PROJECT FINAL REPORT

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4.1 Final publishable summary report

Executive summary

Chronic obstructive pulmonary disease (COPD) is a lung disease caused by smoke (cigarettes, open fire) in susceptible individuals. The disease is of worldwide importance in that based on the most recent survey COPD is number four on the global mortality list.
The main objective of the present project is to define markers of **E**mphysema **v**ersus **A**irway disease (EvA) in COPD at the DNA, mRNA and protein level. This is based on the hypothesis that there are two distinct subphenotypes of COPD, i.e. emphysema and airway disease (bronchitis). The ten clinical partners of the EvA consortium have successfully recruited a total of 534 patients and 280 controls to the study. Computed tomography of the lung was done on these patients and a limited number of controls. These CT scans were analyzed by image analysis for the degree of emphysema and for the extent of airway disease. Patients and controls were then subjected to bronchoscopy in order to obtain epithelial cells and alveolar macrophages. The lung material was used to determine the transcriptome (all genes, which are expressed by the cells) and genes specifically expressed in emphysema and airway disease patients are currently being identified. These are then confirmed and the respective proteins are determined. Markers identified with this strategy can in the future be used for diagnosis and as targets for novel therapies.

Summary description

COPD is a chronic inflammatory disease of the lung. It is a global problem in that in 2004 it was the 4th leading cause of death worldwide (www.who.int/respiratory/copd/burden). The disease is related to smoking and in the developing world to exposure to indoor smoke from cooking fire. When patients with COPD avoid further exposure, e.g. when they stop smoking the disease process continues. The mechanisms driving this are unknown. There is, however, evidence to suggest a contribution of genetics to the COPD phenotype since only 15% of smokers develop COPD and since COPD runs in families (Silverman *Proc Am Thorac Soc.3:405, 2006)*.
Current therapies can improve lung function and reduce frequency of exacerbations but this has little impact on mortality (Calverly, *N Engl J Med. 2007;356:775*). Therefore new therapies based on an understanding of the mechanisms of disease are required.
The current ERS/ATS guidelines point out that in COPD there are two features:
"Chronic bronchitis is defined clinically as chronic productive cough for 3 months in each of 2 successive years in a patient in whom other causes of productive chronic cough have been excluded. Emphysema is defined pathologically as the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls and without obvious fibrosis. In patients with COPD either of those conditions may be present."

Both of these forms (or sub-phenotypes) of COPD lead to airway obstruction, which is due to collapse of the airways during expiration in emphysema and to inflammatory thickening of the airways in chronic bronchitis. Both of these distinct processes will lead to reduced forced expiratory volume in 1 second (FEV_1), which is the unifying feature of the sub-phenotypes in COPD. Emphysema and airway disease co-exist in the majority of patients but patients with only emphysema and patients with only inflammatory obstruction can be identified in about 10% of the COPD cases. This observation suggests that there may be two separate patho-physiological processes: one process is acting in the lung parenchyma and one in the airways. This is supported by evidence of differential biochemical parameters in COPD patients without and with emphysema (Boschetto et al, Thorax 61:1037, 2006).

The majority of studies into COPD have, however, not distinguished between the 2 pathologies. The current Emphysema versus Airway Disease study (EvA study) will focus on the analysis of these subphenotypes. Definition of these subphenotypes can be done via analysis of chest CTs as suggested by Nakano et al (Am J Resp Crit Care Med, 162:1102, 2000). Here emphysema is assessed via lung density and airway disease via the thickness of the airways and this approach has been used in the EvA study.

An important prerequisite for a successful project is to study a well-defined group of patients and controls. This requires exclusion of patients and controls with current infection, antibiotics, oral glucocorticoids and concomitant inflammatory diseases because these conditions will impact on gene expression and therefore will blurr the effects of the COPD inflammatory process. Therefore the EvA study puts emphasis on recruitment of stringently defined probands.

