university of groningen / faculty of science and engineering

Is time all we need for COPD?

Undergraduate Thesis
Presented to the College of Science and Engineering,
Rijksuniversiteit Groningen
In partial fulfilment of the requirements for the
Pharmacy undergraduate program

By

Klaas de Vries
S3636615

6th of April 2021

Research faculty mentor,
Reinoud Gosens

Abstract

COPD is a prime cause of death worldwide with many elderly people suffering from it. While this is a big problem no efficient treatment is available. The only treatment so far is to relieve the symptoms which are wheezing, cough, mucus production and difficulty breathing. The main issue is the destruction and impaired repair of lung epithelial cells, specifically in the lung alveoli. Due to the epithelial damage the elasticity of the lungs decrease and so increase the force necessary to inhale and exhale. The alveoli are important for the gas exchange between CO_2 and O_2 with air and blood, when these are severely damaged or inflamed there is emphysema. All of these factors contribute to the symptoms of COPD.
While no current treatment is efficient for COPD a new novel mechanism might be the solution. The biological master clock in the brain has been known for quite some time however, a more recent finding is that peripheral cells individually also contain a biological clock. This biological clock shows disruptions in COPD with most noticeable a general decrease in NR1D1 and overall increase in SIRT1. This postulates the hypotheses that NR1D1 and SIRT1 are key in the aberrant epithelial mesenchymal cell interaction with fibroblasts driving emphysema. So maybe the best medicine is simply time.

COPD

Clinical representation of COPD

COPD is known as chronic obstructive pulmonary disease and is a chronic inflammation of lung tissue. It is a prime cause of death worldwide and is characterized by limitation of the expiratory airflow cannot be reversed fully, destruction of the lung and chaotic chronic inflammation. The chronic inflammation will cause the airflow in the lungs to be obstructed. Symptoms characteristic for COPD are cough, mucus production, wheezing and difficulty breathing [1].
In normal lungs the epithelium is made up of various cell types which maintain a balanced interaction with microbiota in the normal lung. Usually only the signals which are regulatory are induced. When necessary progenitor cells and mesenchymal stem cells can be mustered from the circulation [1].
With COPD there are changes which could be induced by pathogenic bacteria, oxidants, air pollutants, cigarette smoke, etc. Due to these factors pro-inflammatory pathways can be activated in the epithelium, this induces the liberation of cytokines and chemokines. Cigarette smoke for example is able to muster neutrophils and macrophages, since it is a lavish source of oxidants. Also the amount of progenitor cells present locally and in circulation are reduced by air pollutants, such as cigarette smoke. At sites of inflammation inflammatory cells (dendritic cells, cytotoxic T-cells, neutrophils, monocytes/macrophages) arrive and will become further source of proteases, oxidants and cytokines. CD8 and CD4-T cells, which are Th1/Tc1 dominant, are attending in this process and they liberate perforins and chemokines. While these processes wreak havoc, representatives of normal microbiota attempt to avert exaggeration of the inflammation by generating relative fragile signals. All of these processed induced by different stimuli develop to extracellular matrix proteolysis, vascular endothelial and alveolar wall cell apoptosis. This inflammation cascade could be aggravated by elastin fragment. The loss of endothelium and epithelium, overall inflammatory tone and structural disintegration of alveoli are seen in COPD [1].
Cigarette smoke is a factor which is highly involved with the onset of COPD, since it can trigger inflammation responses in the lung. The inflammation can be at different sites in the lung, the large and the small airways. There are different phenotypes with COPD, chronic bronchitis and emphysema [2]. Bronchitis is the inflammation of conducting airways, emphysema is the destruction of the alveoli [3].In this Bachelor project the focus will be on emphysema.

Pathogenesis of COPD

Emphysema is the unusual persistent dilatation and demolition of cell walls of alveoli, alveolar ducts and alveolar sacs [4]. This will cause loss of lung elastic recoil and intraluminal pressure, as a

consequence of this the alveoli can collapse and so hinder the ability for air to access them. This is especially noticeable when forced expiration occurs [5]. The alveolar surface is covered by 2 types of cells, the flat alveolar type 1 (AT1) which will die through apoptosis when damaged and the cuboidal alveolar epithelial type 2 (AT2) which are more resilient to damage. AT1 cells cover most of the alveolar surface (95%) and are responsible for the gas exchange in the lung , when these cells get damaged the AT2 cells adjacent to the site of damage will be activated into revitalisation and transdifferentiate to AT1 cells. Furthermore studies have found that secretory club cells in mice can behave as genealogy confined progenitors to keep up or repair mild damage to the alveoli [6], [7]. AT2 cells are not only the precursors for AT1 cells, they also secrete surfactant proteins which prevent the collapse of the alveoli after exhalation and they maintain lung fluid homeostasis. Furthermore are AT2 cells involved in repair and anti-inflammatory processes [7].
With emphysema there is destruction of gas-exchange tissue due to insufficient repair of the alveolar tissues [8]. The alveoli contain multiple mesenchymal and myeloid cells which can aid the repair of the alveoli. The mesenchymal cells and alveolar epithelial cells both modulate secretion and expression of receptors and ligands following injury, this allows an increased and directed cell-cell interactivity in the alveolar space [9,10].

Pathways involved in COPD

If lung cells are exposed for prolonged time with smoke, inflammatory cells (neutrophils, T lymphocytes and macrophages) will be employed.
Macrophages are activated first, these release neutrophil chemotactic factors (examples leukotriene B4, interleukin-8). When the neutrophils are involved they will release together with macrophages a myriad of proteinases, causing hyper secretion of mucus [11].
Extracellular matrix is necessary to keep the wholeness of small airways and lung parenchyma, elastin is a crucial component of the extracellular matrix. An imbalance in the elastase/anti-elastase increases likelihood for lung destruction, which causes enlargement of airspace. Neutrophil derived proteases and cathepsins will combat elastin and also demolish connective tissue of the lung parenchyma. The cytotoxic T cells will release perforins and TNF-alpha, these will demolish epithelial cells of the alveolar wall [11]. Not only release of neutrophilic proteolytic enzymes and hyper secretion of mucus, but also inhibition of anti-proteolytic enzymes and alveolar macrophages. Polymorphisms genes contribute in the insufficient production of these antiproteases in smokers. It is known that alpha one antitrypsin (AAT) is produced by lung parenchyma. This protein is able to inhibit neutrophil elastase and trypsinize activity in the lung. A deficiency in this protein can lead to emphysema [11].
Certain signalling pathways in the cell are associated with the impaired lung regeneration, these are WNT/beta-catenin, fibroblast growth factors (FGFs), transforming growth factors TGF-beta and inflammation [6].
WNT/beta-catenin pathway modulates the fate of progenitor cells in lung development in addition to damage [10,12]. Especially in the distal part of the lung where emphysema is present the WNT/beta-catenin pathway plays a crucial role in the formation of AT1 cells which contribute most to the surface in the alveolus responsible for gas-exchange and AT2 cells which produce surfactants [10]. Alveolar epithelial organoid forming capability is kept in cells that response to activity of WNT/beta-catenin pathway [12]. In COPD patients it is seen that the WNT/beta-catenin pathway is decreased in the AT2 cells [13]. Further evidence for dysfunction of WNT/beta-catenin pathway in emphysema induced by elastase is gained from mouse models. The progenitor cells of distal lung epithelium showed decreased capacity in organoid forming, in addition restoring the WNT/beta-catenin pathway substantially restored the organoid forming capacity [12]. Also activation of WNT/beta-catenin pathway by GSK3 inhibition recovers lung function and tissue integrity in animal models with COPD [13,14]. From the WNT ligands some are able to activate beta-catenin independent pathways in specific contexts like Jun N-terminal kinase (JNK), p38 MAP kinase and Rho-kinase. These beta-

catenin independent pathways are typically affected by non-canonical WNT ligands, for example WNT-5A and 5B. This beta-catenin independent pathway is a feature of the chronic and declining lung diseases such as COPD [15]. In COPD it is seen that non canonical WNT-4, WNT-5A, WNT-5B, FZD2 and FZD8 are all over expressed [16]. In lung fibroblast, which can cause fibrosis, from patients with COPD the WNT-5A seemed increased. This increase was induced by stimuli like cigarette smoke (CS), cellular senescence and TGF-beta, which are stimuli related to COPD. In vitro the complete WNT-5A weakens the alveolar epithelial cell wound healing and transdifferentiation which is canonical WNT driven. In vivo the over expression of WNT-5A in the lung would aggravate enlargement of the airspace in emphysema induced by elastase, while inhibition of WNT-5A showed protective effects [17].

