Appendix I – Rennard RFA-HL-12-22

Prostaglandin Inhibition for Emphysema
(PIE)
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Abstract

The Prostaglandin Inhibition for Emphysema (PIE) study will determine if a currently available therapy, ibuprofen 600 mg three times daily, can block PGE production in the lower respiratory tract and if this results in improvement in measures of lung repair function. This is a proof-of-concept study. The PIE study will set the stage for novel therapy to modify the course of chronic obstructive pulmonary disease (COPD).

COPD is the third leading cause of death in the United States. No currently available treatment can meaningfully restore lung function that is lost in this disease. Emphysema is a major component to COPD and results when lung damage exceeds the ability of the lung to repair. Recent evidence indicates that the repair processes present in the normal lung are deficient in patients with emphysema and that this is due, in part, to suppression of repair by an inflammatory mediator: prostaglandin E (PGE). Currently available therapies can block PGE production, but whether this can be achieved in the lung in COPD is unknown. The PIE study will answer that question.

This will be accomplished by performing a randomized, double blind, placebo-controlled, parallel group study that will compare a widely used and well-tolerated non-steroidal anti-inflammatory drug, ibuprofen 600 mg three times daily, with placebo. PGE will be measured directly in the lower respiratory tract by sampling the lung with the technique of bronchoalveolar lavage. Secondary measures will be made, quantifying PGE in induced sputum and quantifying PGE metabolites in blood and urine. In addition, the current proposal will determine if biochemical measures of lung repair are restored by treatments that block PGE production. Additional outcomes will also be assessed including the effect of treatment on PGD and other eicosanoids and assessing IL-8 and neutrophils in sputum and BAL fluid and selected inflammatory biomarkers present in serum that may be associated with lung function decline. Finally, in an accompanying Ancillary Study, the current proposal will determine if alveolar macrophages over-produce PGE and/or PGD in COPD and will determine if the microRNA miR-146a plays modulates the production of these prostaglandins, as we have demonstrated for lung fibroblasts. The Ancillary Study will also determine if genetic variation in a miR-146a is related to differential expression.

The proposed research will, therefore, determine if inhibition of PGE production can be achieved in the lung, if this appears to restore lung repair mechanisms and will help determine who would benefit from such a therapeutic approach. This is a highly novel approach to the treatment of emphysema and has the potential to restore lost lung function, a crucial unmet medical need for a major public health problem.

1. Background and Rationale

1.1 COPD and emphysema. COPD is currently the third leading cause of death in the United States[1]. It is characterized by progressive loss of lung function that can result either from small airways disease or from emphysema. Current therapy is largely symptomatic. To date, medical treatments have been without meaningful effect in slowing lung function loss[2-4].

Smoking is the most important etiologic factor leading to COPD. Smoking cessation improves symptoms, particularly those of bronchitis. If cessation is achieved early enough, disease progression is slowed[5]. However, once disease is sufficiently advanced, progression occurs despite cessation[6,7]. This may be particularly true for emphysema[7,8], which leads to the concept that, in susceptible individuals, smoking initiates a process leading to the development of emphysema and that the process remains active despite subsequent cessation.

Abundant animal and human data support the concept that inflammation, which is induced by smoking, leads to lung damage and the formation of emphysema[3,9]. To have tissue loss and emphysema, however, damage must exceed the capacity of the lung to repair.

Several lines of evidence support the concept that compromised tissue repair also contributes to the pathogenesis of emphysema[10].

1.2 Tissue repair and emphysema. Inadequate tissue maintenance due to loss of growth factors has been demonstrated to lead to emphysema in animal models[11]. Similarly, severe calorie restriction, sufficient to induce weight loss, can cause emphysema in animals[7,12,13]. Emphysema has been reported in young non-smokers with bulimia[7,14] and in young individuals who died from starvation[7,15]. Finally, in animal models, inhibition of repair mechanisms greatly worsens emphysema[16-20]. Thus, it is likely that impaired repair can contribute to the development of emphysema, both as a sole risk factor and as a co-factor potentiating the effects consequent to tissue damage.

In addition, lung repair following the development of emphysema can be induced in animal models. Retinoic acid, which drives the formation of alveolar septa during neonatal development, can induce formation of new alveolar wall in emphysema models in the mouse and rat [21,22]. In addition, growth factors, including hepatocyte growth factor (HGF) [23,24], granulocyte colony stimulating factor (G-CSF) [25], KGF [26] and adrenomedulin [27,28] have all been reported to stimulate new alveolar wall formation following the development of emphysema.

Augmentation of repair mechanisms in the lung, therefore, has the potential to both slow the development of emphysema and restore lost function. Improved repair within the lung is a reasonable target, as recent evidence had demonstrated that lung repair mechanisms are actively INHIBITED by a pharmacologically targetable mechanism. Specifically, repair processes in fibroblasts, the major lung mesenchymal cells responsible for the production and maintenance of extracellular matrix, are inhibited by the inflammatory mediator prostaglandin E (PGE), which is produced in greater amounts by lung fibroblasts and is present in the lower respiratory tract at higher concentrations than in controls. Inhibition of PGE production, therefore, has the potential to restore lung fibroblast repair functions. This, in turn, could improve the

balance of destruction and repair, thus slowing lung function loss and potentially restoring lost function.

Several lines of investigation have demonstrated reduced repair functions in cells cultured from the lungs of patients with emphysema. Compared to fibroblasts from controls, fibroblasts cultured from the lungs of patients with emphysema proliferate more slowly[29,30], are more sensitive to inhibition by smoke[31] and fail to express elastin mRNA[32] and the growth factors HGF and KGF when stimulated[33]. We have demonstrated that COPD fibroblasts are deficient in their chemotactic response and in their ability to contract collagen matrices[34]. These differences persist with passage in culture, consistent with an alteration in cellular phenotype in COPD.

1.3 Prostaglandins, tissue repair and emphysema. *In vitro* human cell culture studies. We have also demonstrated a key role for PGE in mediating the reduced repair function in fibroblasts from emphysema patients. PGE is an inflammatory mediator that is a potent inhibitor of fibroblast-mediated repair responses[35]. A role for PGE in the modulation of tissue remodeling in the lung has been suggested in idiopathic pulmonary fibrosis[36,37]. In this condition, PGE levels are reduced in the lower respiratory tract, and this has been suggested to contribute to excessive fibroblast-mediated repair and fibrosis[37,38]. The converse may contribute to emphysema. As detailed below, PGE levels are increased in the lungs of COPD patients[39-41], which would be expected to inhibit fibroblast repair functions. In addition, we have shown that fibroblasts from COPD patients over-produce PGE and that inhibition of fibroblast production of PGE can partially correct their repair defects *in vitro*[34].

Animal studies. Animal studies also support the potential for inhibition of PGE production as a therapy for emphysema. COX inhibition can reduce smoke-induced inflammation in the mouse[42]. More importantly, inhibition of PGE production in smoke-exposed rats results in reduced severity of emphysema[43].

Human studies. PGE levels. Although published data are limited, three studies have demonstrated increased PGE levels in the lower respiratory tract in COPD. Monuschi et al. reported increased PGE levels in exhaled breath condensate of subjects with COPD[39]. Chen et al.[40] and Profita et al.[41] have reported increased PGE levels in induced sputum from patients with COPD. Chen also reported a significant relationship between PGE levels and reduced FEV_1, although the number of subjects evaluated, 45, was relatively small, resulting in large confidence intervals in the estimated relationship. While these studies demonstrate increased PGE in the lung, they do not obviously assess the alveolar compartment. However, we have utilized the technique of bronchoalveolar lavage (BAL), which does sample the alveolar space. Moreover, we have used the method of fractional BAL processing [44]. When 20 ml aliquots are sequentially infused and immediately aspirated, the first aliquot is relatively enriched for bronchial material, while the subsequent are enriched for alveolar material. By separately processing these samples, partial separation of alveolar and bronchial samples can be obtained. With this method, we demonstrated that “bronchial” samples have higher PGE concentrations than “alveolar” but that for both, COPD patients have increased PGE compared to normals (Figure 1). This study thus confirms the published results and specifically demonstrates that alveolar PGE concentrations are increased

compared to control. In addition, even though the number of subjects in our study was also limited, we also confirmed the association of PGE level with FEV1 (p=0.0285).

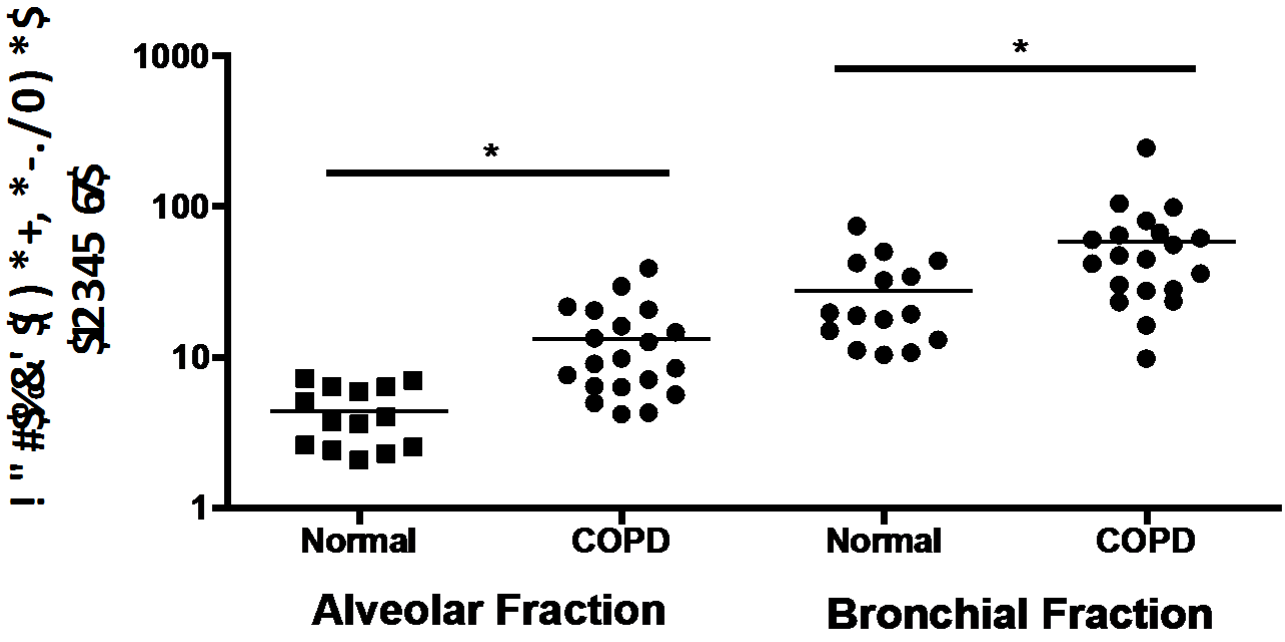

Figure 1. Bronchoalveolar lavage was performed as described[44,45] with five serial 20 ml aliquots, which allows partial separation of the "alveolar" and "bronchial" contents. Levels were four- to five-fold higher in the bronchial fractions. BAL PGE concentration was greater in both alveolar and bronchial fractions in patients with COPD. Note that the vertical axis is a log scale. *p<0.05.