The inflammatory process in COPD in the airways and in the alveoli can best be studied by sampling the alveolar space via lavage and the airways via brush biopsy. This material can then be analysed for gene expression and for candidate proteins. Genome wide gene expression is done by high throughput strategies with RNA sequencing being the most sensitive and comprehensive approach and therefore is used for the EvA samples. Bioinformatic analysis is then used to link genes or groups of genes with COPD versus healthy controls and with subphenotypes of COPD. The genes, which are found to be associated with the E and A subphenotypes can then be studied in detail and the respective proteins can be determined. The results are expected to improve diagnosis and in the long run therapy of COPD.

Change of strategy:

The original strategy for the EvA project was to recruit patients, who are subjected to CT of the lung. Based on image analysis patients with emphysema-predominant and airway disease predominant disease were to be selected. These patients were to be offered bronchoscopy with the aim of obtaining material from 300 patients. Alongside 300 controls were to be recruited for bronchoscopy but no CT.

Also, DNA was proposed to be used for genome wide association studies (GWAS). In order to identify disease specific mutations it was planned to sequence 1 MB of DNA for 60 samples.

The new strategy was implemented by the consortium because of difficulties in patient compliance with a long waiting period between CT and bronchoscopy. The new steps were to recruit 500 patients, perform CT and offer bronchoscopy to all of the patients irrespective of the result of the CT image analysis. A recruitment-above-target was necessary because of a 20% drop-out rate, originally calculated to be only 10%. All of these changes required some reallocation of budget in order to cover the associated costs. In the genetic studies priority was given to transcriptome analysis using the powerful RNA sequencing approach on the epithelial cells and alveolar macrophages from the lung. In addition, with novel technology available, the consortium decided to perform whole genome sequencing on 21 samples from blood and lung.

Taken together the EvA study has been able to recruit a tremendous number of patients and controls to this invasive study and this will allow for informative analysis in a disease, which comes in different stages and consists of sub-phenotypes.

The ambitious targets required dedicated efforts by all partners of the project and it required extension of the recruitment period to 3 years and 3 months such that with recruitment complete only 3 months remained until the end of the funding period. Still the genetic assays with DNA and RNA sequencing and the protein assays have been executed, but the analysis and exploitation of the data is still ongoing.

Description of the main S&T results

Recruitment

534 patients and 280 controls have been recruited by the 10 clinical partners of the EvA project. The total patient number was more than projected (500) while for controls this was somewhat less (300). Stringent exclusion criteria (see also Deliverable 17; SOP 1-2) were applied to ensure that the COPD population is not contaminated by other diseases, which may affect the gene expression.

Table 1 Exclusion criteria for EvA study

COPD Stage IV, FEV1 < 1.0 L, LTOT*, age > 75	increased risk for bronchoscopy AEs
oral glucocorticoids	may attenuate inflammation
active smokers	induces acute inflammation
frequent severe exacerbations	may interfere with CT, bronchoscopy
age < 45, < 5 PY	other causes likely
> 80 PY	low susceptibility to COPD
AAT, IgG deficiency	will mimic COPD phenotype
post-bronchodilator FEV1 increase > 400 ml	asthma as cause of fixed obstruction
serious co-morbidities, anti-coagulation	precludes bronchoscopy
cardiac pacemaker implants	generates CT artefacts
major lung surgery	lung function test, CT is distorted

*long-term oxygen therapy

While many other studies into COPD can include any stage of the disease the EvA group is different because of the invasive bronchoscopy procedure. Because of that the most severe forms of COPD with very low FEV1 and oxygen levels and patients of older age are excluded from participation (Table 1). Also, since in EvA we study the inflammatory process in the lung, current smokers are excluded in order to avoid the smoke induced inflammation. Patients with frequent exacerbations leading to hospitalization are excluded, as well since the sequelae of such acute events may still impact on the inflammatory response at the time of bronchoscopy. With this approach we want to ensure that the markers, which we will identify in the COPD lung, are due to COPD and not to any other inflammatory process. We also exclude patients on oral glucocorticoids since these drugs may substantially reduce inflammation in the lung.