FGF pathway is important in the paracrine modulation of AT2 cells by adjacent cells. There are different type of FGF's and different kind of FGF's receptors (FGFR) [18]. The FGF2 otherwise called basic fibroblast growth factor (bFGF) is a strong mitogen for fibroblasts. This FGF2 and its corresponding receptors FGFR1 and FGFR2 are over expressed in COPD patients there bronchial tissue [19]. However FGF2 was reduced in lungs exposed to cigarette smoke of mice and reduced in the serum of COPD patients [20]. FGF2 which has been modulated, known as recombinant FGF2 is able to arrest development of emphysema in mice with elastase induced emphysema [20]. FGF7 is up regulated in COPD lung tissue [21], however FGF7 in conjunction with FGF10 are growth factors that encourage differentiation and preservation of the alveolar epithelium and avoid fibrosis [22]. Based on this, the idea occurs that the amplified expression indicates an attempt to repair, but failure of completion of this repair [23].

TGF-beta pathway role in the pathology of COPD is established. In the alveolar epithelium of COPD patients the expression of TGF-beta isoforms is amplified [24]. The upregulation of TGF-beta pathway and its ligands in COPD are at the mRNA level in the lung [25]. The differentiation of myofibroblast by TGF-beta will result in the distortion of WNT/beta-catenin pathway and in impairs the ability of fibroblasts to aid in the organoid formation of alveolar epithelial [26,27]. This interference is connected to the upregulation of WNT/beta-catenin independent pathway ligands like WNT-5A/5B, FZD8 and connective tissue growth factor (CTGF), these effectors disturb normal repair. Contrary to the fact that factors like FGF7, FGF10 and hepatocyte growth factor (HGF) which protect normal repair are down regulated due to TGF-beta [26]. This implies that while TGF-beta is necessary for certain phases of damage repair cycle, it has harmful effects on advancement of repair. Specifically the communication of epithelial-mesenchymal related to epithelial repair [23].

Hedgehog (HH) pathway acts on cells expressing the receptor PTCH. The HH pathway leads to an intracellular signalling which stabilizes the transcription factor of Gli family proteins which allows the gene transcription of the target. Two gene variants in the HH pathway (PTCH1 and HHIP) are regularly found in COPD [28]. It appears that HH pathway signalling controls proliferative quiescence in adult lung homeostasis and is pivotal for the phase which resolves lung injury [29]. Also the oxidant defence in controlled by HH pathway, since haploinsufficiency for HHIP leads mice to become sensitive to emphysema development and age-reliant oxidative stress [30].

There are publications which purpose the idea that NF-keppaBeta dependent inflammation has aiding effects on regulation of alveolar epithelial repair [31]. It could be that acute inflammation promotes regeneration, but that consistent inflammatory response actually suppresses the repair [23]. This is proven by the fact that IL-1beta promotes the differentiation from AT2 into AT1, while persistent IL-1beta inflammation the differentiation into AT1 cells arrests and cause impairment of the alveolar regeneration [32].

The switch in COPD from the canonical WNT/beta-catenin dependent pathway to noncanonical WNT/beta-catenin independent pathway could present an extra link between altered repair and inflammation in COPD [23].

Biological clock

What are the circadian rhythm, general biological clock and master clock

Waking up, being active and going to sleep again, this is a rhythm, specifically the circadian rhythm. Circadian rhythms are mental/behaviour/physical changes in a rotation of 24 hours. This cycle primarily responds to the dark-light cycle of nature and affects most living creatures. An example of dark-light dependent circadian rhythm is being awake/active during the day and sleeping/resting at night [33].

This circadian rhythm has to be timed by an organism/creature, this is done by its own biological clock, also known as the circadian clock. This biological clock consists out of several specific proteins which can induce or halt processes in the body by interacting with cells. Almost every organ and tissue contains these biological clocks [33].

Naturally it is necessary that if almost every organ/tissue has a biological clock that they need to by synchronized to prevent chaos. This synchronisation occurs via the suprachiasmatic nucleus, also called SCN. The SCN is a collection of circa 20,000 neurons which are located in the hypothalamus and receive their information directly from the eyes [33].

This system ensures that organism/creatures are able survive. Synchronisation between the external environment and the organism allows the organism to adapt to changes in their environment due to the changes from day to night and vice versa. While synchronisation with its own internal environment ensures good health [34].

What is the biological clock and how does it work

For the biological clock to be able to adjust to the changes in the environment it needs to entrained. The most powerful entrainment is the change in light throughout the day/night. This entrainment is done via photoreceptors in the eyes [35,36].

This idea is further supported by the findings of Czeisler who found that eye loss in humans blocked circadian responses to light [37].

Also some work has been dedicated to revealing the mechanism of photoentrainement in rodents this did not produce any evidence for extra ocular receptors being able to entrain to light [35].

However the threshold levels required for a circadian rhythm response are significantly greater than light levels for visual responses [38].

The biological clock has three components: the entrainment pathway which will transmit the signals from the environment towards the timekeeping apparatus; the time keeping apparatus also known as oscillator which operates without cues from the environment and is the central component of the biological clock; last the output pathway which become activated at specific time points in the circadian rhythm by the timekeeping apparatus.

The biological clock does not only operate at the SCN level, but also on smaller levels like cell-cell interactions and even single cell levels. All the different biological clocks in tissues throughout the body are guided and governed eventually by the SCN [39,40].

When conflicting signals are created in the biological clocks it is possible for peripheral clocks to become desynchronized from the central biological clock, the SCN. This shows that the peripheral clocks can modulate themselves based on the desired rhythm in the specific tissue [41].

Important parts are the genes responsible for the expression of these components, when clock genes are mentioned they are mostly genes that encode for parts of the timekeeping apparatus [42].

Molecular mechanism of the biological clock

The molecular mechanism of the circadian clock arises from transcriptional-translational feedback loops which are approx 24 hours long and it is cell autonomous [43,44].

The biological clock can be divided in a core loop and stabilizing loop. Inside the core loop a heterodimer of CLOCK, (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and muscle Arnt-Like protein) binds to enhancer elements on E-box to initiate transcription of their targeted genes [45].

CLOCK and BMAL1 are “positive” factors that positively regulate the expression of the “negative” factors Period (PER1, PER2) and Cryptochrome (Cry1, Cry2) at the start of the cycle. CRY and PER work as inhibitors for CLOCK/BMAL1 complex. When CRY and PER proteins are translated and dimerized together it translocates into the nucleus to interfere with the transcription of the CLOCK/BMAL1 heterodimer. As a result CRY and PER decrease their own transcription and protein synthesis. Also CRY and PER are degraded via 26S proteasomal pathway, this then releases the auto-inhibition of CRY and PER on themselves [46].
This closes the feedback cycle and will start a new cycle of transcription, this whole cycle takes approx 24 hours.
The stabilizing loop is crucial for proper functioning of the biological clock since dysfunction can induce biological clock arrhythmicity [47].
The stabilizing loop regulates the expression of CLOCK-BMAL1, however BMAL1 more so then CLOCK [39].
The regulation is done by nuclear orphan receptors ‘REV-ERBs’ and nuclear receptors ‘RORs. REV-ERBs inhibit expression of BMAL1, while RORs compete with REV-ERBs for binding sites on DNA. This competition will lead to promotion of the BMAL1 expression. This balance closes the stabilizing loop, both REV-ERBs and RORs are managed by the core loop [48].
The proteins of the biological molecular clock are strongly influenced by modifications after translation like phosphorylation and acetylation. These modifications influence the stability and the activity of the proteins/complexes. These modifications are primarily done by sirtuin (SIRT1), it promotes deacetylation of BMAL1 and PER2. So SIRT1 regulates the transcription of clock genes. The activity of SIRT1 has variations throughout the day in the lungs. In lungs which are exposed to CS there is a decrease in SIRT1 activity as well as in COPD lungs. This decrease of SIRT1 implies less deacetylation of BMAL1 and PER2 [49].
Since BMAL1 needs to be acetylated to be in active form the decrease in deacetylation indicates over-activation of BMAL1 [50].
For PER2 it is that SIRT1 binds to the CLOCK-BMAL1 complex and this complex promotes the degradation and deactylation of PER2 [51,52].