The cellular source(s) of the increased PGE in COPD is(are) undetermined. We have demonstrated that fibroblasts cultured from the lungs of patients with COPD produce more PGE than do fibroblasts from control subjects[34,46]. In addition, PGE production is inversely related to airflow and to the diffusion capacity, which is a measure of the severity of emphysema. Importantly, inhibition of PGE production in the COPD fibroblasts partially restores repair functions[34], confirming the role of PGE and suggesting that improving repair functions is a reasonable therapeutic goal.

We also demonstrated that COPD fibroblasts under-express the microRNA miR-146a[46]. This microRNA targets the PGE biosynthetic enzyme cyclo-oxygenase-2 (COX-2). We demonstrated that under-expression of miR-146a leads to over-expression of COX-2 and contributes to over-production of PGE in COPD fibroblasts, thus establishing its mechanistic role[46]. Detailed sequencing of the miR-146a gene from 40 fibroblast strains (17 controls and 23 COPD patients), including 5000 bp of the 5' regulatory region, exons 1 and 2 and 2000 bp of the 3' untranslated region revealed that polymorphisms in the gene were relatively common. We found 24 polymorphisms, 23 of which were novel, which had a minor allele frequency greater than 5%. Importantly,

fibroblasts with the promoter polymorphism miR146a/-3147AG/GG under-expressed miR-146a compared to cells with the AA genotype. In a population of 280 COPD subjects and controls, this same promoter polymorphism was found to be associated with a decreased FEV1/FVC ratio (p=0.01). Interestingly, this polymorphism affected a putative AP1 transcription factor binding site at miR146a/-3147A that was disrupted with risk allele G. These pilot data, therefore, strongly support the concept that polymorphisms in the miR-146a gene may be related to COPD [47].

Both fibroblasts and alveolar macrophages are major sources of PGE in the lung[35,48,49]. Whether macrophages contribute to the increased production of PGE in COPD patients will be addressed in the Ancillary Study (see Ancillary Study). In addition, whether miR-146a modulates PGE production in alveolar macrophages and whether polymorphisms in the miR-146a gene predict PGE levels in the lung and production by alveolar macrophages will also be assessed. Thus, the Ancillary Study will advance understanding of the mechanisms that underlie increased PGE levels in the lower respiratory tract in COPD. In addition, the Ancillary Study will explore a potentially diagnostic biomarker (miR-146a gene polymorphisms) that has the potential to identify individuals at risk for increased PGE levels.

1.4 NSAIDs, PGE inhibition and emphysema, observational studies.

Currently available medications can inhibit PGE production. PGE is derived from membrane phospholipids in a three-step process[50]. First, a phospholipase A2 cleaves arachidonic acid from phospholipid. Second, arachidonic acid is oxidized to form PGG then PGH by a cyclo-oxygenase (COX). Finally, PGH is converted to PGE by a prostaglandin E synthase (PGES). Several distinct enzymes can mediate each step. In addition, at each step, other enzymes can produce alternate products.

The most commonly used inhibitors of PGE production are COX inhibitors, sometimes termed non-steroidal anti-inflammatory drugs (NSAIDs)[50]. Non-selective agents that inhibit both COX enzymes, COX-1 and COX-2, and agents that are selective for COX-2 are in use clinically. They differ in their adverse side effects, which are mediated by several mechanisms. These include inhibition of COX in cells other than the desired target cell, effects due to inhibition of COX- derived mediators other than PGE and, possibly, to shunting of substrate to alternate products. More selective inhibition of PGE production is possible as specific inhibitors of PGE synthases have been developed, although none are available clinically. Which PGE synthase(s) are most important in COPD, however, is as yet undetermined. Thus, it is possible that a selective PGE synthase inhibitor may be of use clinically. However, non-selective inhibition of PGE production provides a more robust test for the proof-of-concept that PGE can be targeted to treat emphysema. Interestingly, while limited, several observational studies have related NSAID use to features of COPD, and these studies provide observations supporting the concept that NSAIDs may reduce the severity of COPD in general and emphysema specifically.

McKeever and colleagues reviewed the NHANES dataset and observed that users of the non-selective NSAID ibuprofen had significantly better lung function, i.e. higher FEV1, than did non-users[51]. Improved lung function was observed among occasional users of ibuprofen. No clear dose response effect was observed in this trial, in part because there were relatively few daily users. Observational studies with a

therapy will be substantially confounded by selection bias: those using NSAIDs on a regular basis are likely to differ in a number of respects from those who use occasionally or not at all. Nevertheless, the observation that ibuprofen use is associated with better lung function provides suggestive information supporting the premise of the current proposal.

The improved lung function associated with ibuprofen observed by McKeever and colleagues was not observed with acetaminophen[51]. While acetaminophen is also a non-selective COX inhibitor, it does not have a marked anti-inflammatory effect. This is thought to be due to blockade of its COX inhibitory effect by high concentrations of peroxides present in an inflammatory milieu[50]. Since persistent inflammation is a characteristic feature of the lung in COPD, it is likely that acetaminophen would be less effective than ibuprofen.

The ECLIPSE study is an observational study that included 2165 COPD subjects who underwent spirometry of whom 1729 had CT scans[52]. Many reported NSAID use on entry. Consistent with the results reported by McKeever[51], NSAID use was marginally associated with better lung function assessed as post-bronchodilator FEV1 (p=0.09). Because CT scans were done, it was possible to assess emphysema. Patients with COPD had greater emphysema than controls, as reflected by lower lung density (data not shown). Emphysema in COPD patients was more marked in the upper lobes, as would be expected (Figure 2). Data from ECLIPSE also suggest that NSAID use may ameliorate the development of emphysema. Specifically, NSAID users had significantly greater lung density suggesting less emphysema bilaterally in the lower lobes (Figure 2).

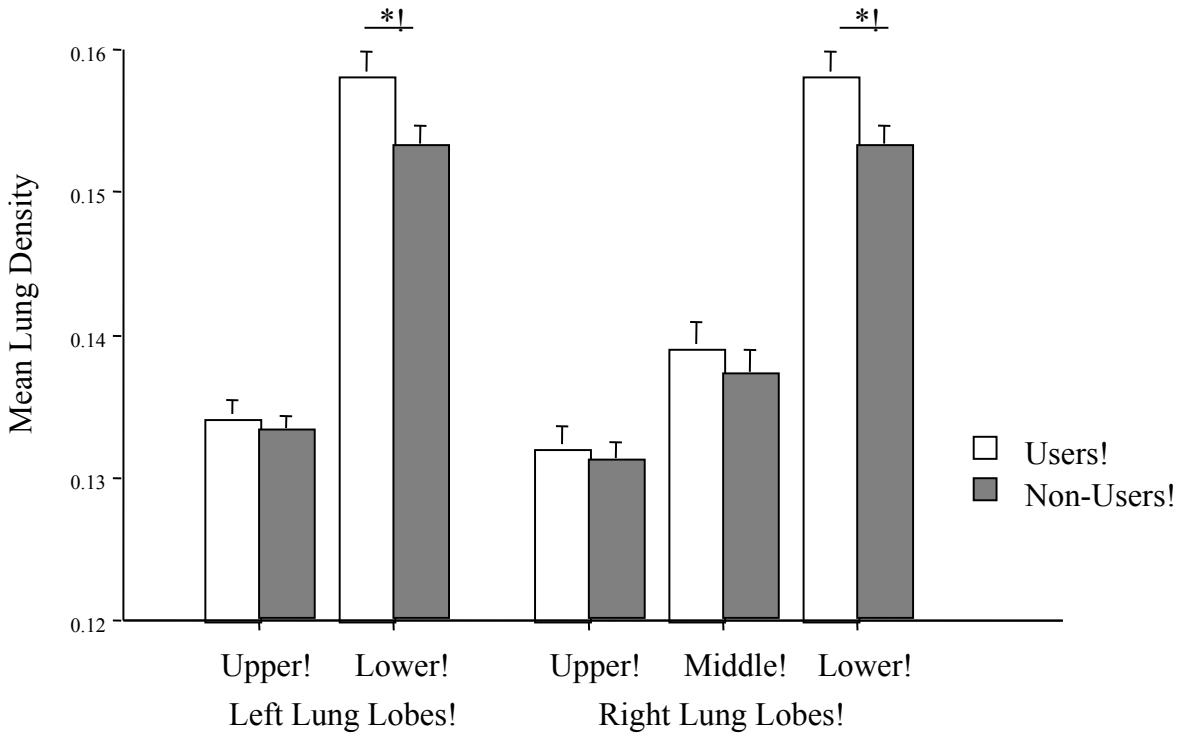

Figure 2. Use of NSAIDs is associated with reduced lower lobe emphysema. Data were evaluated from the ECLIPSE study[52]. Quantitative CT scan was

performed on 1729 subjects enrolled in the trial. At the time of entry, 427 reported using NSAIDs on a regular basis. CT scan density was quantified as a measure of emphysema[53-55]. Those who reported using NSAIDs (clear bars) had significantly greater density (less emphysema) in the lower lobes than those not using NSAIDs (grey bars). * $p < 0.05$.

The mean age of COPD subjects of all severities in ECLIPSE was about 63 years. While it is likely that NSAID use began several years before enrollment, it is highly likely that cigarette smoking and the development of emphysema preceded the use of NSAIDs by several decades. Thus, an effect of NSAIDs on the lower lobes, where emphysema is less severe, is consistent with an effect in mitigating an early stage in the disease pathogenesis. This might be the expected effect of improving repair, which may be able to sustain or repair lung structure in the face of mild injury, but may be much less effective in the presence of severe lung destruction.

1.5 *In vivo* assessment of lung fibroblast repair activity. The studies noted above demonstrate that PGE levels are increased in COPD, that fibroblast repair processes are inhibited by COPD, that inhibition of PGE production can improve fibroblast repair functions *in vitro* and can mitigate emphysema in animal models. The current proposal is a proof-of-concept study designed to determine if inhibition of PGE can be achieved in the lung of patients with emphysema as a means to restore repair functions. This concept can be tested by quantification of PGE levels following use of treatments that can inhibit PGE production and that are available for clinical use. It is also possible, in this proof-of-concept study, to obtain information that inhibition of PGE production can restore repair functions.

Collagen is a major component of extracellular matrix, and fibroblasts are the major source[56,57]. Collagen is synthesized as a precursor, procollagen, that has terminal non-triple helical regions. These must be cleaved so that the triple helical portion can form mature collagen fibers, a process that takes place after collagen is secreted into the extracellular space. Because of this, the procollagen terminal peptides can serve as a marker of collagen synthesis. This biomarker has proved very useful in gauging bone synthesis[58-60]. It has also proved useful as a measure of collagen production in the lung. A number of investigators[61-63], including ourselves[64,65], have demonstrated increases in procollagen peptide associated with interstitial lung disease, which is believed to reflect increased collagen production. Similar increases have been reported following the development of ARDS[66-68].

