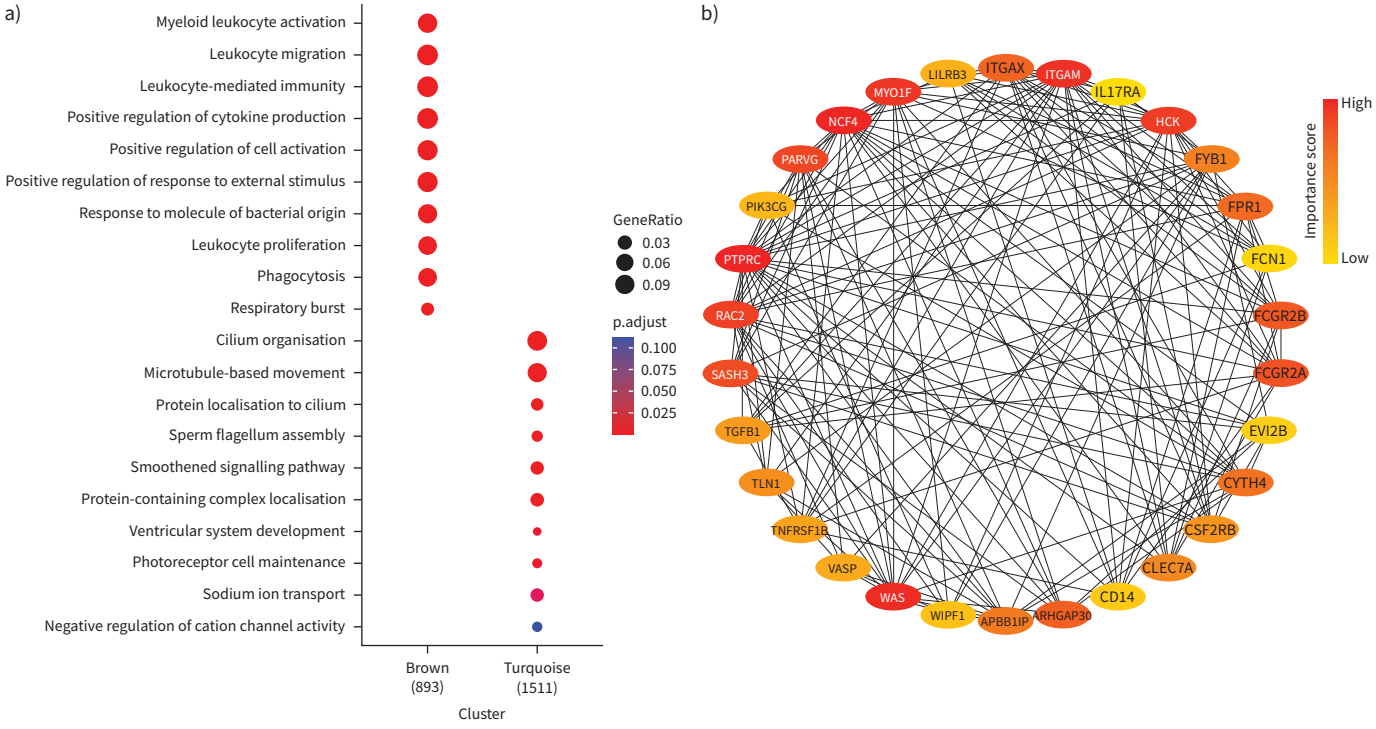

FIGURE 6 Weighted gene coexpression network analysis of specific transcriptome networks associated with core clock gene score expression in bronchial brush samples. a) The top 10 significantly enriched Gene Ontology (GO) annotations of Biological Process in the turquoise and brown modules. Bubble plot size indicates the gene ratio, the ratio of genes mapped to the GO terms; colour indicates p-value. b) PPI network of the 30 hub genes with the highest importance scores based on the maximal clique centrality algorithm in the brown module. Many inflammatory genes were identified, such as *ITGAM*, *TGFB1*, *IL17RA*, *TNFRSF1B*, *FCN1*, *FCGR2A*, *FCGR2B* and *FPR1*.

the expression of several pro-inflammatory genes is inversely correlated with the expression of CCGs in bronchial brushes of 26 COPD stage GOLD 2–3 patients. Furthermore, we found clock signature genes and cytokine signature genes were differentially expressed in the lung tissues of COPD patients compared with healthy smokers. Our results reinforce the notion that inflammation can interfere with the normal functioning of the circadian clock in COPD patients, and provide a detailed analysis of all coexpressed genes.

