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Original Article

AGTR2 absence or antagonism prevents cystic fibrosis pulmonary manifestations

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Abstract

Background: Pulmonary disease remains the primary cause of morbidity and mortality for individuals with cystic fibrosis (CF). Variants at a locus on the X-chromosome containing the type 2 angiotensin II receptor gene (*AGTR2*) were identified by a large GWAS as significantly associating with lung function in CF patients. We hypothesized that manipulating the angiotensin-signaling pathway may yield clinical benefit in CF.

Methods: Genetic subset analysis was conducted on a local CF cohort to extend the GWAS findings. Next, we evaluated pulmonary function in CF mice with a deleted *AGTR2* gene, and in those who were given subcutaneous injections of PD123,319, a selective *AGTR2* antagonist for 12 weeks beginning at weaning.

Results: The genetic subset analysis replicated the initial GWAS identified association, and confirmed the association of this locus with additional lung function parameters. Studies in genetically modified mice established that absence of the *AGTR2* gene normalized pulmonary function indices in two independent CF mouse models. Further, we determined that pharmacologic antagonism of *AGTR2* improved overall pulmonary function in CF mice to near wild-type levels.

Conclusions: These results identify that reduced *AGTR2* signaling is beneficial to CF lung function, and suggest the potential of manipulating the angiotensin-signaling pathway for treatment and/or prevention of CF pulmonary disease. Importantly, the beneficial effects were not CF gene

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mutation dependent, and were able to be reproduced with pharmacologic antagonism. As there are clinically approved drugs available to target the renin-angiotensin signaling system, these findings may be quickly translated to human clinical trials.

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Keywords: Pulmonary function; GWAS follow up; Angiotensin signaling; Mouse models of airway disease

1. Introduction

While cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) [1], there is large natural variability in CF pulmonary disease. Heritability studies indicate that 50–80% of this variation is due to genetic differences not related to *CFTR* [2]. A genome-wide association study (GWAS) conducted by the International Cystic Fibrosis Gene Modifier Consortium identified variants within an interval on the X chromosome containing the type 2 angiotensin II receptor gene (*AGTR2*) as significantly associating with a longitudinal measure of lung function based on forced expiratory volume in 1 s (FEV_1) in individuals with CF. [3] This analysis identified multiple single nucleotide polymorphisms (SNPs) in the interval on the X chromosome containing *AGTR2* that were in linkage disequilibrium, all associating with CF pulmonary disease severity. The most significantly associating SNP in this region was rs5952223, a C/T polymorphism with a minor allele frequency of 0.26.

AGTR2 is part of the renin-angiotensin signaling (RAS) pathway that has been widely studied for its role in blood-pressure regulation and in renal and cardiovascular health. Much less is known about its involvement in pulmonary function and disease. In the airway, the precursor, angiotensinogen, is secreted by airway smooth muscle cells and apoptotic epithelial cells, and cleaved to angiotensin I by renin. Angiotensin converting enzyme (ACE) further cleaves angiotensin I to the biologically active angiotensin II, which can then signal through two receptors, the type-1 angiotensin II receptor (*AGTR1*) and *AGTR2*.

In addition to the GWAS identification of *AGTR2*-linked loci as modifiers of CF pulmonary disease, there is evidence that the RAS pathway is altered in CF. For example, CF patients demonstrate increased activation of RAS in response to salt deprivation that is thought to compensate for salt depletion and prevent dehydration [4]. However, increased serum ACE levels are associated with a more rapid decline in pulmonary function in CF patients [5], suggesting that decreasing angiotensin signaling may be beneficial. To establish the viability of manipulating the angiotensin signaling pathway for clinical benefit in CF, we extended the GWAS findings by confirming the association of this X-chromosome locus with additional lung function parameters. Using genetically modified mice, we examined the effect of absence of the *AGTR2* gene on pulmonary function indices in CF mice to identify whether loss of *AGTR2* would be beneficial or deleterious. Subsequently, we examined the impact of *AGTR2* pharmacologic intervention for CF lung disease treatment, establishing the translational relevance of manipulating the

angiotensin-signaling pathway for treatment and/or prevention of CF pulmonary disease.