Link between the biological clock and lung disease

COPD and asthma exacerbations and hospitalizations are mostly in the early morning or at night, at times when the lung function is at its lowest [53,54,55,56,57]. The biological clock propels daily alterations of airway resistance, airway calibre, mucus hyper secretion, immune-inflammatory responses and respiratory symptoms. These alterations are the underlying reason for the daily variation in frequency and occurrence of the exacerbations. So far a significant number of studies are supportive of the fact that environmental effects like cigarette smoke, hypoxia, shift work, jetlag etcetera can disturb the biological clock functioning in lungs and so speed up potential development of lung pathophysiology [58,59,60,61,62,63,64,65,66,67,68].
Any significant disruption of the biological clock can affect physiological processes which are downstream of the biological clock, this has also been suggested in chronic diseases [69,70]. In the lungs it has been shown in mouse models that the expression of clock genes and clock-controlled genes are altered due to exposure to cigarette smoke and other mediators that promote inflammation [59,71]
In animal studies the extent of the biological clock in regulation of immune-inflammatory responses has been brought under attention [72]. Several other studies in mice showed the impact dysfunction in the biological has in correlation to inflammatory responses and lung pathophysiology. Deletion of CLOCK in lungs promoted the formation of fibrotic like structures and oxidation of proteins. Mice with BMAL1 deletion showed fastened aging and other pathologies, all of which are connected to increased levels of inflammation and reactive oxygen species [73,74,75].
In response to cigarette smoke the levels of SIRT1 are reduced in the lungs. This reduction in SIRT1 has emphysema, senescence and lung inflammation as consequences in the lung [76]. Currently data suggests that core biological clock genes levels are altered in COPD and that the circadian rhythm is

dampened in COPD. The alterations in clock genes levels and in their posttranslational modification are associated with abnormal inflammation of the airways induced by cigarette smoke.
However while studies have been done towards understanding the biological clock in COPD the actual underlying molecular mechanism is not fully understood properly. Also most studies so far are only in animal models and so the actual mechanism in humans is still reasonable novel. That's why in this project a comparison will be made between the expressions of biological clock genes mentioned previously above in normal lung cells versus COPD affected cells.

Results from Human COPD Cell Atlas

The data gathered originates from the Human COPD Cell Atlas. This is an online data mining tool where RNA sequencing of single cells has been done. This sequencing can be used to identify atypical transcriptional profiles of cells and changed signalling in the alveolar proximity in Chronic Obstructive Pulmonary Disease (COPD). The human COPD Cell atlas uses a fixed cell type data base containing
The Human COPD Cell Atlas has four features which can be used to explore data.
UMAP Explorer, can be used to plot the gene expression of certain gene to give a graphical representation of cells which might express the gene of interest.
Gene explorer, can be used for quantitative observation of a gene's expression in specific cells. This pattern is then compared between COPD and control samples. Different forms of visualizations are possible for this feature; Bar graph, Violin plot, Box plot and Density plot.
Multi-Gene Query, can be used to observe up to 8 genes at a time in certain cells. This observation can be compared between COPD and control samples. This feature uses a dot plot format to visualize the data.
Interactive Connectome, can be used to indicate ling-receptor interactions at intracellular level. This data is plotted on an interactive network plot and COPD and control samples can be compared.

As mentioned earlier, epithelial cells and fibroblasts play an important role in the pathophysiology of COPD. For that reason the vocal point is the expression of main biological clock genes in epithelial cells and fibroblasts.

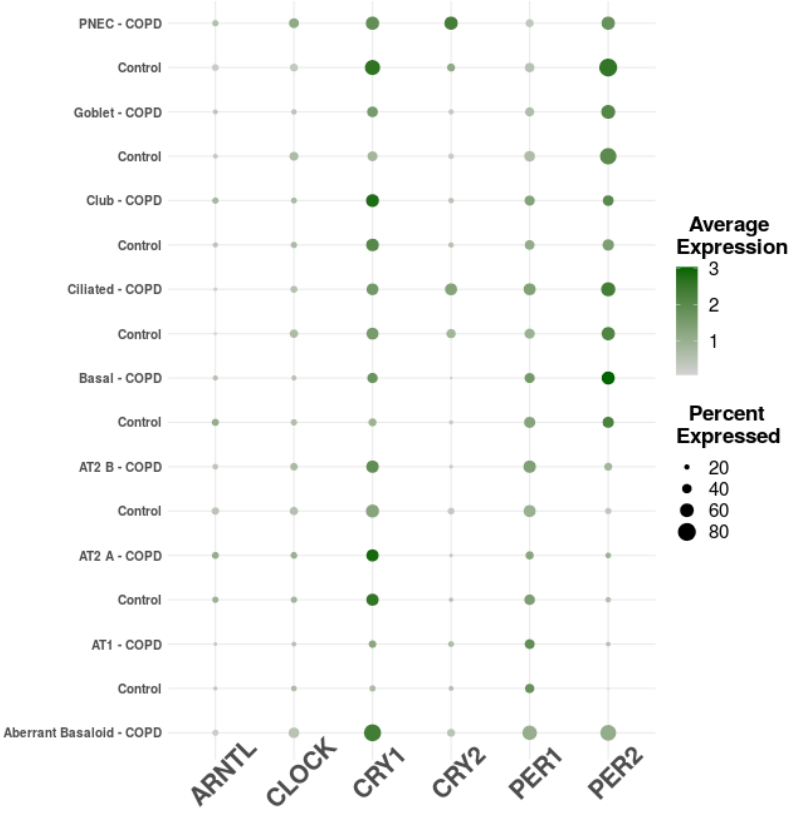

Figure 1: Expression of core loop clock genes in epithelial cells. The colour of the circle indicates the average expression of the gene per cell. The size of the circle indicates the percentage of cells that express the gene.

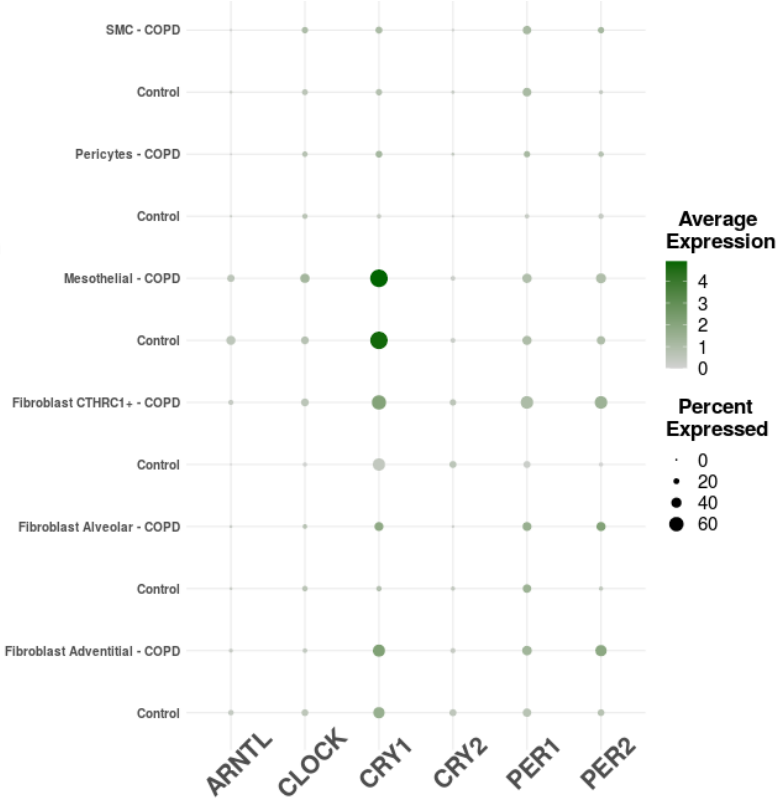

Figure 2: Expression of core loop clock genes in stromal cells. The colour of the circle indicates the average expression of the gene per cell. The size of the circle indicates the percentage of cells of that express the gene.

For overview purposes are the biological clock genes divided in core loop clock genes and stabilizing/modification loop clock genes.
The core loop clock genes are: ARNTL (BMAL1), CLOCK, CRY1, CRY2, PER1 and PER2.
As seen in figure 1, in the epithelial cells little differences can be noticed in the expression of the core clock genes. However these differences are very small. The only potential difference could be seen in the basal cells where the expression of CRY1 is slightly elevated in COPD compared to the control groups.
In figure 2 it can be seen that for the alveolar fibroblast, which are the main focus in the stromal cells, no significant difference could be seen in the genes. Exceptions are CRY1 and PER2 which showed an increase in alveolar fibroblast in COPD compared to the control groups.