In order to focus on prototypic smoke induced COPD we exclude patients with low exposure (< 5 PY), excessive exposure (> 80 PY) and young age. Also, other diseases that lead to lung destruction like alpha-1-anti-trypsin deficiency and IgG deficiency are excluded. Patients with a pronounced asthmatic component (> 400 ml improvement of FEV1 post bronchodilation) are excluded since there is the possibility that the fixed obstruction in an ex-smoker is not due to COPD but is the result of chronic asthma. For control donors the same exclusion criteria apply, but asthma is allowed for.

These many exclusion criteria have made recruitment quite a challenge and many patients, who had been invited for analysis, had to be rejected after detailed interview and lung function analysis.
Lung function analysis was the crucial test for diagnosis with the requirement of a post-bronchodilator ratio < 0.7 for FEV1/FVC.
When EvA patients and controls are analysed for reversibility of the FEV1 we noted a substantial proportion in both groups, which had a substantial improvement by more than 200 mL (Table 2).

Table 2 Reversibility of FEV1 in EvA patients and controls

Reversibility with deltaFEV1	cases	controls
> 400 ml	14	n= 3
>200ml < 400 ml	170	n=55
>200ml	184	n=58

As can be seen there is quite a number of probands with a pronounced reversibility and this will have to be taken into account in the analysis of gene expression.
Patients can be classified via the extent of FEV1 reduction and there are two approaches: a) according to the GOLD criteria b) according to the lower limit of normal (LLN). There is some discussion on which approach should be used and therefore we have applied both approaches to our patient group.

The dominant grade is II in the EvA patients. When the LLN approach is taken, which essentially considers that with increasing age there is a decrease of lung function also in healthy individuals, then there is a decrease mainly in grade I group with mild disease.

In addition, the GOLD initiative has recently proposed to use a refined approach that takes into account the number of exacerbations and the dyspnea score. When applying this to the EvA patients then we find the majority of patients to have grade A.

Medication

For the analysis of gene expression in the lung material it is important which drugs are being used, since they can be expected to up- and downregulate certain genes. In the EvA cohort there is extensive use of inhaled GCs (yellow), even in patients with grade 1, a grade for which the guidelines do not recommend this type of drug. In most patients GCs are combined with long-acting bronchodilators. Patients receiving oral, systemic GCs were excluded in EvA such that major effects

of GCs (downregulation of inflammatory genes like TNF, upregulation of IL-10) should not be seen. Still inhaled GCs do have a clinical effect and can be expected to have a local effect. Therefore any GC induced transcript signature will have to be subtracted from the gene expression seen in patients and the sub-phenotypes.

Computed tomography scanning of the lung

After patients had passed visit 1 and had been included into the study, they were invited to have a CT. These scans were done according to specific protocols for determination of lung density and airway wall analysis.

The scans were recorded with a rotation time of 0.5 sec, a tube voltage of 120 kV, a tube current of 40 (EvA-E) or 50 mAs (EvA-A) and this dose was kept fixed for all patients [i]. The EvA-E protocol is optimal for density resolution at the expense of spatial resolution. Conversely for the EvA-A protocol pitch and speed are lower, which allows for a better spatial resolution for the airway analysis. This, however, goes along with a higher radiation dose. In order to keep the radiation exposure low the EvA-A scan covers a limited part of the lung, (including the right S1 segmental bronchus) such that this protocol comes at a dose of 0.42 mSv only. The total radiation exposure for both scans is 2.1 mSv. Approval had been obtained from the relevant radiation protection authorities. Specific settings were used for the different instruments.

Using these settings the EvA study has performed CT scans on 532 patients and on 34 control donors.

In order to normalize the images obtained from the 10 different centers we have used phantoms that were scanned at every one of the 10 scanners. These phantoms contained objects with different densities and tubings with different wall thickness (see Figure 1).

Figure 1

With reference to a standard scanner a correction factor for the readings from the different scanners was determined.