Measurement of procollagen peptide, therefore, can provide a measure of fibroblast activity. Inhibition of PGE production would be predicted to increase collagen production and generation of procollagen peptides. To demonstrate the feasibility of this approach, we quantified one of the procollagen peptides, pro-collagen I C-terminal peptide in the alveolar fraction of BAL from COPD patients and controls. This study not only confirms the ability to quantify pro-collagen peptide in BAL fluid of COPD patients, but levels in COPD patients were significantly reduced compared to controls, consistent with the concept of inhibited repair (Figure 3).

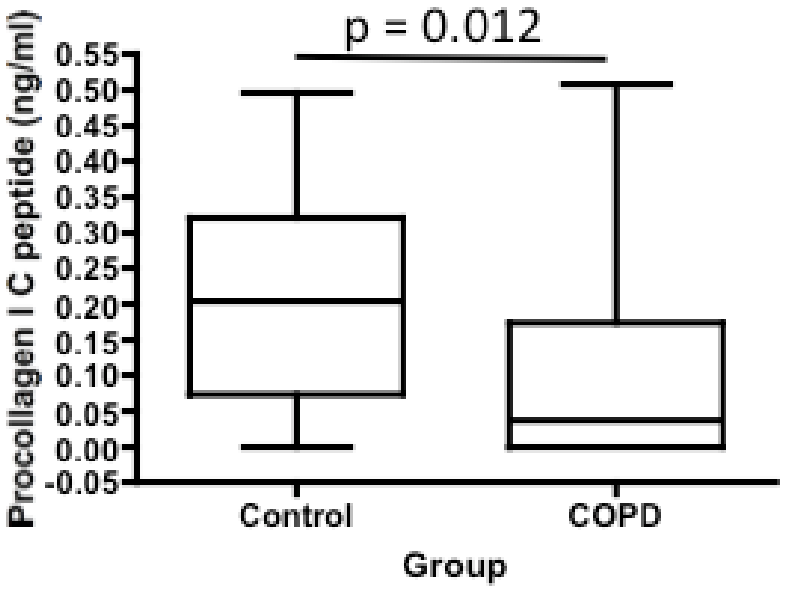

Figure 3. Pro-collagen I C-terminal peptide levels in COPD. BAL samples (alveolar fraction) from COPD subjects and normal controls were evaluated for pro-collagen I C-terminal peptide by ELISA. Levels were significantly lower (Mann-Whitney, two-tailed, p=0.012) in the COPD subject BAL fluids compared to control, although there was considerable overlap. Values were undetectable in a substantial number of subjects (this does not affect the rank order non-parametric statistical test used to compare the groups).

1.6 PGE and inflammation in COPD. COPD is an inflammatory disease characterized by increased numbers of macrophages and neutrophils in the lower respiratory tract[3]. Associated with these cells, several chemotactic factors, including interleukin-8 (IL-8), are also increased in COPD[69,70]. PGE is an inflammatory mediator that is increased in COPD. Interestingly, we have observed that PGE levels are significantly related to IL-8 levels in both the bronchial and alveolar portions of BAL fluid (Figure 4).

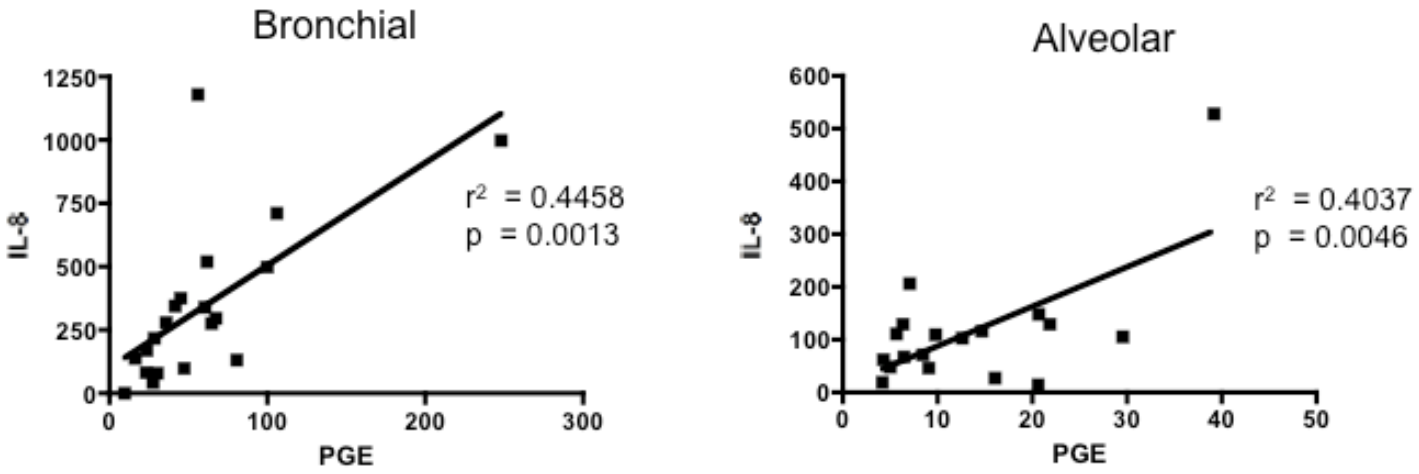

Figure 4. Bronchoalveolar lavage PGE and IL-8 levels. BAL was performed in patients with COPD with 5 X 20 ml aliquots as described[44,45] and the first aliquot, enriched for bronchial material, processed separately from the subsequent four aliquots, enriched for alveolar material. PGE and IL-8 levels are higher in the bronchial portion than the alveolar, but correlate significantly in both portions.

One interesting possibility is that increased PGE levels in COPD may contribute, in part, to driving the increased levels of IL-8. In support of this concept, we have demonstrated that PGE can stimulate IL-8 production from fibroblasts (Figure 5).

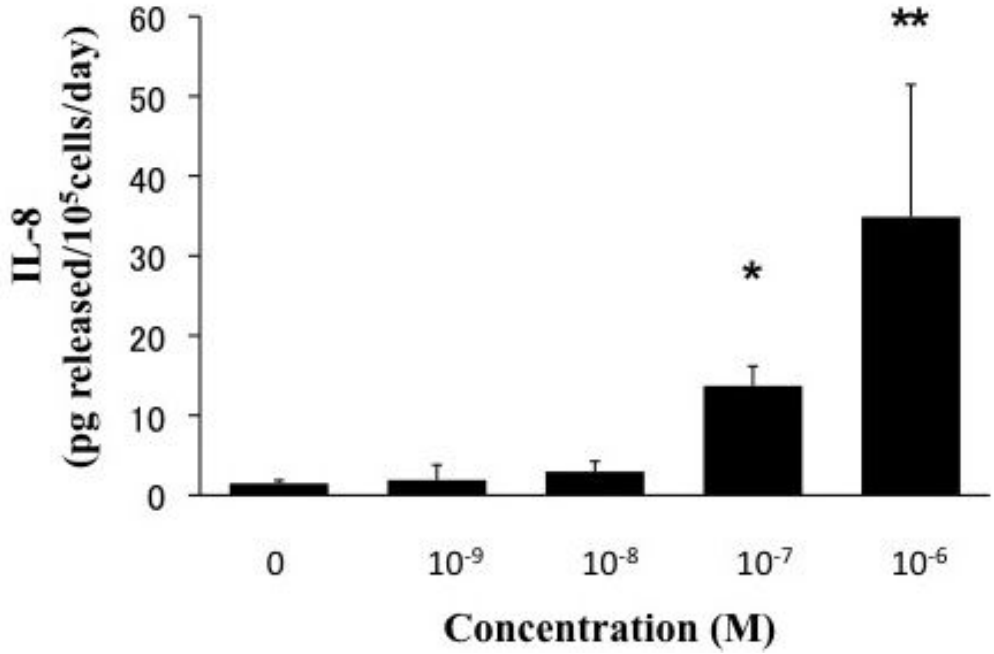

Figure 5. PGE stimulates IL-8 production by human lung fibroblasts. Human lung fibroblasts (HFL-1) were cultured to confluence *in vitro* and stimulated with PGE for 24 hours. PGE stimulation resulted in a concentration-dependent induction of IL-8 production. Similar results were obtained with adult lung fibroblasts. *p<0.05; **p<0.01.

While the current study will focus on restoration of repair functions, reduction in inflammation could also be beneficial in COPD, and its demonstration would be a measure of a pharmacodynamic drug effect. Moreover, reduced inflammation can provide an independent measure of proof-of-concept that PGE inhibition could be beneficial in emphysema.

1.7 Other prostaglandins. Prostaglandins other that PGE may also play a role in COPD. Prostaglandin D2 (PGD) is present in the lower respiratory tract and has been related to recruitment of inflammatory cells including lymphocytes and eosinophils[71,72]. IBlockade of the D-prostaglandin (DP) receptor reduced inflammation in smoke exposed mice[73]. PGD also has the potential to modulate fibroblast-mediated repair responses [74,75]. Whether PGD or other prostanoids might also contribute to altered repair in emphysema is unknown. Montuschi et al. observed no difference in PGD levels in exhaled breath condensate in contrast to the increased levels of PGE that were observed [76]. The current study will determine if PGD and other prostanoid levels are altered in the lungs of COPD patients using BAL and induced sputum.

1.8 Choice of interventional drug. Drug choice. Several considerations have influenced the choice of interventional drug for the current proof-of-concept study, which is designed to determine if PGE production can be inhibited effectively in the lower respiratory tract and if this is associated with biomarkers suggestive of improved tissue repair and reduced inflammation. To this end, a non-selective COX inhibitor is more likely to be effective, as both COX enzymes may play a role. While subsequent studies may demonstrate advantages for more selective approaches, the approach used is appropriate for the initial proof-of-concept study. In this regard, a variety of NSAIDs that vary in their selectivity for COX-1 or COX-2 are in clinical use[50]. COX-2 selective agents have reduced risk of gastrointestinal side effects, but are associated with an increase in cardiac events.

Ibuprofen is a widely used, non-selective COX inhibitor. It is not associated with an increased risk for cardiac events[77,78], although risks may be greater among individuals with a history of myocardial infarction[79]. Ibuprofen can cause gastritis, but monitoring for this adverse event is possible by following symptoms, stool blood and hemoglobin levels. Naproxen is also a non-selective COX inhibitor that is slightly more selective for COX-1. It has a reduced cardiovascular risk compared to COX-2 selective agents, but an increased risk of gastrointestinal side effects[79,80]. It has a much longer half-life than ibuprofen and is used clinically on a twice-daily regimen. In contrast, ibuprofen is often dosed four times daily. However, twice daily dosing with ibuprofen has been used in cystic fibrosis and has resulted in significant lowering of inflammatory measures[81] and slowed deterioration in lung function[82,83]. In addition, the effect of conventional doses of ibuprofen has been assessed on BAL levels of PGE; in contrast, to our knowledge, there are no data on the effect of naproxen in the lung. For these reasons, ibuprofen has been selected for use in the current short-term, proof-of-concept trial.