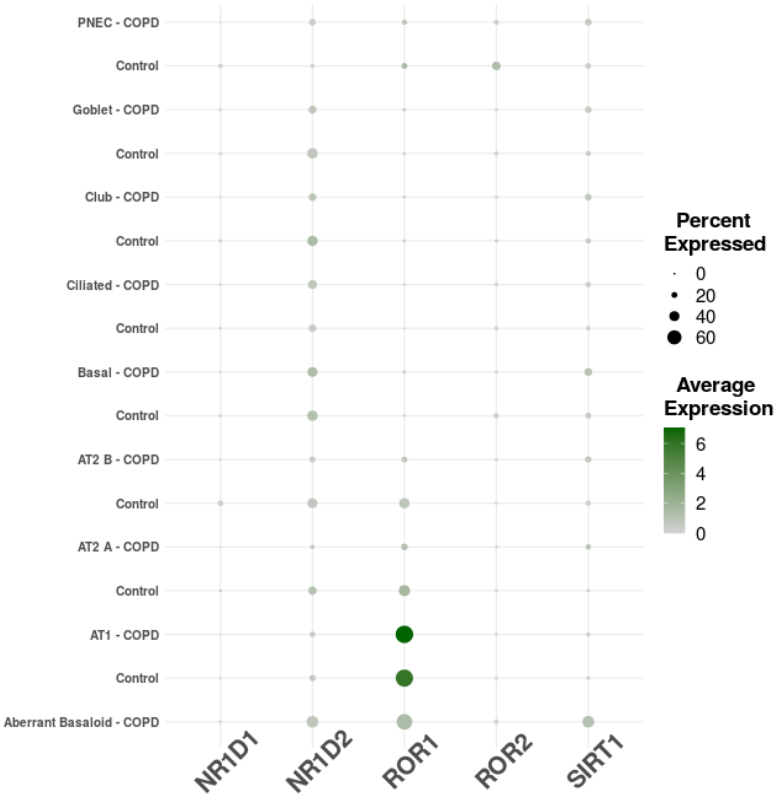

Figure 3: Expression of stabilizing loop clock genes in epithelial cells. The colour of the circle indicates the average expression of the gene per cell. The size of the circle indicates the percentage of cells that express the gene

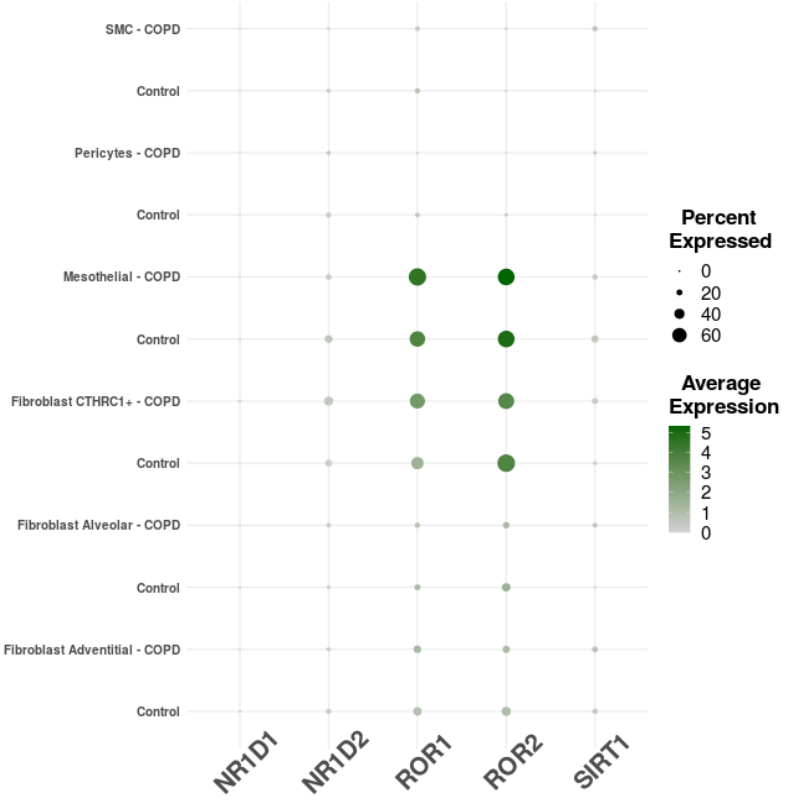

Figure 4: Expression of stabilizing loop clock genes in stromal cells. The colour of the circle indicates the average expression of the gene per cell. The size of the circle indicates the percentage of cells of that express the gene.

The stabilizing loop clock genes are: NR1D1 REV-ERBA-alpha), NR1D2 (REV-ERBA-beta), ROR1(ROR-alpha), ROR2(ROR-beta) and SIRT1.

In figure 3 and 4 an overview differences in the expression of the stabilizing loop clock genes can be seen. This overview however is not sufficient to indicate how a gene is expressed. To support this, a bar diagram has been made for every individual gene of the stabilizing loop of the biological clock. This is then also done for epithelial cells and for the stromal cells, more specifically the alveolar fibroblasts.

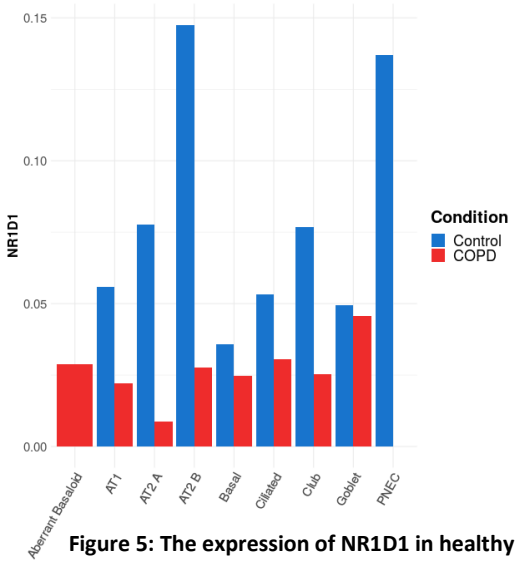

Figure 5: The expression of NR1D1 in healthy lung epithelial cells compared to COPD lung epithelial cells.

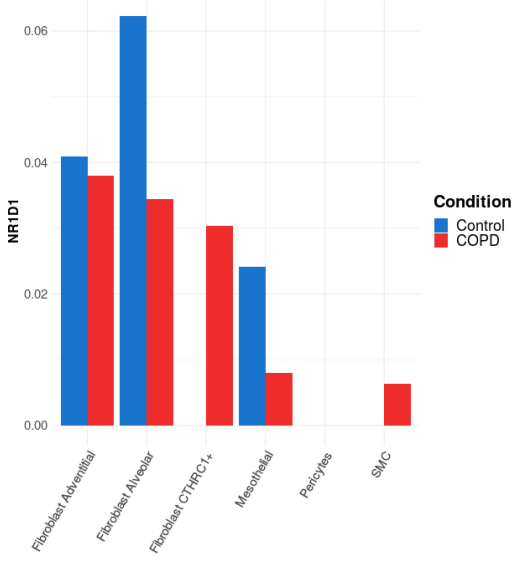

Figure 6: The expression of NR1D1 in healthy lung stromal cells compared to COPD lung stromal cells. The focus is on the alveolar fibroblasts.

NR1D1:

The NR1D1 gene shows a general decrease in its expression in all types of epithelial cells compared to COPD (fig 5). The biggest gaps in expression are in the AT2A, AT2B and club cells. A decrease in the expression of NR1D1 in COPD can also be detected in the alveolar fibroblasts (fig 6).

NR1D2:
In the AT2B, AT2A, AT1, Club and goblet cells the NR1D2 is less expressed in COPD compared to the control. While in basal and ciliated cells NR1D2 seems to have an increase in expression in COPD compared to control groups. For the alveolar fibroblasts it can be seen that NR1D2 has an increase in expression slightly compared to the control groups.