CT image analysis

Emphysema: All imaging series were analyzed using a semi-automated software program (Pulmo-CMS; Medis Medical Imaging, Leiden, Netherlands) by a single operator (DS). Analysis consisted of 3 parts: internal image calibration utilising measurements of air and blood density as previously described (Stoel BC, Stolk J. Optimization and standardisation of lung densitometry in the assessment of pulmonary emphysema. Invest Radiol 2004;39:681-688, Parr et al. Influence of calibration on densitometric studies of emphysema progression using computed tomography. Am J Respir Crit Care Med 2004;170:883-890.); semi-automated, threshold-based lung segmentation; derivation of lung densitometric indices from the voxel frequency distribution histogram, as previously described (Parr et al. Exploring the optimum approach to the use of ct densitometry in a randomised placebo-controlled study of augmentation therapy in alpha 1-antitrypsin deficiency. Respir Res 2009;10:75.). As a main read-out the percentile for the lowest 15% in lung density Perc15 (HU) is used. An illustration of the regions of low density is given in the left hand part of Figure 3 below.

Airways disease: Airway cross-sectional geometry of the right upper lobe apical bronchus (RB1) was determined with a semi-automated software program (Emphylx-J V 1.00.01 (6)) by a single operator (SG) utilising the full width at half maximum (FWHM) method (Nakano et al. Quantitative assessment of airway remodeling using high-resolution ct. Chest 2002;122:271S-275S). Following manual placement of a seed point in the airway lumen of RB1, 64-128 radial trajectories were cast across the airway wall and the boundaries of the airway wall were defined by the mid-point of the profile of CT numbers across each radial trajectory. Lumen area (LA) and wall area (WA) were measured as described previously (Gupta S,et al. Qualitative analysis of high-resolution ct scans in severe asthma. Chest 2009;136:1521-1528). The airway wall area is expressed as percent of the entire airway area. An example is given in the right hand image in Figure 2, below.

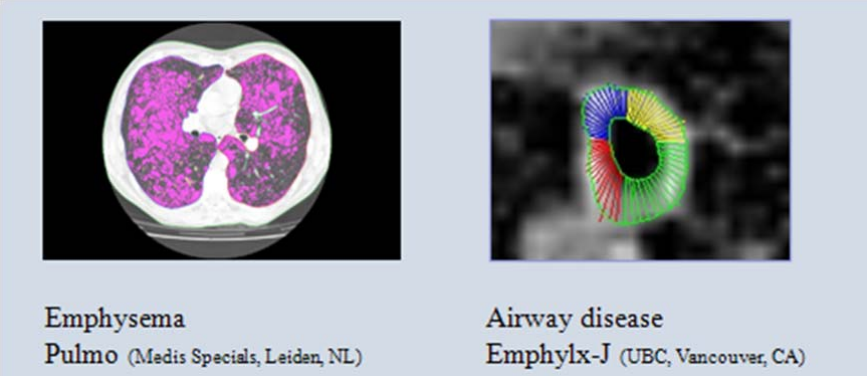

Figure 2

The results obtained for the EvA patients have demonstrated that it is possible to identify patients with emphysema-predominant disease and with airway predominant disease.

Bronchoscopy

Patients having undergone CT scanning were then invited for bronchoscopy. Here many patients withdrew their consent for this final step of the study. The main reason being the anxiety connected with this invasive approach. More than 100 such patients with CT did not go forward to bronchoscopy. Among controls, where CT was not part of the standard protocol, only 2 withdrew consent. The main reason being that the decision to undergo bronchoscopy had to be taken earlier in the study and control donors not willing to have a bronchoscopy were not included into EvA in the first place.

Bronchoscopy was done with a flexible instrument and mild sedation. Using a 5mm brush at bristle level (see Figure 4, left hand) bronchial epithelial cells were obtained from the right lung and lavage with up to 150 mL volumes was done in the left lung (see Figure 3, right hand) .