Circadian rhythms play an important role in regulating various physiological processes, including immune responses, tissue repair and oxidative stress. Interestingly, in previous analysis of RNA sequencing data of mouse lung tissues, it was found that about 10% of the transcriptome is correlated with expression of clock genes [19]. This is consistent with the results we obtained from the clinical data analysed here. This stresses how extensive the biochemical processes that clock genes participate in are in living organisms.

BMAL and PER/CRY mRNA expression tends to follow anticyclic expression patterns in lung tissue [20]. On the basis of this notion, one would expect opposite gene expression associations between positive and negative regulators of circadian clock signalling. However, smoking and the presence of COPD broadly suppress clock pathway signalling and clock pathway gene expression. For example, it has been demonstrated that *in vivo* smoke exposure dampens the expression of most CCGs [20]. In COPD and in smokers, there also seems to be a general suppression of clock gene expression, including both positive and negative regulators [10, 14]. The basis of this change is most probably related to oxidative stress [21]. Our findings show that within patient samples, the expression of individual CCGs (both positive and negative regulators) correlates quite well. Thus, high CCG-score expressers tend to have high expression of both positive and negative regulators and *vice versa*, this is lower across the board for low-CCG expressers. Accordingly, the most likely explanation for our findings is that the gene expression associations that we find in our study should be interpreted as being related to the broad suppression of clock pathway signalling that is observed in COPD. These associations may be different in healthy subjects in which the cyclical oscillations of individual clock genes are more outspoken.

Inflammation and clock pathway signalling influence each other in a bidirectional manner. For instance, REV-ERBα agonists can inhibit the expression of *IL6*, *IL19*, *CXCL6*, *CXCL11* and *CCL2* [22]. Our study reveals that CCGs are inversely correlated with the expression of several pro-inflammatory genes, such as *CXCL8*, *IL1A*, *IL1B*, *IL7*, *TGFB1* and *IL17RA*, in COPD patients. This suggests that inflammation and the circadian clock can disrupt each other. Therefore, understanding how these systems interact may provide new insights into the pathophysiology and treatment of COPD, for example by modifying the action of core clock components. This may reduce inflammation and benefit COPD patients. CXCL8 is a chemokine that attracts and activates neutrophils and is linked to COPD severity and exacerbation [23]. IL-1A and IL-1B are pro-inflammatory cytokines that bind to IL-1R1. They can induce CXCL8 and IL-6 expression, and activate the nuclear factor (NF)-κB pathway, which regulates the transcription of genes related to inflammation and immunity [24]. This contributes to airway inflammation and remodelling in COPD patients. IL-17RA activates the NF-κB pathway and induces CXCL8 and IL-6 expression. IL-17RA signalling also promotes neutrophil recruitment and mucus production in the airways [25]. TGF-β1 is a cytokine that regulates various cellular processes, such as growth, differentiation, apoptosis, migration, adhesion and extracellular matrix production. TGF-β1 inhibits the activation of other cytokines such as TNF-α [26]. TGF-β1 plays a role in emphysema formation and airway fibrosis. It is possible that the expression of clock genes may be regulated with the expression of IL-1 family cytokines, CXCL8, IL-17RA and TGF-β1 and that these genes have a complex mutual regulatory network.