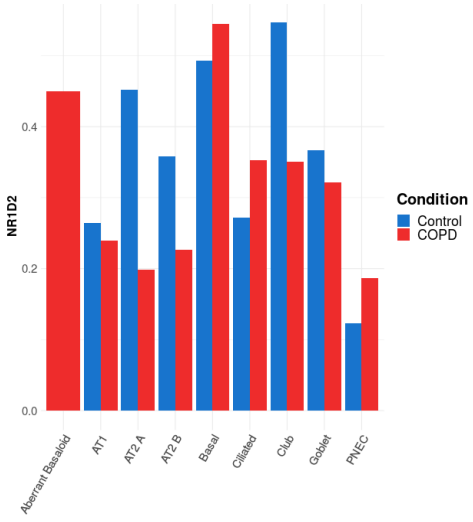

Figure 7: The expression of NR1D2 in healthy lung epithelial cells compared to COPD lung epithelial cells.

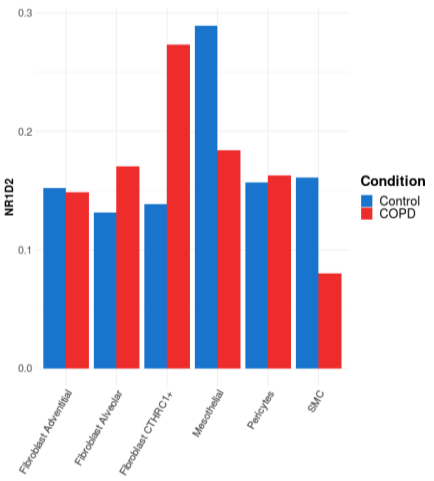

Figure 8: The expression of NR1D2 in healthy lung stromal cells compared to COPD lung stromal cells. The focus is on the alveolar fibroblasts.

ROR1:
The ROR1 gene is clearly less expressed in AT2B and AT2A cells in COPD compared to the control. ROR1 is also less expressed in the alveolar fibroblast in COPD compared to the control groups.

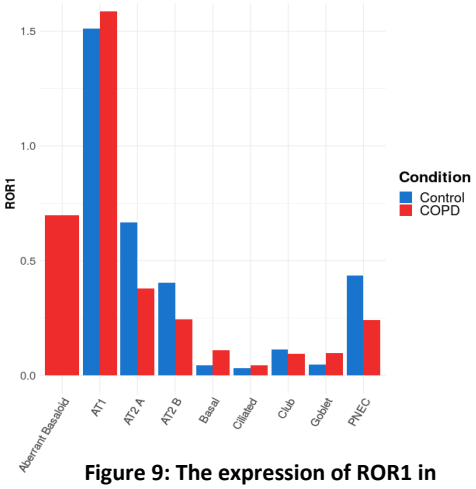

Figure 9: The expression of ROR1 in healthy lung epithelial cells compared to COPD lung epithelial cells.

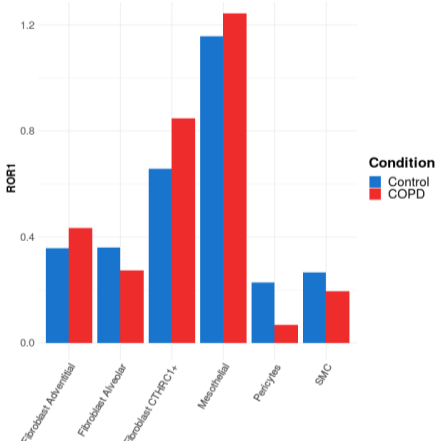

Figure 10: The expression of ROR1 in healthy lung stromal cells compared to COPD lung stromal cells. The focus is on the alveolar fibroblasts.

ROR2:
ROR2 is relatively stable across COPD groups and control groups, however a significant decrease in expression can be seen in basal cells of COPD groups compared to the control groups. Another interesting finding is that ROR2 has an enormous decrease in its expression in COPD vs control groups, while in control groups ROR2 seems to be much more prominent in the PNEC then any other stabilizing clock gene. In the alveolar fibroblast the ROR2 gene is less expressed in COPD compared to the control group.

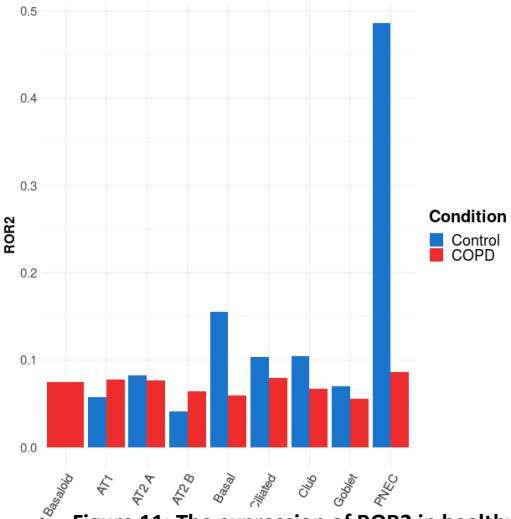

Figure 11: The expression of ROR2 in healthy lung epithelial cells compared to COPD lung epithelial cells.

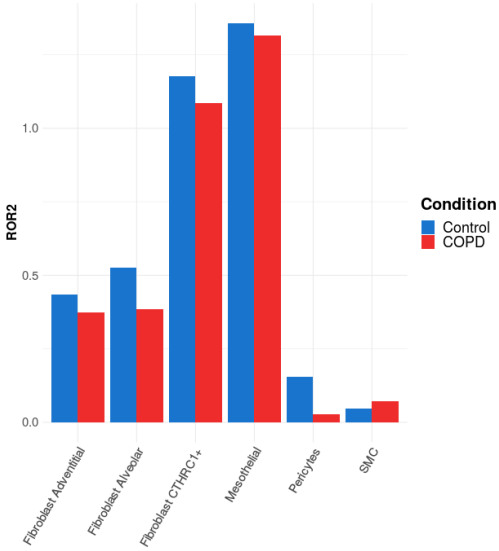

Figure 12: The expression of ROR2 in healthy lung stromal cells compared to COPD lung stromal cells. The focus is on the alveolar fibroblasts

SIRT1:
The SIRT1 gene has an interesting pattern in the epithelial cells, it is the exact opposite of NR1D1. It has increased expression in every type of epithelial cell of interest. The increase is highest in the AT2A and basal cells. In the alveolar fibroblast a huge increase in the expression of SIRT1 in COPD compared to the control can be observed.

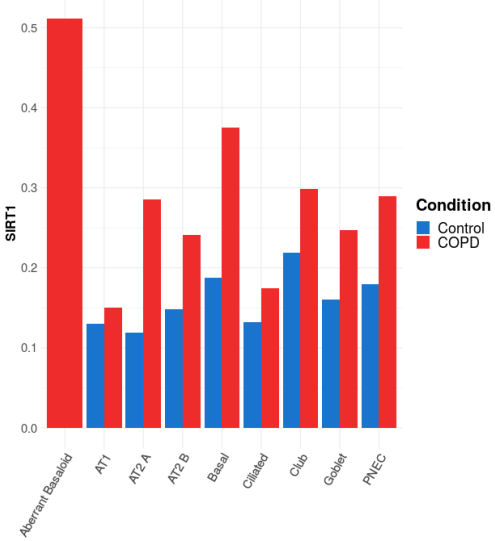

Figure 13: The expression of SIRT1 in healthy lung epithelial cells compared to COPD lung epithelial cells.

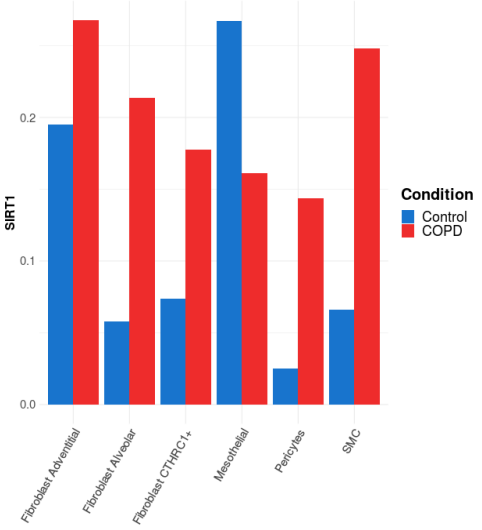

Figure 14: The expression of SIRT1 in healthy lung stromal cells compared to COPD lung stromal cells. The focus is on the alveolar fibroblasts

When evaluating the data presented a noticeable trend can be observed. It seems that the genes of the stabilizing loop are more affected in their expression than the core loop clock genes. An interesting result is the inverse relation between NR1D1 and SIRT1 in epithelial cells. Whereas NR1D1 is less expressed, SIRT1 is over expressed in COPD.
For ROR2 the huge decrease in expression in PNEC is very noticeable. For the alveolar fibroblast the decrease in NR1D1 and increase in SIRT1 are the most prominent differences seen. For the PNEC (pulmonary neuroendocrine cell) a huge expression of ROR2 in control groups can be seen while a very big decrease in ROR2 in COPD groups is noticed. This could indicate a disturbance in the sensor function of the PNEC, also the PNEC are involved inducing tissue remodelling and shaping the immune response. So a dysfunction in the PNEC could contribute to the development of emphysema.
The trend that in AT2 cells a decrease in expression of NR1D1 and an increase in SIRT1, together with the increase of SIRT1 and decrease of NR1D1 expression in alveolar fibroblasts is very interesting in context of emphysema development. The AT2 cells are destroyed in emphysema and in this data a clear link with NR1D1 decrease and SIRT1 increase in these cells can be seen. Also for the fibroblasts, which try to repair the damage in emphysema but fail, a link with the NR1D1 and SIRT1 expression and failure of the fibroblasts could be possible. Arising from this data the following hypothesis is derived: NR1D1 and SIRT1 are key in the aberrant epithelial mesenchymal cell interaction with fibroblasts driving emphysema.