Dose. Like most NSAIDs, ibuprofen is a competitive inhibitor of arachidonic acid oxidation[50]. As such, at clinical doses, NSAIDs do not completely prevent the formation of eicosanoids, and their degree of inhibition is concentration dependent. After single doses of 400 and 600 mg, ibuprofen resulted in a 10 and 15% reduction in the blood level of the PGE metabolite 13,14-dihydro-15-keto-PGE 2 (PGEM)[84]. The effects may be greater with chronic dosing or with assessments over longer time frames. McAnulty and colleagues assessed marathon runners who were chronic users of ibuprofen compared to non-users[85]. Although chronic dosing was not controlled, users took 600 mg the evening before and 200 mg the morning of a race and then 200 mg every four hours. Urinary levels of PGEM were about 50% of the levels observed in non-users both before and after running (treatment effect $p=0.016$)[85].

Pulmonary effects. Effects of ibuprofen may be greater in the lung. Hazucha and colleagues measured PGE levels in bronchoalveolar lavage (BAL) fluid before and after exercise in normal men while breathing ozone[86]. Pretreatment with 800 mg ibuprofen followed by 200 mg during the exercise reduced PGE levels by 60% ($p<0.05$)[86]. Similarly, in a COPD population, Montuschi et al. reported a 77% reduction ($p<0.0001$) in exhaled breath condensate PGE following treatment with 400 mg ibuprofen every six hours for two days[87].

Because of its anti-inflammatory effect, ibuprofen has been tested in patients with cystic fibrosis. Reduction in the rate of decline of FEV1 has been observed in two placebo-controlled trials[82,83]. Both studies employed relatively high doses of ibuprofen (20-30 mg/kg), but the drug was administered twice daily. Konstan and colleagues have also demonstrated a relationship between neutrophil recruitment *in vivo* and blood levels[81]. With twice daily administration, reduction in neutrophil recruitment was observed in both normal volunteers and cystic fibrosis patients, but only when the Cmax exceeded 50 ug/ml. None of these studies quantified eicosanoid production.

Rationale for current study design. The current study is a proof-of-concept study to demonstrate inhibition of PGE production and associated biological effects in the lower respiratory tract. Because ibuprofen is a competitive inhibitor, at conventional doses, it only partially inhibits PGE production. Nevertheless, at doses that are within usual clinical practice, reduction in lower respiratory tract PGE levels by greater than 50% seems a reasonable target[85-87]. In addition, at these doses, ibuprofen has been observed to reduce the rate of lung function loss in cystic fibrosis. It is likely that the effect in cystic fibrosis is due to an effect on airways inflammation. While not the primary goal of the present trial, this provides evidence that is helpful in confirming the choice of dose and in establishing tolerability for treatment in patients with lung disease and provides support for an important secondary outcome measure.

Based on this prior experience, the current trial will use ibuprofen at a dose of 600 mg three times daily vs. placebo. The primary analysis will be by intention-to-treat. However, blood levels will be determined after four weeks of use one to two hours after dosing. This will not be used as a measure of compliance, as ibuprofen has a short half-life. However, it will be used in a secondary analysis to evaluate variable absorption or metabolism, which may be particularly important in a proof-of-concept trial. Because of the logistical complexity that would require dummy adjustments in the placebo and the generally good response to clinical doses of ibuprofen, a dose adjustment strategy was not felt warranted.

1.9 Subject identification and recruitment. A major limitation to the study of interventions affecting repair in emphysema has been access to subjects with relatively limited disease. Because COPD is diagnoses relatively late in the course, and because emphysema is not specifically detected by spirometry, individuals with mild disease are most often undiagnosed and, in most COPD patients, the extent of emphysema is unknown. The current study will specifically enroll subjects with emphysema who do not have severe or very severe COPD. This has several important theoretical and practical advantages. First, it is likely that improvement in repair can mitigate emphysema progression more if the disease is not markedly advanced. In addition, restoration of lost structure and function may be more readily achieved if abnormalities are not severe. That is, it may be easier to fix a mild anatomic defect rather than a severe one. The current study is possible because of the opportunity of accessing a previously identified cohort of appropriate study subjects from the COPDGene Study.

COPDGene (NCT00608764) is an NIH-funded study of more than 10,000 subjects who have undergone lung function measurement, CT scanning and collection of blood for DNA analysis[88]. Subjects have provided consent to be recontacted, and several studies have been initiated that utilize this cohort (e.g. NCT01253473). The four clinical centers participating in the current proposal are all COPDGene centers. The anticipated number of subjects at each center with emphysema (>5% of voxels <-950 Hounsfield Units) for each level of COPD severity is shown in Table 1. A telephone screen of 10 subjects was able to contact nine, and seven of these expressed interest in participation. Thus, the available population of 919 potential subjects should represent an adequate pool of potential subjects for the current proposal. If needed, it would be possible to augment these numbers with GOLD 3 subjects.

Table 1. Potential Subject at Each Participating Site

	Number of subjects with >5% emphysema (<-950 Hounsfield Units)				
Center	**GOLD 0**	**GOLD 1**	**GOLD 2**	**GOLD 3**	**GOLD 4**
National Jewish	23	48	168	206	130
Harbor	5	15	20	36	35
Harvard	18	18	48	35	15
Temple	61	26	83	108	70
Totals	**106**	**108**	**329**	**385**	250

Total number of potential subjects (GOLD 0, 1,2 and 3) = 919

Another advantage of recruiting COPDGene subjects is that, when possible, study protocols will use COPDGene methods. This will greatly simplify training and quality control, which have already been implemented at each study site as part of COPDGene.

1.10 Summary of background and preliminary data supporting the concept of the current proposal. COPD is the third leading cause of death in the United States. It progresses despite smoking cessation, and this appears to be particularly true for emphysema. No currently available therapy has been demonstrated to meaningfully slow disease progression or to restore lost lung function. The destruction of lung tissue that characterizes emphysema results from tissue damage that exceeds the capacity of tissue repair. Both play roles in emphysema. Importantly, impaired mesenchymal cell, i.e. fibroblast, repair functions result, in part, from over-production of PGE. *In vitro* and animal studies suggest that inhibition of PGE can restore repair functions to COPD fibroblasts from human patients and can ameliorate the development of emphysema in animal models. In addition, data from human observational studies suggest that the use of NSAIDs, which can inhibit PGE production, is associated with reduced severity of emphysema. PGE, moreover, may contribute to the chronic inflammation that characterizes COPD, the inhibition of which could be of therapeutic benefit. Moreover,

pro-collagen peptide, a biochemical measure that reflects tissue repair, has been used in several clinical settings, including lung disease, and its use to gauge fibroblast-mediated repair activity in emphysema is practicable. Finally, the appropriate population for a proof-of-concept study, individuals with definite emphysema but with mild or moderate COPD, has been identified.

Taken together, these observations suggest that inhibition of PGE production has the potential to slow the development of emphysema and potentially may allow tissue repair to correct anatomic defects and that a proof-of-concept study is readily feasible.

2. Objectives and hypotheses

2.1 Primary clinical objective. Previous studies support the concept that impaired lung repair contributes to the pathogenesis of emphysema and that PGE present in the lower respiratory tract inhibits repair function of lung mesenchymal cells, i.e. fibroblasts. The primary goal of the current proposal is to test, as a proof-of-concept, that inhibition of PGE production will reduce PGE levels in the lower respiratory tract of patients with emphysema and will this lead to increased biochemical markers of tissue repair. This will be tested by intention-to-treat comparing the ibuprofen-treated subjects with placebo treated subjects and is essential to demonstrate prior use of PGE inhibition strategies as therapy to slow disease progression or to restore lung function in emphysema. Several secondary questions will also be pursued.

2.2 Clinical study hypotheses

Primary hypothesis:

H1: Will ibuprofen, 600 mg three times daily, decrease PGE concentrations in the alveolar portion of BAL fluid in subjects with emphysema in comparison to placebo?

H2: Will ibuprofen, 600 mg three times daily, increase pro-collagen peptide fragment concentrations in the alveolar portion of BAL fluid in subjects with emphysema in comparison to placebo?

Other hypotheses:

Effects on PGE

H3: Will ibuprofen, 600 mg three times daily, reduce prostaglandin E concentrations in the alveolar portion of BAL fluid in subjects with emphysema to those present in smoking, former smoking and healthy non-smoking controls?

H4: Will ibuprofen 600 mg three times daily, reduce prostaglandin E concentrations in the bronchial portion of BAL fluid in subjects with emphysema compared to placebo?

H4.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H5: Will ibuprofen 600 mg three times daily, reduce prostaglandin E concentrations in induced sputum from subjects with emphysema compared to placebo?

H5.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H6: Will ibuprofen 600 mg three times daily, reduce the PGE metabolite PGEM in the peripheral blood of subjects with emphysema compared to placebo?

H6.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H7: Will ibuprofen 600 mg three times daily, reduce the PGE metabolite PGEM in the urine of subjects with emphysema compared to placebo?

H7.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

Effects on biomarkers of repair

H8: Will ibuprofen, 600 mg three times daily, increase pro-collagen peptide fragment concentrations in the alveolar portion of BAL fluid in subjects to the range observed in normal individuals or to higher levels?

H9: Will ibuprofen 600 mg three times daily, increase the procollagen III amino peptide excreted into the urine of subjects with emphysema compared to placebo?

Effects on inflammation

H10: Will ibuprofen, 600 mg three times daily, decrease IL-8 concentrations in the alveolar portion of BAL fluid in subjects with emphysema in comparison to placebo?

H10.1: Will levels be reduced to those observed in former smoking and healthy non-smoking controls?

H11: Will ibuprofen, 600 mg three times daily, decrease neutrophil concentrations in the alveolar portion of BAL fluid in subjects with emphysema in comparison to placebo?

H11.1 Will levels be reduced to those observed in former smoking and healthy non-smoking controls?

H12: Will ibuprofen 600 mg three times daily, reduce IL-8 concentrations in the bronchial portion of BAL fluid in subjects with emphysema compared to placebo?

H12.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H13: Will ibuprofen 600 mg three times daily, reduce IL-8 concentrations in induced sputum from subjects with emphysema compared to placebo?

H13.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H14: Will ibuprofen 600 mg three times daily, reduce PMN concentrations in the bronchial portion of BAL fluid in subjects with emphysema compared to placebo?

H14.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H15: Will ibuprofen 600 mg three times daily, reduce PMN concentrations in induced sputum from subjects with emphysema compared to placebo?

H12.1 Will levels reduce to those present in former smoking and healthy non-smoking controls?

H16: Will ibuprofen 600 mg three times daily, alter LTB4 concentrations in subjects with emphysema compared to placebo?

H16.1: Alveolar portion of BAL

H16.2: Bronchial portion of BAL

H16.3 Induced sputum

H17: Will ibuprofen 600 mg three times daily, reduce concentrations of CC-16 in serum from subjects with emphysema compared to placebo?

H18: Will ibuprofen 600 mg three times daily, reduce concentrations of CRP in serum from subjects with emphysema compared to placebo?

H19: Will ibuprofen 600 mg three times daily, reduce concentrations of SP-d in serum from subjects with emphysema compared to placebo?