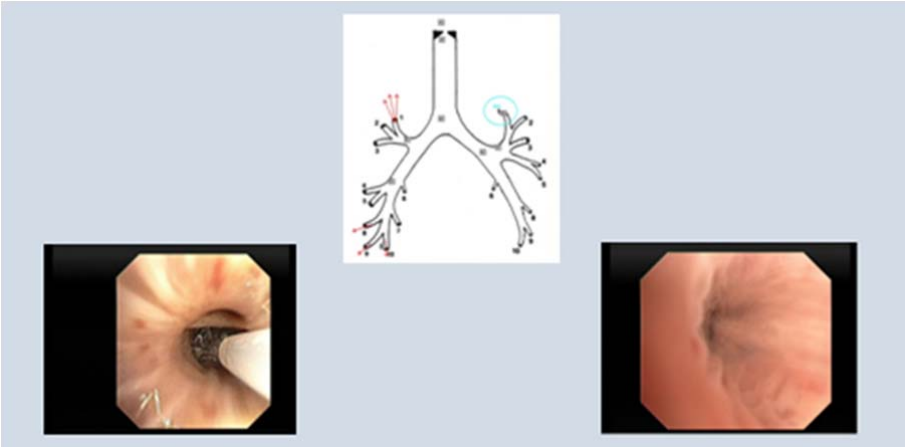

Figure 3

The total number of bronchoscopies for patients was 421 and for controls it was 278. Samples from these probands contain bronchial epithelial cells and alveolar macrophages (Fig 5). The material was resuspended in a fixative and aliquots are store at -80°C for later processing.

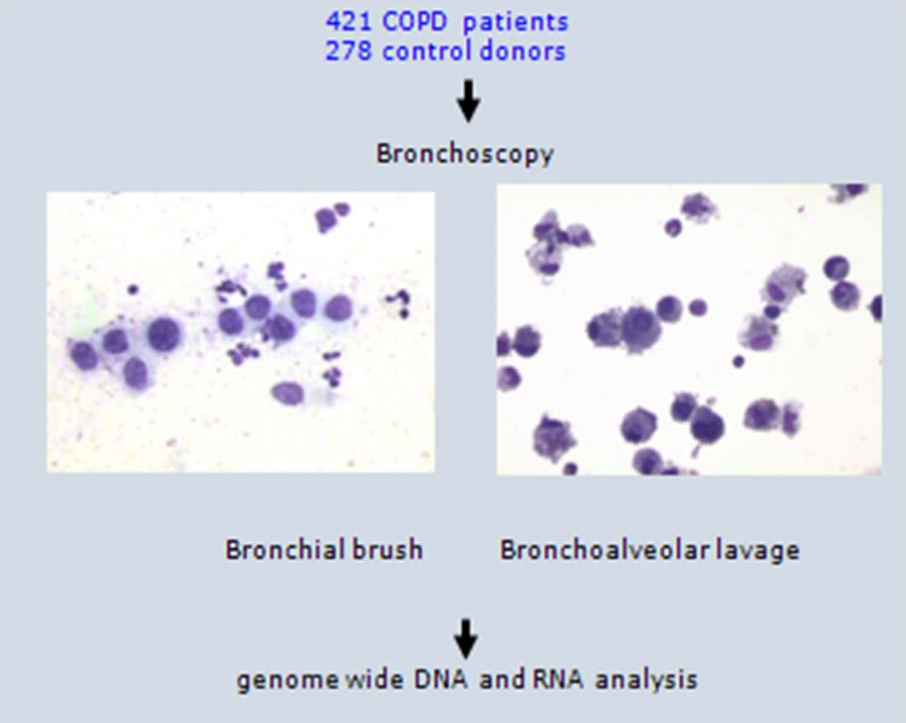

Figure 5

All processing of samples for DNA and RNA analysis was done by partner 9 (CNG, Paris). Whole genome sequencing of DNA from blood and lung epithelium from altogether 10 individuals was done by partner 15 (CNAG, Barcelona). This included 3 healthy never smokers, 3 healthy ex-smokers and 4 COPD ex-smokers. Sequencing was done as multiplexed paired- end run on Illumina Hiseq 2000 systems. Data were collected in an automated way, processed by GEM mapper and SAM tools for variant detection before being filtered for quality criteria and tabulated by R software. The data show that there are many somatic mutations in the epithelial cells and an impact of smoking and disease development is currently being studied.