2. Methods

2.1. Human CF cohort

All research subjects were cared for at the Rainbow Babies and Children's Hospital Cystic Fibrosis Center in Cleveland, Ohio between 2005 and 2013. Individuals with CF who chose to participate were consented for study enrollment during routine outpatient clinic visits and provided buccal swabs for research purposes. Specimens were matched with pulmonary function data available from a curated research database which contains phenotypic data (including spirometry values). All available values of FEV_1 percent predicted, FVC percent predicted, and FEF_{25-75} percent predicted were included for each participant; the number of individual measurements for each subject varied.

2.2. Human genotyping

DNA extraction was performed immediately upon collection of the buccal swab using a Qiagen DNeasy kit (Qiagen) following the manufacturer's protocol. Resultant DNA was stored at -20 °C for batch analysis. Genotyping was performed using custom Taqman SNP genotyping assays to the *AGTR2* SNP rs5952223 (AB Life Technologies) on a Taqman StepOne plus thermocycler per manufacturer instructions. Genotype assignments were made using Applied Biosystems Taqman Genotyper software.

2.3. Statistical analysis of human pulmonary function

All spirometric variables and changes over time were examined using box plots and LOWESS line plots over time. Frequency analysis was used to check and validate data quality. Distributions of spirometry measurements (FEV_1 percent predicted, FVC percent predicted, and FEF_{25-75} percent predicted) were found to be skewed and unable to be transformed into symmetrical distributions. Thus, we used simultaneous quantile (median) regression analysis for studying effects of *AGTR2* genotype on these longitudinal outcome variables. For this analysis, *AGTR2* genotype was coded as risk genotype (C [males] or CC [females]) or low-risk genotypes (CT [females], T [males] or TT [females]). To capture the correlations among the repeated measurements, we used clustering in time of the measurements and computed the standard errors of the estimates

using a bootstrap method. We performed 500 bootstraps for estimating the standard error of the estimates. Point estimates are presented as well as normal-based 95% confidence intervals (CI) of the AGTR2 genotype coefficient and other regression coefficients in the multivariable or adjusted quantile regression models. All the analyses were performed using statistical software Stata 15.0.

2.4. Mouse models

Several genetically altered mouse models were utilized in these studies. The mice harbored either the F508del *Cftr* mutation (*Cftr*^{tm1kth}) [6], the R117H *Cftr* mutation (*Cftr*^{tm2Uth}) [7] or an *Agtr2* mutation (*Agtr2*^{tm1Tin}) [8]. In addition, the *Agtr2* and *Cftr* mutations were bred on the same background to give an *F508del/Agtr2* strain and the *R117H/Agtr2* strain. Wild type (WT) littermates from each strain were used as controls. Mice were allowed unrestricted access to chow (Harlan Teklad 7960; Harlan Teklad Global Diets, Madison, WI) and sterile water and were maintained on a 12 h light/dark cycle at a mean ambient temperature of 22 °C. Mice with *Cftr* mutations were given access to sterile water with an osmotic laxative, Colyte (Schwarz Pharma, Milwaukee, WI), to decrease the incidence of intestinal obstruction due to loss of CFTR function.

2.5. Respiratory mechanics

Lung mechanics measurements were made using automated maneuvers and analysis algorithms as described previously using a computer-controlled mechanical ventilator (flexiVent system, SCIREQ) [9]. Briefly, following induction of anesthesia via intraperitoneal injection of ketamine (150 mg/kg) and xylazine (15 mg/kg), mice were intubated and mechanically ventilated at a rate of 150 breaths/min with tidal volumes of 10 mL/kg and positive end-expiratory pressure of 3 cmH₂O. Single-frequency forced oscillation maneuvers were used to calculate overall respiratory system compliance (Crs). Automated measurements made during a broadband low-frequency forced oscillation maneuver with a mixed frequency forcing function comprised of multiple prime frequencies ranging from 0.25 to 20 Hz were fit to a constant phase model to determine tissue damping (G) and tissue elastance (H). Parameters from individual data sets were included in the analysis if the coefficient of determination assessing the fit of the model to the experimental data was ≥ 0.95 . Pressure-volume curves were produced by stepped increases and subsequent decreases in airway pressure. Static lung compliance (Cst) was calculated from the slope of each curve and a shape parameter (K) describing the deflation limb of the pressure-volume loop was quantified.