Other prostanoids

H20: Are levels of PGD increased in the alveolar component of BAL fluid in patients with COPD.

H20.1 Are levels of PGD in alveolar lavage fluid related to FEV1

H20.2 Are levels of PGD in alveolar lavage fluid related to severity of emphysema

H 21. Are levels of TXB2 (the primary metabolite of thromboxane) increased in the alveolar component of BAL fluid in patients with COPD.

H21.1 Are levels of TXB2 (the primary metabolite of thromboxane) in alveolar lavage fluid related to FEV1

H21.2 Are levels of TXB2 (the primary metabolite of thromboxane) in alveolar lavage fluid related to severity of emphysema

H22: Are levels of 6kPGF1a (the primary metabolite of prostacyclin) increased in the alveolar component of BAL fluid in patients with COPD.

H22.1 Are levels of 6kPGF1a (the primary metabolite of prostacyclin) in alveolar lavage fluid related to FEV1

H22.2 Are levels of 6kPGF1a (the primary metabolite of prostacyclin) in alveolar lavage fluid related to severity of emphysema

H23: Will ibuprofen 600 mg three times daily, reduce the eicosanoids other than PGE in subjects with emphysema compared to placebo in BAL and induced sputum?

H23.1 PGD

H23.2 TXB2 (the primary metabolite of thromboxane)

H23.3 6kPGF1a (the primary metabolite of prostacyclin) and LTB4 in the bronchial and alveolar portions of BAL

Exploratory clinical measures

H24: Will ibuprofen 600 mg three times daily alter the rate of change of emphysema progression as assessed by CT scan?

H25: Will ibuprofen 600 mg three times daily alter the rate of change of DLCO?

H26: Will ibuprofen 600 mg three times daily alter the rate of change of FEV1?

H27: Will ibuprofen 600 mg three times daily alter the rate of COPD exacerbations?

H28: Will ibuprofen 600 mg three times daily, alter health status assessed by the SGRQ or COPD status assessed by the CAT in subjects with emphysema

Emphysema phenotype

H29: Are there differences between subjects with upper lobe vs. lower lobe emphysema

H29.1 Do PGE levels in BAL fluid, sputum and metabolites in blood and urine differ between subjects with upper lobe vs. lower lobe emphysema

H29.2 Do procollagen peptides differ in BAL fluid between subjects with upper lobe vs. lower lobe emphysema

H29.3 Do their eicosanoids (PGD, 6kPGF1a, TXB2) urine differ between subjects with upper lobe vs. lower lobe emphysema

H29.4 Do measures of inflammation differ between subjects with upper vs. lower lobe emphysema

H29.5 Is the response to 600 mg ibuprofen three times daily different in subjects with upper lobe vs. lower lobe emphysema

H29.5.1 Response of PGE

H29.5.2 Response of measures of repair

H29.5.3 Response of other eicosanoids

H29.5.4 Response of measures of inflammation

H30: Are there differences between subjects with clustered vs. diffuse emphysema

H30.1 Do PGE levels in BAL fluid, sputum and metabolites in blood and urine differ between subjects with clustered vs. diffuse emphysema

H30.2 Do procollagen peptides differ in BAL fluid between subjects with clustered vs. diffuse emphysema

H30.3 Do the eicosanoids (PGD, 6kPGF1a, TXB2) urine differ between subjects with clustered vs. diffuse emphysema

H30.4 Do measures of inflammation differ between subjects with upper vs. lower lobe emphysema

H30.5 Is the response to 600 mg ibuprofen three times daily different in subjects with clustered vs. diffuse emphysema

H30.5.1 Response of PGE
H30.5.2 Response of measures of repair
H30.5.3 Response of other eicosanoids
H30.5.4 Response of measures of inflammation

Note: for all questions evaluating the effect of ibuprofen, a secondary analysis will be performed to determine the relationship with individual subject ibuprofen levels.

Progression of COPD

H31: Will BAL PGE levels assessed at study initiation be related to the rate of emphysema progression determined by the change in CT quantified lung density from the time of assessment in COPDGene to the current study (estimated 3-6 years)?

H32: Will BAL PGE levels assessed at study initiation be related to the rate of emphysema progression determined by the change in FEV1 from the time of assessment in COPDGene to the current study (estimated 3-6 years)?

H33: Will measures of prostanoids other than PGE assessed at study initiation be related to the rate of emphysema progression determined by the change in CT quantified lung density from the time of assessment in COPDGene to the current study (estimated 3-6 years)?

H34: Will measures of inflammation assessed at study initiation be related to the rate of emphysema progression determined by the change in FEV1 from the time of assessment in COPDGene to the current study (estimated 3-6 years)?

2.3 Ancillary Study Objectives. Prior studies have shown that fibroblasts from patients with COPD over-produce PGE, that this is due to under-expression of the microRNA miR-146a and that genetic polymorphisms may contribute to miR-146a under-expression. The Ancillary Study will determine if alveolar macrophages contribute to PGE over-production in emphysema and will determine if miR-146a plays a role in these cells. We will also determine if the other major prostanoid produced by alveolar macrophages, PGD, is over-produced in COPD and if miR-146a modulates its production.

Ancillary Study Hypotheses

Primary Hypothesis

AH1: Do alveolar macrophages from patients with emphysema over-produce PGE compared to alveolar macrophages from controls?

Secondary Hypotheses

AH2: Do alveolar macrophages from patients with emphysema over-produce PGD compared to alveolar macrophages from controls?

AH3: Does miR-146a expression differ in alveolar macrophages from emphysema subjects compared to controls?

AH3.1 Are alveolar macrophage miR-146a levels related to macrophage PGE production?

AH3.2 Are alveolar macrophage miR-146a levels related to macrophage COX-2 mRNA and protein expression levels?

AH4: Does miR-146a modulate COX-2 mRNA and protein expression and PGE production in alveolar macrophages?

AH5: Does miR-146a modulate COX-2 mRNA and protein expression and PGE production in alveolar macrophages?

AH6: Are polymorphisms in the miR-146a gene, including nearby regulatory sequences and the large intron, related to miR-146a expression in alveolar macrophages?

AH7: Are the polymorphisms related to COX-2 mRNA and protein expression?

AH8: Are the polymorphisms related to PGE production?

3. Study design

3.1 Overview. The Prostaglandin Inhibition for Emphysema (PIE) trial is a proof-of-concept trial designed to determine if a conventional dose of a well-tolerated NSAID can reduce PGE levels in the lower respiratory tract of subjects with emphysema. The study is a randomized, placebo controlled, parallel group study that will compare ibuprofen, 600 mg three times daily, with placebo, administered for 12 months. The primary outcome will be PGE levels quantified in the alveolar portion of BAL fluid. The primary comparison will be intention-to-treat comparing the difference between pre- and post-treatment in the treated group to pre- and post-treatment in the placebo group.

One hundred forty emphysema subjects will be randomized at a 1:1 ratio (70 subjects in each group). This based on a sample size estimate based on preliminary data from our center and from the literature that 52 subjects with paired BAL samples in each group would provide 90% power to detect a 50% reduction in PGE levels at a significance level of 0.05, estimates that we believe are conservative. To have 52 evaluable subjects with paired BAL samples in each group, 70 randomized subjects in each group, 140 total subjects, will be required if there is a 25% drop-out rate, an estimate we believe conservative.

The secondary objective is to determine if ibuprofen 600 mg three times daily compared to placebo increases lower respiratory tract pro-collagen peptide levels. Conditional power analyses suggest our sample size will have a 69% power to detect a standardized effect size of 0.485 (which was the difference observed in pilot studies), comparing the effect of treatment to that of placebo and a 92% power to detect a difference of treatment before to after within the treated group.

Additional objectives are to determine if treatment with ibuprofen alters PGE concentration in induced sputum, if there are changes in other eicosanoids, in particular PGD, and eicosanoid metabolites in BAL, sputum, blood and urine and, by using previously collected data required for subject enrollment, if PGE, other eicosanoid and measures of inflammation levels are related to the rate of emphysema progression. Control subjects will also be assessed on one occasion so that levels in the emphysema subjects before and after treatment can be compared to control. Control subjects will not receive treatment with ibuprofen. Finally, the primary goals will be achieved by assessing subjects with bronchoscopy and BAL after 3 months of treatment. Subjects will continue to receive randomized treatment for a total of 48 months. At the end of that time, emphysema assessed by CT-scan, lung function and health status will be assessed to provide an estimate of effect size that can help with the design of Phase 3 efficacy studies.

The study will be conducted at least three clinical centers including: Temple University Hospital, Philadelphia, National Jewish Health, Denver and Harbor/UCLA Hospital, Los Angeles. All three centers were participants in COPDGene. The University of Nebraska Medical Center (UNMC) will serve as the Data Coordinating Center (DCC). Samples will be shipped to the DCC where they will be banked and where the biochemical and cellular study-specific analyses will be performed. Clinical tests required for subject enrollment and to assure safety for bronchoscopy will be performed at the study sites. National Jewish Health (NJH), which served as the radiology reading center for COPDGene, will serve as the radiology reading center.

Annonomized CDs with the CT data will be shipped to NJH. Where possible, standardized procedures adopted by COPDGene will be used.

All emphysema subjects will be prior COPDGene participants or other subjects who have had CT scans demonstrating emphysema as determined by the radiology center at NJH. Smoking and former control subjects will also be COPDGene participants or subjects who have had a CT scan reviewed and accepted by the radiology center at NJH. No non-smokers were enrolled in COPDGene. For this reason, 20 healthy non-smoking non-COPDGene subjects will also be recruited.

After randomization, subjects will be treated for 48 weeks. following which they will be reassessed.

Because of the invasive nature of the BAL, an interim analysis with a formal futility stopping rule is include to minimize unnecessary exposure of study subjects to risk.