Experiment

To test this hypothesis an experiment can be carried out with the aims to determine to what extend NR1D1 and SIRT1 contribute towards emphysema development. A proposal is an experiment which will be a combination of in vivo and ex vivo. For the creation of the in vivo part, fertilized one celled oocytes of mice can be microinjected with CRISPR-cas9.
CRISPR is an abbreviation for Clustered regularly interspaced short palindromic repeats. CRISPR system contains fragments of foreign DNA which are protospacers and integrated in between spacers (repetitive DNA sequences) which are in tandem in the DNA of the host. When this region is transcripted it forms CRISPR RNA also known as crRNA. This crRNA consists out of both spacer and protospacer sequences. This crRNA can hybridize with transactivating CRISPR RNA also known as tracrRNA. The tracrRNA allows the crRNA to form complexes with nuclease Cas9. The spacers of crRNA guide Cas9 to its target on the DNA sequence of interest. On this spot the Cas9 introduces a double stranded break in the DNA. This break causes the cell to repair this mainly via NHEJ (non homologous end joining) this can induce a deletion of a gene. However the CRISPR-Cas9 system can also be used to induce a gene. This can be done by creating the double stranded break and pasting a promoter in front of the gene of interest. So this allows for the down regulation of NR1D1 and the

over expression of SIRT1 [77]. So for our experiment it is necessary to design or order a CRISPR Cas9 system which can edit NR1D1 gene and SIRT1 gene.
After the microinjection of the fertilized one celled oocytes they will be transferred to female mice which are pseudo pregnant. When the offspring is born they all carry the germ line of interest.
In total 12 groups with different conditions will be created. See table 1 for the conditions of the groups. The addition of elastase is used to speed up the process of emphysema, since it can be a time consuming process for the formation of emphysema in the lungs. Apart from the above mentioned conditions, the mice will live comfortably in healthy and normal conditions. After 4 months the mice will be sacrificed and the lungs will be harvested.

Table 1: Shows the condition to which the mice are exposed. In total 12 different groups are present.

Air		Cigarette smoke	
Addition of elastase	**No elastase**	**Addition of elastase**	**No elastase**
NR1D1 knockout.	NR1D1 knockout.	NR1D1 knockout.	NR1D1 knockout.
SIRT1 knock in.	SIRT1 knock in.	SIRT1 knock in.	SIRT1 knock in.
NR1D1 knockout & SIRT1 knock in.	NR1D1 knockout & SIRT1 knock in.	NR1D1 knockout & SIRT1 knock in.	NR1D1 knockout & SIRT1 knock in.

To prevent fast degradation of the lungs and to ensure proper production of precision cut lung slices (PCLS) the lungs are infused with low melting point agarose. Before PCLS are made the whole lung is examined on morphological characteristics. Also the air space size of the alveoli are determined, if the alveolar air space size increases then the degree of emphysema also increases. These can be compared to control which consists out of healthy lungs without modifications and lungs that developed emphysema due to exposure to cigarette smoke and had no modifications.
After this examination and after the agarose has become gel, cylindrical cores are prepared from the harvested lungs. These cores are transferred to a cylindrical holder after which they will be cut with a tissue slicer.
The PCLS will be incubated in DMEM which is Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM, pH 7.2-7.4 and in L-glutamine and HEPES which is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The culture is protected from bacteria by supplementing with penicillin and streptomycin [78]. Cell culture conditions will be as normal.
To determine if and how much NR1D1 and SIRT1 levels were influenced by the CRISPR Cas9 system RNA sequencing of the PCLs can be done via standard RNA isolation protocols. However due to the agarose used the yield of mRNA can be lower and of less purity. To overcome this by excluding the phase separation step and by an additional step to remove DNA from the mixture, this is possible via DNAase for example [79].
To see and to count the abundance of epithelial cells in the PCLs staining can be used. Since we are interested in the epithelial cells and fibroblasts EpCAM can be used for the membrane of epithelial cells and SiR-DNA can be used to stain the nuclei of the cells [80].
For fibroblasts it remains hard to have very specific markers. A marker that can be used for the fibroblasts in lung fibrosis, something that occurs in COPD, is alpha-smooth muscle actin (αSMA) [81]. This staining can make cellular form and quantification more feasible.
The RNA sequencing and the cellular count and morphology can all be compared to control groups and with the different groups themselves. The results should give an indication of the amplitude of involvement of NR1D1 and SIRT1 in the development of emphysema. No real expectations can yet be made beforehand due to the novelty of the subject. All of the results which will come forward from this experiment will contribute towards more knowledge about the importance of NR1D1 and SIRT1 involvement in COPD.

References

[1] Aldonyte, R., Bagdonas, E., Raudoniute, J., & Bruzauskaite, I. (2015). Novel aspects of pathogenesis and regeneration mechanisms in COPD. *International Journal of Chronic Obstructive Pulmonary Disease*, 995. https://doi.org/10.2147/copd.s82518

[2] Cruz, C. M. S. D. S. (2019, 23 januari). *COPD: Clinical Manifestations and Management (include pulmonary rehabilitation)*. Pulmonology Advisor. https://www.pulmonologyadvisor.com/home/decision-support-in-medicine/pulmonary-medicine/copd-clinical-manifestations-and-management-include-pulmonary-rehabilitation/

[3] *Emphysema*. (z.d.). Www.Lung.Org. https://www.lung.org/lung-health-diseases/lung-disease-lookup/emphysema.

[4] Pahal P, Avula A, Sharma S. Emphysema. [Updated 2021 Feb 23]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482217/

[5] Mannino DM. Chronic obstructive pulmonary disease: definition and epidemiology. Respir Care. 2003 Dec; 48(12):1185-91; discussion 1191-3. PMID: 14651759.

[6]Ng-Blichfeldt JP, Gosens R, Dean C, Griffiths M, Hind M. Regenerative pharmacology for COPD: breathing new life into old lungs. Thorax. 2019; 74(9):890-7.

[7] Mercer, B. A., Lemaître, V., Powell, C. A., & D'Armiento, J. (2006). The Epithelial Cell in Lung Health and Emphysema Pathogenesis. *Current respiratory medicine reviews*, *2*(2), 101–142. https://doi.org/10.2174/157339806776843085

[8] Hogg JC, Timens W. The pathology of chronic obstructive pulmonary disease. Annu Rev Pathol. 2009; 4:435-59. doi: 10.1146/annurev.pathol.4.110807.092145. PMID: 18954287.

[9]Zepp JA, Zacharias WJ, Frank DB, Cavanaugh CA, Zhou S, Morley MP, et al. Distinct Mesenchymal Lineages and Niches Promote Epithelial Self-Renewal and Myofibrogenesis in the Lung. Cell. 2017; 170(6):1134-48 e10

[10] Lee JH, Tammela T, Hofree M, Choi J, Marjanovic ND, Han S, et al. Anatomically and Functionally Distinct Lung Mesenchymal Populations Marked by Lgr5 and Lgr6. Cell. 2017; 170(6):1149-63 e12

[11] Pahal P, Avula A, Sharma S. Emphysema. [Updated 2021 Feb 23]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482217/

[12] Hu Y, Ng-Blichfeldt JP, Ota C, Ciminieri C, Ren W, Hiemstra PS, et al. Wnt/betacatenin signaling is critical for regenerative potential of distal lung epithelial progenitor cells in homeostasis and emphysema. Stem Cells. 2020.