Our study has several strengths and limitations. One strength is that we used clinical samples from COPD patients with a severe disease stage, which increased the relevance and validity of our results. Another strength is that we used multiple analyses to identify coexpressed genes and pathways related to clock genes and inflammation, which enhanced the robustness and comprehensiveness of our findings. One limitation is that we used healthy smokers as the control group for differential expression analysis, which may introduce confounding factors due to the effect of smoking on gene expression and circadian rhythm. This was a deliberate choice as using the same dataset, we have previously shown that there is disruption of clock pathway genes in COPD compared with nonsmoking controls [10]. Others have previously shown that smoking reduces protein expression of CCGs in the lung sections of patients [14]. While the comparison of COPD with nonsmoker controls therefore maximises the window to observe changes in clock pathway expression and its associations, it is not possible to disentangle the effect of smoking from that of the presence of established COPD.

Future studies should aim to uncover the mechanisms behind the observed inverse associations between inflammatory gene expression and clock gene expression and to identify whether these can be reversed. In view of the previously reported role of oxidative stress in the disruption of circadian signalling in response to smoke exposure [21], antioxidants are an interesting path to pursue. Equally, in view of the reported roles of corticosteroids and cAMP-elevating agents in synchronising circadian signalling [10, 27], drugs targeting these pathways are also of interest. For example, we have previously demonstrated the capacity of PGE_2 and PGI_2 in restoring circadian signalling and epithelial repair responses *in vitro* and *in vivo*.

To advance our understanding and treatment of COPD, it is crucial to employ animal models or bronchial epithelial cell models to dissect how circadian disruption impacts inflammatory responses and disease progression. Key challenges include elucidating the molecular pathways by which clock genes such as *CRY2* and *BMAL1* influence inflammation in COPD, devising and validating chronotherapy strategies tailored to enhance treatment efficacy and safety in COPD patients, and addressing individual variability in circadian rhythms to develop personalised chronotherapy protocols. Longitudinal studies are imperative to assess how modulating circadian rhythms affects COPD progression, inflammation levels and patient quality of life. Addressing these challenges will deepen our mechanistic insights and pave the way for more-effective therapeutic interventions in COPD.

In conclusion, we identified a broad panel of pro-inflammatory genes inversely correlated with the expression of a subset of CCGs that is potentially involved in COPD. Our study provides a new perspective for understanding the pathophysiology and treatment of COPD. By regulating the circadian rhythm, we may be able to reduce inflammation and improve the quality of life of COPD patients. However, more research is needed to explore the molecular mechanisms underlying the interaction between inflammation and circadian rhythm, and to test the efficacy of chronotherapy in COPD patients.

Provenance: Submitted article, peer reviewed.

Ethics statement: This study concerns the re-use of data previously gathered in ClinicalTrials.gov NCT02058459. All local ethics committees approved the study. All patients provided written informed consent.

Author contributions: X. Li, A. Nagelkerke and R. Gosens conceived and designed the study. K. Srikanthan, D-J. Slebos, P.L. Shah, P.J. Johnson and L.E.M. Kistermaker organised and oversaw the collection of the bronchial brushes. X. Li and K. Srikanthan performed the analyses. X. Li, S.F. Rahmawati and R. Gosens interpreted the data. X. Li drafted the main manuscript text and prepared all figures. All authors revised the manuscript for important intellectual content and approved the final version.

Conflict of interest: D-J. Slebos reports receiving clinical trial expenses from Pulmonx Corp USA, Nuvaira USA, PulmAir USA, Apreo USA and FreeFlowMedical, outside the submitted work; consulting fees form Nuvaira USA, MoreAir USA, Apreo USA and PulmonX USA, outside the submitted work; payment or honoraria for lectures, presentations, speakers' bureaus, manuscript writing or educational events from PulmonX USA and Nuvaira USA, outside the submitted work; and support for attending meetings and/or travel from PulmonX USA, outside the submitted work. P.L. Shah reports that Nuvaira have reimbursed the institution for clinical trial expenses incurred during the AIRFLOW-1, -2 and -3 trials, outside the submitted work. P.J. Johnson reports support for the present manuscript from Nuvaira Inc. The author is a current paid employee of Nuvaira Inc. with stock options., disclosures made outside the submitted work. Reinoud Gosens reports support for the present manuscript from Nuvaira Inc.; and grants or contracts from Chiesi, Boehringer Ingelheim and Aquilo, outside the submitted work. The remaining authors have nothing to disclose.

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