Figure 6. Study visits

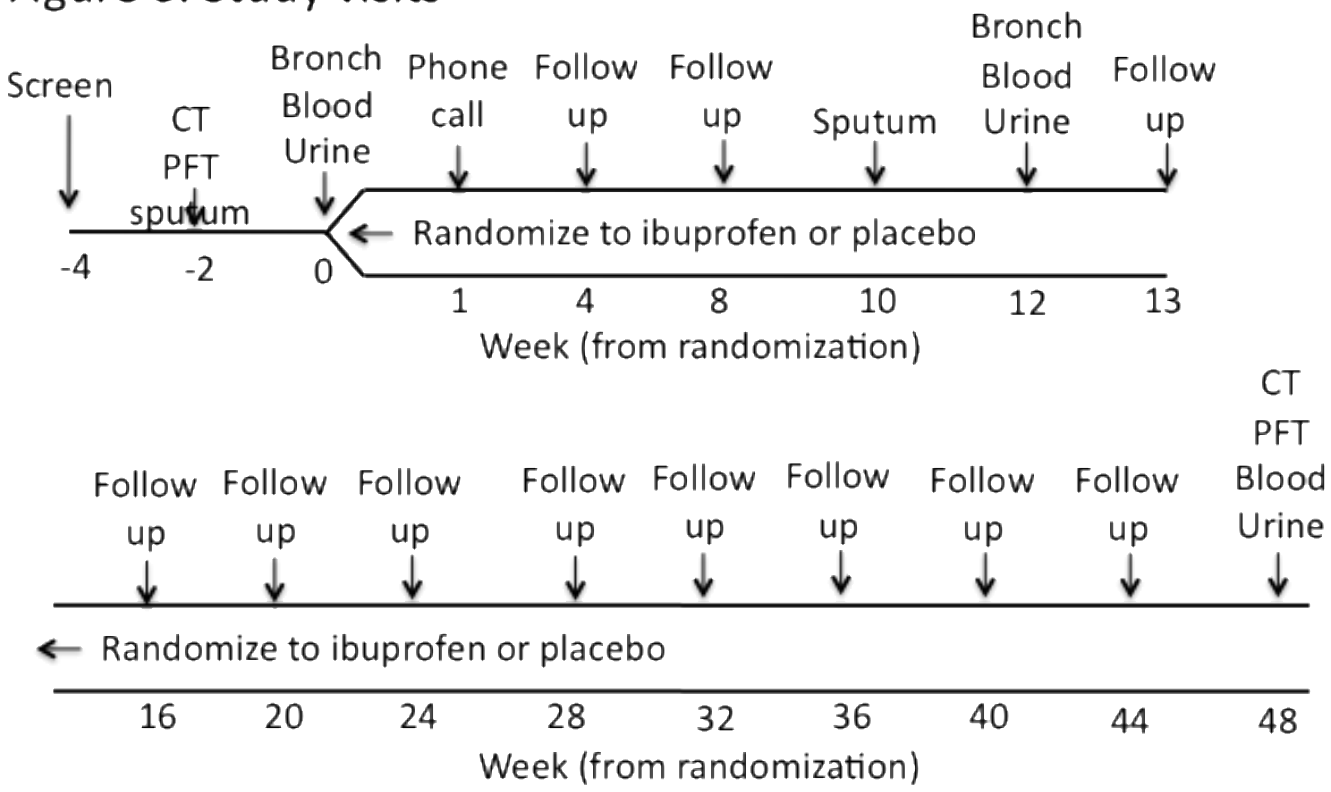

Figure 6. Schematic time line of key study events.

The study will include 18 visits (Figure 6)

Visit 1: Screening will include a history and physical, demographics, stool for occult blood, blood chemistry, CBC, spirometry, DLCO, SGRG and CAT.

Visit 2 (within two weeks of screening): CT scan, induced sputum

Visit 3 (2-3 weeks after visit 2): Bronchoscopy, blood and urine for biomarkers; randomize to ibuprofen or placebo

Visit 4 (phone call): follow-up after one week of medication

Visit 5* (4 weeks after randomization): follow-up including stool for occult blood

Visit 6* (8 weeks after randomization): follow-up including stool for occult blood

Visit 7* (10 weeks after randomization): sputum

Visit 8* (12 weeks after randomization, at least 2 weeks after visit 7): Bronchoscopy, blood and urine for biomarkers

Visit 9* (1 week after bronchoscopy phone call): follow up

Visit 10*-17* (every 4 weeks from week 16 to week 44 after randomization) follow-up including stool for occult blood

Visit 18* (48 weeks after randomization): CT-scan, blood and urine for biomarkers, spirometry, D_LCO, SGRG and CAT.

*Emphysema subjects only.

3.2 Enrollment criteria, emphysema subjects. At each study site, emphysema subjects will be recruited from prior COPDGene subjects or from other subjects who have had CT scans demonstrating emphysema. All must have consented to be recontacted for additional studies. All must meet the following criteria

Age > 45 years

Emphysema (>5% of voxels <-950 Hounsfield Units determined on a CT scan quantified at the COPDGene radiology center, i.e. NJH)

Post-bronchodilator FEV1 > 35% predicted

Smoker or ex-smoker (10 pack years minimum)

Subjects who meet the entry criteria will be contacted by phone. If interested in participating, subjects will be informed of the following exclusion criteria by phone:

Contraindication to bronchoscopy or other study procedures

Pregnancy or plans to become pregnant within six months

Aspirin-sensitive asthma

Regular use of systemic glucocorticoid

Regular use of an NSAID

Unstable medical condition

History of myocardial infarction or unstable angina within six months

Allergy to or history of adverse effect from ibuprofen or other NSAID

History of gastrointestinal bleeding within one year

Any condition that, in the opinion of the investigator, places the subject at untoward risk

Inability to provide informed consent

Subjects who meet the entry criteria and who believe they do not have any exclusions and who are interested in participating will be invited to the study site.

3.3 Study visits, emphysema subjects

Visit 1. Day 0.
Informed consent. Consent will be obtained by a study physician who is qualified to explain all study procedures including bronchoscopy.
Medical history
Physical examination
Clinical laboratory
Blood chemistry panel including Na, K, Cl, HCO3, albumin, glucose, creatinine, BUN
CBC
Urine pregnancy test (women only) to be repeated in serum if positive
Stool occult blood assessment
Spirometry, pre- and post-bronchodilator
DLCO
SGRQ
CAT
Smoking history

Confirmation that entry criteria are met and that no exclusions are present; confirm that subject continues to meet study criteria by spirometry
Schedule CT Scan, induced sputum and bronchoscopy

Visit 2. (can be two separate days, but CT scan must precede sputum. To be performed 0-14 days after screening)
CT scan: this will not be repeated is an acceptable CT scan has been performed within 1 year. A CT scan will be deemed acceptable if the radiology center confirms that emphysema can be quantified.
Induced sputum

Visit 3. (two to three weeks after visit 2)
Subjects will be called within one week of visit to review schedule and instructions for bronchoscopy.
Review of study and procedure and consent for bronchoscopy and BAL
Limited interval history and physical
Collect 3 tubes of blood for research study. Tube 1 (DNA); Tube 2 (serum); Tube 3 (plasma)
Urine for biomarkers
Bronchoscopy and BAL
Distribute study medication*
Call one day after procedure to assure post bronchoscopy recovery

*Subject randomization will be determined by the DCC. This will be done in blocks for each site. Study medication will be prepared by a vendor (tentatively the Temple University Pharmacy) and shipped to study sites with a subject number-specific code. Subjects will be given acetaminophen for PRN use.

Visit 4. (one week +/- 4 days after randomization)
Phone call to check use of study medication, potential problems

Visit 5. (four weeks +/- one week after randomization)
Call one week and one day prior to visit to remind about appointment
Visit to occur 1-2 hours after morning dose of study medication
Interval history
Stool for occult blood
Distribute study medication
Collect 1 tube of blood for ibuprofen level

Visit 6. (eight weeks +/- one week after randomization)
Call one week
Interval history
Stool for occult blood
Distribute study medication
Schedule induced sputum

Visit 7. (10 weeks +/- four days after randomization)
Induced sputum

Visit 8. (12 weeks +/- 1 week after randomization, at least 2 weeks after visit 7)
Subjects will be called within one week of visit to review schedule and instructions for bronchoscopy
Review of study and procedure and consent for bronchoscopy and BAL
Limited interval history and physical
Urine pregnancy test (women only) to be repeated in serum if positive
Collect 2 tubes of blood for research study. Tube 1 (serum); Tube 2 (plasma).
Urine for biomarkers
Bronchoscopy and BAL
Call one day after procedure to assure post-bronchoscopy recovery

Visit 9. (one week +/- 4 days after visit 8)
Phone call follow-up

Visit 10-17. (Every 4 from week 16 until week 44 after randomization).
Clinical assessment
Stool for occult blood
Distribute study medication

Visit 18 (48 weeks after randomization)
Clinical assessment
Stool for occult blood
CT-scan
Spirometry (pre and post bronchodilator)

DLCO
SGRQ
CAT
Blood (serum and plasma) for biomarkers
Urine for biomarkers

At each visit, all study information will be recorded in the study binder. All fields in the study report forms must be completed for each visit. Sample inventory forms must be completed when samples are initially stored. Sample shipping forms must be completed when samples are shipped, which will be batchwise.

3.4 Enrollment criteria smoking and ex-smoking control subjects. At each study site, smoking and ex-smoking control subjects will be recruited from prior COPDGene subjects or other subjects that have had a CT scan reviewed and accepted by the radiology center at NJH. All must have consented to be recontacted for additional studies. All must meet the following:

Age > 45 years

No emphysema (<3% of voxels <-950 Hounsfield Units determined on a CT scan quantified at the COPDGene radiology center, i.e. NJH)

Normal lung function (pre bronchodilator FEV1/FVC > 0.7 and FEV1 > 80% predicted)

Smoker or ex-smoker (10 pack years minimum).

Subjects who meet the entry criteria will be contacted by phone. If interested in participating, subjects will be informed of the following exclusion criteria by phone:

Contraindication to bronchoscopy or other study procedures
Pregnancy or plans to become pregnant within six months
Aspirin-sensitive asthma
Regular use of systemic glucocorticoid
Regular use of an NSAID
Unstable medical condition
History of myocardial infarction or unstable angina within six months
Allergy to or history of adverse effect from ibuprofen or other NSAID
History of gastrointestinal bleeding within one year
Any condition that, in the opinion of the investigator, places the subject at untoward risk
Inability to provide informed consent

Subjects who meet the entry criteria and who believe they do have any exclusions and who are interested in participating will be invited to the study site.

3.5 Entry criteria healthy non-smoker controls. Because COPDGene does not include healthy non-smokers, these subjects will be recruited de novo. Subjects must be healthy without a chronic medical problem that requires treatment and without

having had an acute medical problem requiring treatment within six months. In addition, all must be non-smokers (less than 100 cigarettes per lifetime) and must meet the following criteria:

Age > 45 years
Normal lung function (pre bronchodilator FEV1/FVC > 0.7 and FEV1 > 80% predicted); lung function criteria will be established at the screening visit

and have none of the exclusions that apply to other subjects:

Contraindication to bronchoscopy or other study procedures
Pregnancy or plans to become pregnant within six months
Aspirin-sensitive asthma
Regular use of systemic glucocorticoid
Regular use of an NSAID
Unstable medical condition
History of myocardial infarction or unstable angina within six months
Allergy to or history of adverse effect from ibuprofen or other NSAID
History of gastrointestinal bleeding within one year
Any condition that, in the opinion of the investigator, places the subject at untoward risk
Inability to provide informed consent

CT scan must show no emphysema (<5% Housfield units). If emphysema is present, a subject will be excluded as a normal non-smoking control.