[13] Kneidinger N, Yildirim AO, Callegari J, Takenaka S, Stein MM, Dumitrascu R, et al. Activation of the WNT/beta-catenin pathway attenuates experimental emphysema. Am J Respir Crit Care Med. 2011; 183(6):723-33.

[14] Baarsma HA, Bos S, Meurs H, Visser KH, Smit M, Schols AM, et al. Pharmacological inhibition of GSK-3 in a guinea pig model of LPS-induced pulmonary inflammation: I. Effects on lung remodeling and pathology. Respir Res. 2013; 14(1):113.

[15] Skronska-Wasek W, Gosens R, Königshoff M, Baarsma HA. WNT receptor signalling in lung physiology and pathology. Pharmacol Ther. 2018; 187:150-66.

[16] van Dijk EM, Menzen MH, Spanjer AI, Middag LD, Brandsma CA, Gosens R. Noncanonical WNT-5B signaling induces inflammatory responses in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2016;310(11):L1166-76.

[17] Baarsma HA, Skronska-Wasek W, Mutze K, Ciolek F, Wagner DE, John-Schuster G, et al. Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD. J Exp Med. 2017;214(1):143-63.

[18] El Agha E, Seeger W, Bellusci S. Therapeutic and pathological roles of fibroblast growth factors in pulmonary diseases. Dev Dyn. 2017;246(4):235-44.

[19] Kranenburg AR, Willems-Widyastuti A, Mooi WJ, Saxena PR, Sterk PJ, de Boer WI, et al. Chronic obstructive pulmonary disease is associated with enhanced bronchial expression of FGF-1, FGF-2, and FGFR-1. J Pathol. 2005;206(1):28-38.
[20] Kim YS, Hong G, Kim DH, Kim YM, Kim YK, Oh YM, et al. The role of FGF-2 in smokeinduced emphysema and the therapeutic potential of recombinant FGF-2 in patients with COPD. Exp Mol Med. 2018;50(11):1-10
[21] El Agha E, Schwind F, Ruppert C, Günther A, Bellusci S, Schermuly RT, et al. Is the fibroblast growth factor signaling pathway a victim of receptor tyrosine kinase inhibition in pulmonary parenchymal and vascular remodeling? Am J Physiol Lung Cell Mol Physiol. 2018;315(2):L248-l52
[22] Correll KA, Edeen KE, Redente EF, Zemans RL, Edelman BL, Danhorn T, et al. TGF beta inhibits HGF, FGF7, and FGF10 expression in normal and IPF lung fibroblasts. Physiol Rep. 2018;6(16):e13794.
[23] Hiemstra, P., Wu, X., Khedoe, P., & Gosens, R. (ter perse). Role of altered stem cell function in airway and alveolar repair and remodelling in COPD. *ERS Educational Publications*.
[24] De Boer WI, Van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, et al. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1998;158(6):1951-7.
[25] Brandsma CA, van den Berge M, Postma DS, Jonker MR, Brouwer S, Paré PD, et al. A large lung gene expression study identifying fibulin-5 as a novel player in tissue repair in COPD. Thorax. 2015;70(1):21-32.
[26] Ng-Blichfeldt JP, de Jong T, Kortekaas RK, Wu X, Lindner M, Guryev V, et al. TGFbeta activation impairs fibroblast ability to support adult lung epithelial progenitor cell organoid formation. Am J Physiol Lung Cell Mol Physiol. 2019;317(1):L14-L28.
[27] McQualter JL, Yuen K, Williams B, Bertoncello I. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. Proc Natl Acad Sci U S A. 2010;107(4):1414-9.
[28] Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, et al. Metaanalyses of genome-wide association studies identify multiple loci associated with pulmonary function. Nat Genet. 2010;42(1):45-52.
[29] Peng T, Frank DB, Kadzik RS, Morley MP, Rathi KS, Wang T, et al. Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration. Nature. 2015;526(7574):578-82.
[30] Lao T, Jiang Z, Yun J, Qiu W, Guo F, Huang C, et al. Hhip haploinsufficiency sensitizes mice to age-related emphysema. Proc Natl Acad Sci U S A. 2016;113(32):E4681-7.
[31] Katsura H, Kobayashi Y, Tata PR, Hogan BLM. IL-1 and TNFalpha Contribute to the Inflammatory Niche to Enhance Alveolar Regeneration. Stem Cell Reports. 2019;12(4):657- 66.
[32] Choi J, Park JE, Tsagkogeorga G, Yanagita M, Koo BK, Han N, et al. Inflammatory Signals Induce AT2 Cell-Derived Damage-Associated Transient Progenitors that Mediate Alveolar Regeneration. Cell Stem Cell. 2020;27(3):366-82 e7.
[33] *Circadian Rhythms*. (z.d.). Www.Nigms.Nih.Gov. https://www.nigms.nih.gov/education/fact-sheets/Pages/circadian-rhythms.aspx
[34] Vitaterna, M. H., Takahashi, J. S., & Turek, F. W. (2001). Overview of circadian rhythms. *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism*, *25*(2), 85–93.
[35] Foster, R. G. (1998). Shedding Light on the Biological Clock. *Neuron*, *20*(5), 829–832. https://doi.org/10.1016/s0896-6273(00)80464-x
[36] *Nelson R.J, Zucker I Comp. Biochem. Physiol. 1981;* ***69A****: 145-148*
[37] Klerman, E. B., Shanahan, T. L., Brotman, D. J., Rimmer, D. W., Emens, J. S., Rizzo, J. F., & Czeisler, C. A. (2002). Photic resetting of the human circadian pacemaker in the absence of conscious vision. *Journal of Biological Rhythms*, *17*(6), 548–555. https://doi.org/10.1177/0748730402238237
[38] Nelson, D. E., & Takahashi, J. S. (1991). Sensitivity and integration in a visual pathway for circadian entrainment in the hamster (Mesocricetus auratus). *The Journal of Physiology*, *439*(1), 115–145. https://doi.org/10.1113/jphysiol.1991.sp018660
[39] Lowrey, P. L., & Takahashi, J. S. (2011). Genetics of circadian rhythms in Mammalian model organisms. *Advances in genetics*, *74*, 175–230. https://doi.org/10.1016/B978-0-12-387690-4.00006-4