2.6. Histology

Following CO₂ euthanasia in separate groups of animals, lungs were pressure inflated to 30cmHg with 10% buffered formalin for 30 min. Resected lungs were fixed in 10% buffered formalin for ten days and subsequently embedded in

paraffin prior to sectioning in 150 μ m increments at a thickness of 5 μ m. Lung sections were mounted on glass slides and stained with hematoxylin/eosin (H&E) for assessments of distal airspaces.

2.7. Distal airspace enlargement

A Mean Linear Intercept (MLI, L_m) calculation was used to quantitatively assess distal airspace enlargement using an indirect method as described previously [9]. Briefly, software generated a test-line across multiple 20 \times fields of view representing $\sim 5\%$ of the total lung area. The L_m was determined by calculating the ratio of line endpoints within parenchyma, including airspaces and ductal spaces [P(A) + P(duct)], to points intersecting the septum [I(A)] across the known-length test line (d) (excluding nonparenchyma structures) [10], as indicated by the following formula [11]: $L_m = d \cdot [P(A) + P(duct)]/I(A)$.

2.8. AGTR2 pharmacologic inhibition

Weanling wild-type and F508del mice were weighed at 3 weeks of age for dosing of AGTR2 inhibitor PD123,319 (Sigma). PD123,319 has been well established as an AGTR2 antagonist [12–14]. Lyophilized PD123,319 was resuspended in filtered 0.9% saline. Injections were performed with 27G TB syringe (BD) subcutaneously in the interscapular region every 24 h with 2 mg/kg/day (5-day-a-week protocol) for 12 weeks. Weights were recorded bi-weekly during weanling growth phases, and once weekly at 6 weeks of age to maintain dosing accuracy. The administered volume was diluted as necessary to deliver an accurate and consistent weight-based bolus between 200 and 400 μ L throughout the treatment course.

2.9. Statistical analysis of murine data

Descriptive statistics were used to summarize pulmonary function measurements and MLI distances in mice. Differences in group means were quantified by analysis of variance (ANOVA) using the statistical software package SigmaStat. When significant differences were detected, a pairwise comparison of the means of each combination of groups was conducted assuming equal variances. The Student-Newman-Keuls procedure was used to account for all pairwise comparisons. Corrected p -values $\leq .05$ were considered significant.

2.10. Study approval

Relevant human studies were approved by the University Hospitals Cleveland Medical Center Institutional Review Board (protocol 11–67-200). Informed consent was obtained from all participants. Mouse studies were approved by the Case Western Reserve University Institutional Care and Use Committee under protocol 2015–0181.

Table 1
Cystic fibrosis patient cohort characteristics.

Characteristic	Total study population	rs5952223 (C/CC) risk genotype	rs5952223 (CT/TT) low risk genotypes
N	126	73	53
Male Sex - n (%)	59 (46.8)	36 (49.3)	23(43.4)
Age - years	9.3 ± 2.3	8.9 ± 1.7	9.9 ± 2.8
FEV ₁ (% predicted)	83.7 ± 23.4	80.8 ± 22.4	87.6 ± 24.4
FVC (% predicted)	96.9 ± 18.4	95.8 ± 16.2	98.6 ± 21.2
FEF _{25–75} (% predicted)	69.6 ± 31.3	65.8 ± 28.3	74.7 ± 34.6

3. Results

The rs5952223 association was evaluated in a local CF cohort of 126 participants in whom additional measures of lung function were available. The mean (±SD) age at enrollment was 9.3 ± 2.3 years, and 46.8% of the participants were male. No subjects were excluded because of race or ethnic background; 96.7% of the patients were self-identified as white. Basic demographic and clinical characteristics of the participants are detailed in Table 1. The risk genotype of rs5952223 that associated with worse pulmonary function in the previous GWAS (C [males] or CC [females]) was also significantly associated with lower FEV₁ percent predicted values in this population (7.1% lower, 95% Confidence Interval (CI), −5.1 to −9.0). In addition, the same risk genotype was significantly associated with a reduction in the percent predicted values of forced vital capacity (FVC) (4.4% lower, 95% CI, −2.5 to −6.2) and forced expiratory flow over the middle one half of the FVC (FEF_{25–75}) (5.3% lower, 95% CI, −2.2 to −8.5). These findings, summarized in Table 2, are consistent with the prior GWAS analyses [3, 15]. Moreover, they extend the association between genetic variation in this region and two additional, functionally related measures of pulmonary function and replicate the genetic findings in a younger cohort.