3.6 Study visits, control subjects

Visit 1. Day 0
Informed consent. Consent will be obtained by a study physician who is qualified to explain all study procedures including bronchoscopy.
Medical history
Physical examination
Clinical laboratory
Blood chemistry panel including Na, K, Cl, HCO3, albumin, glucose, creatinine, BUN
Urine pregnancy test (women only); followed by serum pregnancy test if positive
CBC
Stool occult blood assessment
Spirometry, pre- and post-bronchodilator
EKG
DLCO
SGRQ
CAT
Smoking history

Confirmation that entry criteria are met and that no exclusions are present; confirm that subject continues to meet study criteria by spirometry
Schedule CT Scan, induced sputum and bronchoscopy

Visit 2. (can be two separate days, but CT scan must precede sputum. To be performed 0-14 days after screening.)
CT scan
Induced sputum

Visit 3. (2-3 weeks after visit 2)
Subjects will be called within one week of visit to review schedule and instructions for bronchoscopy.
Review of study and procedure and consent for bronchoscopy and BAL
Limited interval history and physical
Collect 3 tubes of blood for research study. Tube 1 (DNA); Tube 2 (serum); Tube 3 (plasma).
Urine for biomarkers
Bronchoscopy and BAL

Call one day after procedure to assure post-bronchoscopy recovery

Visit 4. (one week +/- 4 days after visit 3)
Phone call follow-up

At each visit, all study information will be recorded in the study binder. All fields in the study report forms must be completed for each visit. Sample inventory forms must be completed when samples are initially stored. Sample shipping forms must be completed when samples are shipped, which will be batchwise.

4. Statistical considerations

4.1 Study design. The PIE is a double blind, placebo controlled, parallel group, multi-center study designed to determine if ibuprofen 600 mg three times daily can reduce lower respiratory tract PGE levels compared to placebo. The primary outcome will be PGE levels in the alveolar portion of BAL fluid.

The primary measure will be difference from beginning to end of treatment comparing treated vs. placebo groups on an intention-to-treat basis. The secondary outcome will be procollagen peptides measured in the alveolar portion of the BAL analyzed by the same plan. All three pro-collagen peptides will be assessed and evaluated separately. Levels in BAL fluid will be compared to levels determined in a set of smoking, ex-smoking and non-smoking controls.

Additional analyses will include PGE in the bronchial portion of the BAL fluid and induced sputum, other eicosanoids and metabolites in BAL fluid, blood and urine and inflammatory markers, including IL-8 and neutrophils measured in BAL and sputum. Ibuprofen levels will be measured, and a secondary analysis based on blood levels will be performed.

An exploratory analysis relating the change in emphysema over time to BAL levels of PGE will be performed. This will be accomplished by using historical data collected in the COPDGene study.

4.2 Sample size considerations. The primary question is: Will ibuprofen treatment reduce lower respiratory tract PGE levels? The question will be tested by comparing the difference in PGE concentration in the alveolar BAL fraction in the treated group to the difference in the placebo group after three months of treatment. Based on preliminary data, the observed standardized effect size for the difference in PGE concentration between COPD and controls was 1.3 (COPD mean=13.13, SD=9.2; Control mean=4.39, SD=1.9). Using a 0.05 level two-sided independent sample t-test, a sample size of 52 per group will have a 90% power to detect an effect half this size (0.65). This difference in effect size equates to a 33% reduction in mean PGE (13.13 to 8.76). Based on the literature, we believe this is a conservative effect size, as inhibition of PGE of 60% or greater has been reported. In prior studies, we have had less than 10% drop-out in studies requiring multiple bronchoscopies over intervals of three months or less [89-92]. Using a conservative estimate of 25% drop-outs, we intend to enroll a total of 140 subjects, 70 in each group.

The study has been powered on the primary outcome, change in PGE level compared to placebo. We have, however, estimated the power to detect a difference in the secondary outcome, pro-collagen peptide. Based on our pilot studies with pro-collagen I N-terminal peptide, our study with an anticipated 52 subjects will have 69% power to detect a standardized effect size of 0.485 (as observed in pilot studies) for the difference between groups using a 0.05 level two-sided independent sample t-test if inhibition of PGE corrects the pro-collagen I peptide levels to that of normal subjects. There is over 92% power to detect an improvement of this size within the treatment group using a 0.05 level two-sided paired t-test. With 52 subjects per group, we will have 80% power to detect an effect of 0.55 (slightly larger than in pilot studies) between groups and 0.396 (slightly smaller than in pilot studies) within group at the 0.05 level.

The same power estimates described above apply to the analyses of PGE in sputum samples. However, we anticipate obtaining satisfactory sputum samples in 75% (39) of subjects. As a result, we will have 80% power to detect the same effect size in the sputum specimens, as there will be fewer subjects with paired sputums than with paired BALs. However, we feel our estimates are conservative, as they are based on a 50% reduction in PGE concentration, which was readily achieved in prior studies.

Preliminary data do not exist to estimate the rate of change of emphysema quantified by emphysema. As a result, we do not have a basis to estimate the conditional power for the exploratory analysis. The analysis planned, however, should provide a basis for estimating sample sizes for follow-on studies that would use CT-quantified emphysema as an outcome measure.

For the Ancillary Study, the primary outcome is macrophage PGE production by COPD macrophages compared to controls. Data from all evaluable subjects will be used. Each subject will be considered as a single data point. The number of data points, therefore, is determined by the sample size considerations of the primary aim of the clinical trial. We anticipate obtaining samples from 140 COPD subjects and 60 controls. Based on the mean (SD) from preliminary studies for PGE production by fibroblasts, which is 641 (409) ng/10_5 cells/day for COPD subjects and 260 (161) for controls, and estimating adequate samples from 80% of subjects, a conservative estimate, and assuming the variance is the same and the difference in production of PGE is half that we observed in COPD fibroblasts, we will have greater than 93% power to detect a difference at the 0.05 level between COPD and control groups.

For the secondary aim of the Ancillary Study evaluating miR-146a levels in COPD subjects vs. controls, again using as pilot data that obtained from fibroblasts that showed the mean(SD) of miR-146a expression following stimulation to be 392(177) for COPD and 681(456) for controls, we will have 80% power to detect a standardized effect size of 0.486 for the difference in miR-146a expression in COPD macrophages compared to controls, at the 0.05 level. This effect is reasonable to assume, as the observed effect size in the pilot data with fibroblasts was 0.868. For assessing the functional role of miR-146a in modulating macrophage COX-2 mRNA, we used a similar analysis based on prior results with fibroblasts that shows the observed means(SD) are for COX-2 mRNA expression in fibroblasts following an miR-146a mimic were 60(25) treated and 130(25) untreated. With the conservative estimate that the role of miR-146a in alveolar macrophages will be 50% that in fibroblasts, samples from eight subjects will have 90% power to detect differences in COX-2 expression in response to miRNA-146a mimic at a 0.05 level. However, because of COPD heterogeneity, sample sizes of less than 10-15 are generally regarded skeptically. Thus, we plan to assess cells from 20 COPD subjects and 20 control smoker, ex-smoker and non-smoker subjects, which we believe will be adequate to assess the functional role of miR-146a.

The final aim of the Ancillary Study is to relate genetic variants in the miR-146a gene to COPD. Conditional power for this aim was estimated for 200 subjects (140 COPD, 60 controls) based on the binomial distribution. Table 2 shows excellent power to detect alleles present in at least twice in the study sample (frequency ≥ 0.5%).

Table 2. Power to detect variants by sequencing

Allele frequency	power
0.25%	0.63
0.50%	0.87
1%	0.98
2%	1
5%	1

Power to detect association between SNPs of allele frequency ≥1% and miR-146a expression levels was calculated using Quanto software[93], at α=0.05 (no multiple testing correction) and at α=0.001 to adjust for 50 SNPs tested (Table 3).

Table 3. Power to detect association between SNPs (allele frequency ! 1%) and miR-146a expression levels

	α=0.05		α=0.001	
Percent of variation explained	COPD cases	All subjects	COPD cases	All subjects
5%	0.76	0.89	0.27	0.47
10%	0.97	1	0.71	0.9
15%	1	1	0.93	0.99

4.3 Interim monitoring and stopping rules. A Data and Safety Monitoring Board (DSMB) will be constituted. It will consist of four members, two pulmonologists, one cardiologist and a statistician, who will review the progress of the study on a quarterly basis. The status of each subject after bronchoscopy and the results of the monthly gastritis monitoring will be reported monthly in summary reports to the DSMB members. In addition, the DSMB will receive reports of all Serious Adverse Events within 24 hours of their being reported to the DCC.

Because this trial involves an invasive procedure (bronchoscopy), formal interim analyses and formal stopping rules were believed to be required to help prevent subject exposure to unnecessary risks. Formal interim analysis of outcomes will be performed once, when approximately 50% of patients have completed the post-treatment bronchoscopy. We will use an O'Brien-Fleming monitoring boundary [94] (truncated at three standard deviations) to assess whether the interim results are sufficient to conclude that ibuprofen is effective at reducing PGE. We will also apply a futility

monitoring rule. The monitoring boundary nominal significance level associated with the interim analysis using the O'Brien-Fleming spending function will be 0.003 and 0.012, with the final analysis being done with α=0.047. Should there be any meaningful prevalence of concerning adverse events, e.g. myocardial infarctions, the DSMB can ask for an unblinded set of data to determine if the effect is related to the intervention. The DSMB is empowered to stop the study should concerns regarding subject safety warrant.

4.4 Analysis plans. The primary analysis will be by intention-to-treat. Descriptive statistics will be computed for all variables to ensure data quality and to evaluate the assumptions of statistical tests. Variable distributions will be described with box plots, histograms, measures of central tendency, measures of variation and frequencies. We will test the assumption of normality and equal variances for continuous outcome measures within each group and transform variables for which these assumptions are not met. Before testing the study hypotheses, we will examine the data for possible confounders. Potentially confounding variables, such as age, gender, and disease severity, will be controlled for by entering these variables as covariates. These variables will be entered as a covariate if the univariate two-sided test (either t-test or Chi-square test) comparing the two treatment groups is significant at the 0.20 level. We will use a large alpha level here so that important potentially confounding effects are not overlooked.

Linear mixed effects modeling will be used to determine the effect of ibuprofen on reducing PGE levels in COPD patients. Linear mixed effects modeling is a regression technique that accounts for the correlation due to multiple measurements on the same subject. This method will allow for the evaluation of differences within group (pre- to post-treatment change in PGE) as well as the difference between groups post treatment. The effects of treatment and time point, as well as any variables found to be significant in the univariate analysis, will be included in the model. Finally, a secondary analysis will be performed using blood levels of ibuprofen as a covariate.

In order to place the magnitude of any reduction observed into perspective, BAL PGE levels in the COPD patients before and after treatment will be compared with the levels present in normals. This comparison will be made estimating mean BAL PGE levels for each group and calculating their associated 95% confidence intervals. As the treatment is expected to lower PGE levels, the post-treatment is expected to be closer to controls.

For the secondary outcome, change in alveolar fraction pro-collagen peptide concentration, we will use the same modeling and analysis approach described above. Pro-collagen peptides, pro-collagen I C-terminal and N-terminal peptides and pro-collagen III N-terminal peptide will be quantified in BAL fluid. The change in each pro-collagen peptide level following ibuprofen treatment will be compared to the change in placebo-treated subjects.