[40] Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol. 2010;72:517-49. doi: 10.1146/annurev-physiol-021909-135821. PMID: 20148687.
[41] Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 2000 Dec 1;14(23):2950-61. doi: 10.1101/gad.183500. PMID: 11114885; PMCID: PMC317100.
An important part are the genes responsible for the expression of these components, when clock genes are mentioned they are mostly genes that encode for parts of the timekeeping apparatus [42].
https://genomebiology.biomedcentral.com/articles/10.1186/gb-2000-1-4-reviews1023
[42] Hardin, P. E. (2000). From biological clock to biological rhythms. *Genome Biology*, *1*(4), reviews1023.1. https://doi.org/10.1186/gb-2000-1-4-reviews1023
[43] Lowrey PL, Takahashi JS. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet. 2004;5:407-41. doi: 10.1146/annurev.genom.5.061903.175925. PMID: 15485355; PMCID: PMC3770722.
[44] Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat Rev Genet. 2008 Oct;9(10):764-75. doi: 10.1038/nrg2430. PMID: 18802415; PMCID: PMC3758473.
[45] Weger, M., Diotel, N., Dorsemans, A. C., Dickmeis, T., & Weger, B. D. (2017). Stem cells and the circadian clock. *Developmental Biology*, *431*(2), 111–123. https://doi.org/10.1016/j.ydbio.2017.09.012
[46] Takahashi JS. Molecular components of the circadian clock in mammals. Diabetes Obes Metab. 2015 Sep;17 Suppl 1(0 1):6-11. doi: 10.1111/dom.12514. PMID: 26332962; PMCID: PMC4560116.
[47] Bugge, A., Feng, D., Everett, L. J., Briggs, E. R., Mullican, S. E., Wang, F., Jager, J., & Lazar, M. A. (2012). Rev-erbα and Rev-erbβ coordinately protect the circadian clock and normal metabolic function. *Genes & development*, *26*(7), 657–667. https://doi.org/10.1101/gad.186858.112
[48] Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature reviews. Genetics*, *6*(7), 544–556. https://doi.org/10.1038/nrg1633
[49] Sundar, I. K., Yao, H., Sellix, M. T., & Rahman, I. (2015). Circadian clock-coupled lung cellular and molecular functions in chronic airway diseases. *American journal of respiratory cell and molecular biology*, *53*(3), 285–290. https://doi.org/10.1165/rcmb.2014-0476TR
[50] Nakahata, Y., Sahar, S., Astarita, G., Kaluzova, M., & Sassone-Corsi, P. (2009). Circadian Control of the NAD+ Salvage Pathway by CLOCK-SIRT1. *Science*, *324*(5927), 654–657. https://doi.org/10.1126/science.1170803
[51] Asher G. et al.. SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* **134**, 317–328 (2008)
[52] Nakahata Y. et al.. The NAD^+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* **134**, 329–340, 10.1016/j.cell.2008.07.002 (2008).
[53] McNicholas WT, Fitzgerald MX. Nocturnal deaths among patients with chronic bronchitis and emphysema. *Br Med J (Clin Res Ed)* 289: 878, 1984.
[54] Petty TL. Circadian variations in chronic asthma and chronic obstructive pulmonary disease. *Am J Med* 85: 21–23, 1988.
[55] Tirlapur VG. Nocturnal deaths among patients with chronic bronchitis and emphysema. *Br Med J (Clin Res Ed)* 289: 1540, 1984.
[56] Traves SL, Proud D. Viral-associated exacerbations of asthma and COPD. *Curr Opin Pharmacol* 7: 252–258, 2007.
[57] Tsai CL, Brenner BE, Camargo CA Jr. Circadian-rhythm differences among emergency department patients with chronic obstructive pulmonary disease exacerbation. *Chronobiol Int* 24: 699–713, 2007.
[58] Davidson AJ, Castanon-Cervantes O, Leise TL, Molyneux PC, Harrington ME. Visualizing jet lag in the mouse suprachiasmatic nucleus and peripheral circadian timing system. *Eur J Neurosci* 29: 171–180, 2009.

[59] Gebel S, Gerstmayer B, Kuhl P, Borlak J, Meurrens K, Muller T. The kinetics of transcriptomic changes induced by cigarette smoke in rat lungs reveals a specific program of defense, inflammation, and circadian clock gene expression. *Toxicol Sci* 93: 422–431, 2006.
[60] Hadden H, Soldin SJ, Massaro D. Circadian disruption alters mouse lung clock gene expression and lung mechanics. *J Appl Physiol* 113: 385–392, 2012.
[61] Hwang JW, Sundar IK, Yao H, Sellix MT, Rahman I. Circadian clock function is disrupted by environmental tobacco/cigarette smoke, leading to lung inflammation and injury via a SIRT1-BMAL1 pathway. *FASEB J* 28: 176–194, 2014.
[62] Lagishetty V, Parthasarathy PT, Phillips O, Fukumoto J, Cho Y, Fukumoto I, Bao H, Cox R Jr, Galam L, Lockey RF, Kolliputi N. Dysregulation of CLOCK gene expression in hyperoxia-induced lung injury. *Am J Physiol Cell Physiol* 306: C999–C1007, 2014.
[63] Logan RW, Zhang C, Murugan S, O'Connell S, Levitt D, Rosenwasser AM, Sarkar DK. Chronic shift-lag alters the circadian clock of NK cells and promotes lung cancer growth in rats. *J Immunol* 188: 2583–2591, 2012.
[64] Sukumaran S, Jusko WJ, Dubois DC, Almon RR. Light-dark oscillations in the lung transcriptome: implications for lung homeostasis, repair, metabolism, disease, and drug action. *J Appl Physiol* 110: 1732–1747, 2011.
[65] Sundar IK, Yao H, Huang Y, Lyda E, Sime PJ, Sellix MT, Rahman I. Serotonin and corticosterone rhythms in mice exposed to cigarette smoke and in patients with COPD: implication for COPD-associated neuropathogenesis. *PLoS One* 9: e87999, 2014.
[66] Vasu VT, Cross CE, Gohil K. Nr1d1, an important circadian pathway regulatory gene, is suppressed by cigarette smoke in murine lungs. *Integr Cancer Ther* 8: 321–328, 2009.
[67] Wolff G, Duncan MJ, Esser KA. Chronic phase advance alters circadian physiological rhythms and peripheral molecular clocks. *J Appl Physiol* 115: 373–382, 2013.
[68] Yang G, Wright CJ, Hinson MD, Fernando AP, Sengupta S, Biswas C, La P, Dennery PA. Oxidative stress and inflammation modulate Rev-erbα signaling in the neonatal lung and affect circadian rhythmicity. *Antioxid Redox Signal* 21: 17–32, 2014.
[69] Evans JA, Davidson AJ. Health consequences of circadian disruption in humans and animal models. *Prog Mol Biol Transl Sci* 119: 283–323, 2013.
[70] Marcheva B, Ramsey KM, Peek CB, Affinati A, Maury E, Bass J. Circadian clocks and metabolism. *Hand Exp Pharmacol* 217: 127–155, 2013.
[71] Cavadini G, Petrzilka S, Kohler P, Jud C, Tobler I, Birchler T, Fontana A. TNF-α suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc Natl Acad Sci USA* 104: 12843–12848, 2007.
[72] . Sato S, Sakurai T, Ogasawara J, Takahashi M, Izawa T, Imaizumi K, Taniguchi N, Ohno H, Kizaki T. A circadian clock gene, rev-erbalpha, modulates the inflammatory function of macrophages through the negative regulation of ccl2 expression. *J Immunol.* 2014;192:407–417.
[73] Pekovic-Vaughan V, Gibbs J, Yoshitane H, Yang N, Pathiranage D, Guo B, Sagami A, Taguchi K, Bechtold D, Loudon A, et al. The circadian clock regulates rhythmic activation of the nrf2/glutathione–mediated antioxidant defense pathway to modulate pulmonary fibrosis. *Genes Dev.* 2014;28:548–560.
[74] Tanimura N, Kusunose N, Matsunaga N, Koyanagi S, Ohdo S. Aryl hydrocarbon receptor–mediated cyp1a1 expression is modulated in a clock-dependent circadian manner. *Toxicology.* 2011;290:203–207.
[75] Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in bmal1, the core componentof the circadian clock. *Genes Dev.* 2006;20:1868–1873.
[76] Yao H., Chung S., Hwang J. W., Rajendrasozhan S., Sundar I. K., Dean D. A., McBurney M. W., Guarente L., Gu W., Ronty M., Rahman I. (2012) SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. *J. Clin. Invest*. 122, 2032–2045

[77] Driehuis, E., & Clevers, H. (2017). CRISPR/Cas 9 genome editing and its applications in organoids. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *312*(3), G257–G265. https://doi.org/10.1152/ajpgi.00410.2016
[78] Niehof, M., Hildebrandt, T., Danov, O., Arndt, K., Koschmann, J., Dahlmann, F., Hansen, T., & Sewald, K. (2017). RNA isolation from precision-cut lung slices (PCLS) from different species. *BMC Research Notes*, *10*(1), 1–5. https://doi.org/10.1186/s13104-017-2447-6
[79] Stegmayr, J., Alsafadi, H. N., Langwiński, W., Niroomand, A., Lindstedt, S., Leigh, N. D., & Wagner, D. E. (2021). Isolation of high-yield and -quality RNA from human precision-cut lung slices for RNA-sequencing and computational integration with larger patient cohorts. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *320*(2), L232–L240. https://doi.org/10.1152/ajplung.00401.2020
[80] Akram, K. M., Yates, L. L., Mongey, R., Rothery, S., Gaboriau, D. C. A., Sanderson, J., Hind, M., Griffiths, M., & Dean, C. H. (2019). Live imaging of alveologenesis in precision-cut lung slices reveals dynamic epithelial cell behaviour. *Nature Communications*, *10*(1), 1–20. https://doi.org/10.1038/s41467-019-09067-3
[81] Heinzelmann, K., Lehmann, M., Gerckens, M., Noskovičová, N., Frankenberger, M., Lindner, M., Hatz, R., Behr, J., Hilgendorff, A., Königshoff, M., & Eickelberg, O. (2018). Cell-surface phenotyping identifies CD36 and CD97 as novel markers of fibroblast quiescence in lung fibrosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *315*(5), L682–L696. https://doi.org/10.1152/ajplung.00439.2017