For transcriptome analysis RNA was extracted and RNA integrity was determined. Samples with 1µg of total RNA or more and with an integrity level of 5 or above were subjected to RNASeq using the Illumina platform. Quality control of data included trimming and deletion of duplexes and transcripts with error rates >2. Data were mapped against the genome and a reference transcriptome. The transcripts were linked to NM numbers and gene names and the level was

expressed as rpkm (reads per kilobase per million mapped reads). Data from 900 samples are now being analysed against clinical and laboratory data and against the CT phenotypes.

Protein levels are being determined in fluids from the lung by partners 5 (Leiden) and 13 (Uppsala). This includes Eosinophil Cationic Protein (ECP), Eosinophil Protein X (EPX) and Myeloperoxidase (MPO), human neutrophil lipocalin (HNL), Lysozyme, Human Phospholipase B-II and sCEACAM8 as well as s well as neutrophil -defensins and the cathelicidin hCAP18/ LL-37.

- **The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results.**

Many studies into genetics of COPD mainly look at association with SNPs as determined in blood DNA. By contrast, the EvA project uses a unique approach not taken previously in that it studies gene expression in lung material obtained from patients and controls via bronchoscopy. Here we directly look at gene expression and we study the level of mRNA for the entire genome. We hypothesize that the gene expression in the lung is different in patients compared to controls. Also, we hypothesize that patients with the emphysema-dominant disease and patients with airway disease-dominant phenotype show a different pattern of gene expression.

In order to avoid that the patterns of gene expression are blurred by noise coming from interfering pathophysiological processes the patient group studied has been selected with stringent criteria. When our hypothesis proves correct then this will have a strong impact on our understanding of the COPD disease process. Also it will open new avenues for diagnosis. When we identify a set of markers associated with emphysema or with airway disease then we may be able to define these subphenotypes by a molecular rather than a clinical or lung function approach. Also, new assays for the respective proteins may be used for diagnosis.
Genes, which are expressed selectively in COPD and its subphenotypes, are very likely to play a role in the pathophysiological process. Therefore they can provide novel targets for therapy.
The potential for the results of the EvA project can be substantial and can impact on various steps in the management of patients with this disease of global relevance. This is relevant to cigarette smoke induced disease. However, it also applies to disease caused by inhalation of smoke from open cooking fires in badly ventilated dwellings. This is a common situation in third world countries, were the affected are mainly females.
The results of the EvA project will initially be disseminated through publication in international journals, be it major journals for the general readership or specialist journals for pneumology. One major paper is in press which describes the aims and strategies of the EvA project and this will be the point of reference for all the following publications (Ziegler-Heitbrock et al, The EvA study: aims and strategy, European Respiratory Journal, in press). We expect a set of about 30 publications coming from this project directly and this includes manuscripts that are currently being written like papers on the CT scanning approach, on the classification of patients, on whole genome sequencing and on proteins in lung fluids.
Dissemination will also include patient groups and this will be through the European Lung Foundation and through presentations given to the patient groups. The general public will be informed via press releases.

Exploitation includes the publication of scientific articles as detailed above and if applicable patents on unique markers and assay kits will be applied for. Also, follow-up project are underway in the area of CT image analysis (AirPROM FP7 grant # 270194) and are being planned for protein assay development and for validation of the novel markers identified in EvA.

The address of the project public website The project web site can be found under http://www.eva-copd.eu/eva/english/ and it uses the following logo:

The web site has been used mainly to assist in recruitment of probands for the EvA project. It now will be used as a platform to inform the public about the relevant novel findings of the project.