A number of additional analyses are planned. The alveolar fraction of the BAL will be assessed for COX-derived eicosanoids in addition to PGE: PGD, TXB2 (a metabolite of thromboxane A2) and 6kPGF1a (a metabolite of prostacyclin) by LC-MS/MS. These metabolites will be quantified under the same chromatographic and

sample preparation conditions used for PGE analysis and using the same LC-MS/MS method. This will confirm the effect of COX inhibition of products other than PGE. In addition, urea will be used to estimate concentrations of PGE in alveolar ELF. Finally, we will assess PGE concentration in the bronchial portion of the BAL fluid and in induced sputum. While these latter two measures will not assess alveolar PGE, they will provide independent measures of the effect of ibuprofen on PGE in the lung. Similarly, quantification of PGEM in blood and urine will provide an independent assessment of the effect on total body PGE production. The same analysis plans described above for the analysis of PGE in the BAL fluid will be used for these secondary analyses.

Use of legacy data from COPDGene will allow us to relate measures made to progression of COPD and emphysema. To accomplish this, we will determine the difference between the legacy COPDGene CT-scan compared to that obtained upon PIE study entry. Correction will be made for change in achieved lung volume[95]. Dividing by the time between the assessments (3-6 years) will estimate a rate of progression of emphysema. The rate of change in emphysema for the entire population will then be related to PGE concentration in the BAL fluid on the initial (without treatment) BAL. This will allow us to determine if PGE is related to the rate of progression of emphysema. A similar analysis will be performed estimating the relationship with the rate of change in FEV1.

We will also assess clinical measures after 48 weeks of treatment. We do not believe our study, which is powered to confirm lower respiratory tract inhibition of PGE, will be adequately powered to rigorously assess disease progression. The sample size of 52 per group will provide 80% power to detect a standardized effect size for the mean change in lung volume of 0.57 between groups (a medium effect as defined by Cohen) and 0.4 within group (slightly smaller than a medium effect). The standardized effect sizes represent a difference between groups 57% as large as the standard deviation and change within group 40% as large as the standard deviation. We expect the change observed during this study to be smaller. However, the results from this trial will be used as assessment of the likely effect size and variance and will be important is the design of future Phase 3 efficacy trials. For this purpose, we will assess several outcome variables.

At present the most accurate way of detecting emphysema severity is CT scanning. CT scan quantification of emphysema has been demonstrated to be sensitive to change over a one-year time frame and has been used as an outcome in several clinical trials[95-97]. We will confirm the diagnosis and severity of emphysema at study entry by CT scan.

Volumetric CT scans will be acquired at end inspiration using multi-detector scanners (≥ 64 detector rows) using the SPIROMICS CT protocol. Phantom scans will be acquired on a monthly basis at each site, to assure scanner stability. Phantom scans will be analyzed as in the COPDGene and SPIROMICS protocol. Any deviations from recommended phantom parameters will be communicated immediately to the sites, and further scans will be suspended until the deviation is corrected. To perform quantitative emphysema analysis, de-identified DICOM images will be segmented using the Apollo image analysis system (VIDA Diagnostics, http://www.vidadiagnostics.com)[98]. The software will divide each lung into lobes as well as three craniocaudal zones of equal z-

axis lengths. The following analyses will be performed on the segmented inspiratory lung images: total inspiratory lung volumes, mean lung attenuation, relative lung volumes (for the whole lung, each lung lobe, and apical and basal lung thirds) falling below attenuation coefficients of -950, -910, and 856 HU. The 15th percentile point of the lung density histogram (PERC15) will also be calculated. Additionally, to evaluate emphysematous "hole size", the alpha value (the negative of the slope from the log-log relationship of hole size versus number of holes) will be measured for the entire lung and for the upper and lower lobes [99].

We will also assess FEV_1, D_LCO and SGRQ as these additional clinical parameters that can also contribute to the design of phase 3 efficacy trials.

Our assessment of CT scan will provide quantitative measures of two distinct patterns of emphysema, upper lobe vs. lower lobe and clustered vs. diffuse. Both of these distinct patterns have been associated with differences in natural history, clinical features and response to treatment. Therefore, we will also conduct a secondary analysis based on these parameters.

The first aim of the Ancillary Study is, do alveolar macrophages from COPD subjects produce more PGE than from controls? Because the data distribution cannot be assumed to be normal, generalized linear modeling will be used. Generalized linear modeling is a regression technique that allows for non-normally distributed outcome variables. In addition, a modeling approach will allow us to adjust for important covariates, as well as smoking status and disease severity. Secondary analyses will be performed similarly, evaluating current and former smoking COPD subjects separately. Each subject will be considered as a single data point.

The second aim of the Ancillary Study will determine if miR-146a levels differ between COPD subjects and controls. This will be approached with the same plan described above. To determine if miR-146a levels are related to BAL fluid PGE and alveolar macrophage COX-2 mRNA, protein levels and PGE production, we will use Spearman's correlation. Similar analyses will be performed with macrophages following culture with and without IL-1ß/TNF-α stimulation. For analyses of functional macrophage studies using miR-146a mimics and inhibitors, each subject will be a single data point, and differences in the paired values will be analyzed using a non-parametric Wilcoxon signed ranks test.

In the third aim of the Ancillary Study, we will test for association between miR-146a polymorphisms and the BAL PGE levels (determined in the Clinical Study) and the immediate *ex-vivo* miR-146a and COX-2 expression in alveolar macrophages. For common variants (allele frequency ≥ 1%), we will use linear regression in all subjects and in COPD cases only, adjusting for smoking status, if that is found to be relevant. For rare variants (<1%), we will employ one of several current methods that can be applied to quantitative traits, such as the Variable Threshold test[100] or the SNP-set Kernel Association Test[101]. We will adapt these methods for analysis of exome/genome sequencing data to the candidate gene analysis, by collapsing SNPs according to location within the gene (e.g. promoter, exon, 3' UTR) and weighting by presumed functional effects, such as SNPs that alter transcription factor binding sites. Methods for rare variant analysis are constantly evolving, and we will adopt best practices as they emerge. In addition, we predict that polymorphisms in miR-146a related to AP-1

regulatory sites will specifically modulate response to IL-1ß/TNF-α, and this will be ascertained by relating miR-146a polymorphisms to the results of the stimulation of PGE production by IL-1ß/TNF-α, using the analysis methods above.

In addition to PGE, alveolar macrophages produce PGD. Our analytical method is able to quantify PGD and, but including an additional internal standard in the LC-MS/MS runs, both PGE and PGD can be accurately determined with little increase in total cost [102].

5. Study organization and conduct

5.1 Data Coordinating Center. The DCC will be located at the University of Nebraska Medical Center (UNMC). The DCC will have the primary responsibility to assure that all study sites are fully equipped and trained to execute the study, will monitor the progress of the study and its compliance with protocol and regulatory requirements, will house all samples, will coordinate sample analyses, will have the responsibility for maintaining the study database and will perform statistical analyses. In addition, the DCC will prepare the Study Procedures Manual and will oversee data entry and quality control.

5.2 Clinical sites. At least three clinical sites will participate: Temple University Hospital, National Jewish Health, and Harbor/UCLA Hospital. All centers are previous COPDGene centers. Study procedures will be harmonized with prior COPDGene procedures. Each center will have a site Principal Investigator, a Study Coordinator and a Laboratory Technician, all of whom will complete formal training for study procedures. The study will be approved by the local Institutional Review Board and will comply with local HIPAA guidelines.

5.3 Radiology reading center. National Jewish Health (NJH) will serve as the Radiology Reading Center. To perform quantitative emphysema analysis, de-identified DICOM images will be sent to NJH for analysis. Data will then be transferred to the DCC.

5.4 Study Procedures Manual, and Data Forms. The Study Procedures Manual and Data Forms will be prepared by the DCC after study approval. We expect this to be completed between notification and accomplished before the funding start date so that all study materials will be in place prior to the official funding date. The Data Forms will be completed at the Study Sites, the Radiology Reading Center and at the analysis laboratories of the DCC. In general, the same procedures used for COPDGene for the performance of CT Scans, for the transfer of CT data and for quality assurance of CT Scans, including staff training, data monitoring and scanning of phantoms will be used. Other procedures, e.g. bronchoscopy and BAL and induced sputum, are currently based on standard UNMC procedures. Should they become available prior to study initiation, these procedures will be harmonized with those of SPIROMICS as much as possible. This will help with cross-study comparisons and will simplify staff training and quality control measures.

5.5 Study drug. Study drug, ibuprofen and matching placebo, will be prepared by an experienced vendor. This is tentatively planned to be Temple University Hospital Pharmacy. Medication will be packaged with a coded label and shipped to each study site. Randomization of subjects at each site will be determined by the statistician at the DCC.

5.6. Study materials. Study materials will be provided to the study sites by the DCC. The materials provided will include data collection forms, sample tubes, coded

labels and consumables. In addition, kits will be prepared for the processing of BAL and induced-sputum specimens. These will include reagents needed for cell culture and harvest. Each site is adequately equipped with the required equipment, e.g. spirometer, nebulizer, endoscopy suite, centrifuge, incubator, CT scanner, etc.

5.7 Quality Control Measures. Quality control measures will be established and included in the Study Procedures Manual. These measures will include real time measures of quality for spirometry, CT Scanning, induced sputum and BAL. The CT quality control measures will be the same as those implemented in COPDGene. The BAL measures will include quantification of fluid return, cell return and cytology of bronchial and alveolar specimens. In addition, although personnel at all study sites are highly experienced, the Study Principal Investigator will directly observe the initial bronchoscopy and BAL, including sample processing that will be performed at each site. Should it be necessary, subsequent procedures will also be directly observed.

5.8 Subject recruitment. COPD subjects and current and ex-smoking controls will be recruited at each study site from eligible participants in COPDGene and other subjects with qualifying CT scans. The COPDGene Data Center (NJH) will be notified of subjects entered in the study using the COPDGene Study code, which is de-identified. The COPDGene Data Center will then transfer the data needed for the PIE study to the DCC at UNMC using only the COPDGene Study Code. Neither the COPDGene Data Center nor the PIE DCC at UNMC will have access to subject identifiers. Healthy non-smoking control subjects will be recruited at the study sites and de-identified data sent to the DCC at UNMC.

6. Regulatory Issues

6.1 IRB approvals. The study will be approved by the human studies committee (Institutional Review Board, IRB) at all study sites. In addition, approval will be obtained from the IRBs at UNMC and at NJH (the COPDGene Data Center and the PIE Radiology Reading Center). Final approval of the protocol will also be obtained from the COPDGene Steering Committee. Preliminary interviews with the IRB at UNMC have indicated that the limited analysis of the miR-146a gene does not constitute genetic testing, and no separate genetics consent will be requested. This question, however, will be revisited with all study site IRBs prior to study initiation.

6.2 FDA approval. Pre-IND consultation with the FDA will be requested. The medication to be used, ibuprofen, is already approved and usage will be within currently approved dosing. However, the indication in the current proposal is significantly different from approved indications, interstate transport of the drug and placebo will be required and the study involves more than minimal risk. Thus an investigator Investigational New Drug (IND) Approval may be required. If so, an investigator IND will be submitted to the FDA. It is anticipated that this process will be completed prior to the scheduled date for funding approval.

6.3 Study registration. The study will be registered with ClinicalTrials.gov.

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