To further establish whether AGTR2 is mechanistically linked to lung function in CF, we crossed a CF mouse model (F508del; *Cftr*^{tm1Kth}) [6] with an *Agtr2* knock out mouse model (*Agtr2*^{tm1Tin}) [8]. Overall survival was not impacted in the resultant CF/*Agtr2* double mutants (*F508del/Agtr2* KO). Our objective was to determine if absence of *Agtr2* would either positively or negatively impact the pulmonary phenotype in a mouse model of early CF lung disease [9, 16, 17]. Using a

Table 2
AGTR2 risk genotype associates with lower spirometry measurements in individuals with CF.

Pulmonary Function	Regression coefficient	Bootstrap standard error	z-score	p-Value	95% confidence interval
FEV ₁ (% predicted)	−7.1 ^a	1.01	7.01	<0.001	−5.09 to −9.04
FVC (% predicted)	−4.4 ^a	0.93	4.67	<0.001	−2.52 to −6.18
FEF _{25–75} (% predicted)	−5.3 ^a	1.6	3.32	0.001	−2.18 to −8.47

^a Risk genotype (C/CC) worse than low-risk genotypes (CT/TT).

computer-controlled mechanical ventilator, automated maneuvers showed that the absence of *Agtr2* restores respiratory system compliance, tissue elastance, tissue damping and static tissue compliance to near wild type (WT) levels in F508del mice (Fig. 1). In contrast, absence of *Agtr2* did not improve distal airspace enlargement or the delayed stepwise lung deflation observed in CF mice [9]. Thus, these results indicate that some but not all CF-specific changes in the distal lung compartment are improved by *Agtr2* inactivation (Fig. 1).

To ensure that the restoration of pulmonary function resulting from the absence of *Agtr2* was not specific to the F508del mouse model, we crossed the *Agtr2* KO mice with a second, independent mouse model with a human CFTR mutation (R117H; *Cftr*^{tm2Uth}) [7]. We observed a similarly beneficial effect on pulmonary function in the *R117H/Agtr2* KO mice (Fig. 2), suggesting that reduction in *Agtr2* may impact lung pathophysiology via a CFTR-genotype independent mechanism. Taken together, these findings support the idea that genetic inactivation of AGTR2 is beneficial to lung function in CF.

To extend these pre-clinical studies and test whether the improvements in lung function could be achieved with pharmacologic inhibition of *Agtr2* function, we treated WT and F508del mice by subcutaneous injections of a selective *Agtr2* antagonist (PD123,319) [12–14]. A 5-day-a-week regimen (2 mg/kg/day) was started after weaning (age 3–4 weeks) and was continued for 12 weeks. Following this pharmacologic intervention, CF mice had lung function that was not significantly different from WT levels, and was similar to what was achieved by complete genetic inactivation of the *Agtr2* gene (Fig. 3). In contrast, *Agtr2* antagonism had no detectable effect on lung function in WT mice.

4. Discussion

The objective of this study was to establish the potential for manipulation of angiotensin signaling as a novel therapeutic strategy to be pursued for the treatment of CF pulmonary disease. Key findings include: 1) replicating the prior GWAS analysis on a younger CF cohort; 2) extending the genetic association of the X-chromosome locus containing *AGTR2* with additional pulmonary function parameters in CF patients (FVC and FEF_{25–75}% predicted); 3) determining that an inactivating deletion of *Agtr2* prevents some but not all pathologic distal lung compartment phenotypes in a mouse model of early CF lung disease; 4) establishing that the beneficial impact of *Agtr2* absence on pulmonary function is independent of the CFTR mutation; and 5) identifying that pharmacologic antagonism of *Agtr2* function yielded similarly beneficial effects in CF mice.

The GWAS analyses that motivated this work included genotyping over 8 million genetic variants in over 6000 individuals with CF from 3 independent cohorts with an average age of 19.5 ± 9.4 years [3]. Our findings, although based on a smaller cohort of CF individuals, extended the association of the X-chromosome locus to additional lung function parameters. This increases the likelihood that this

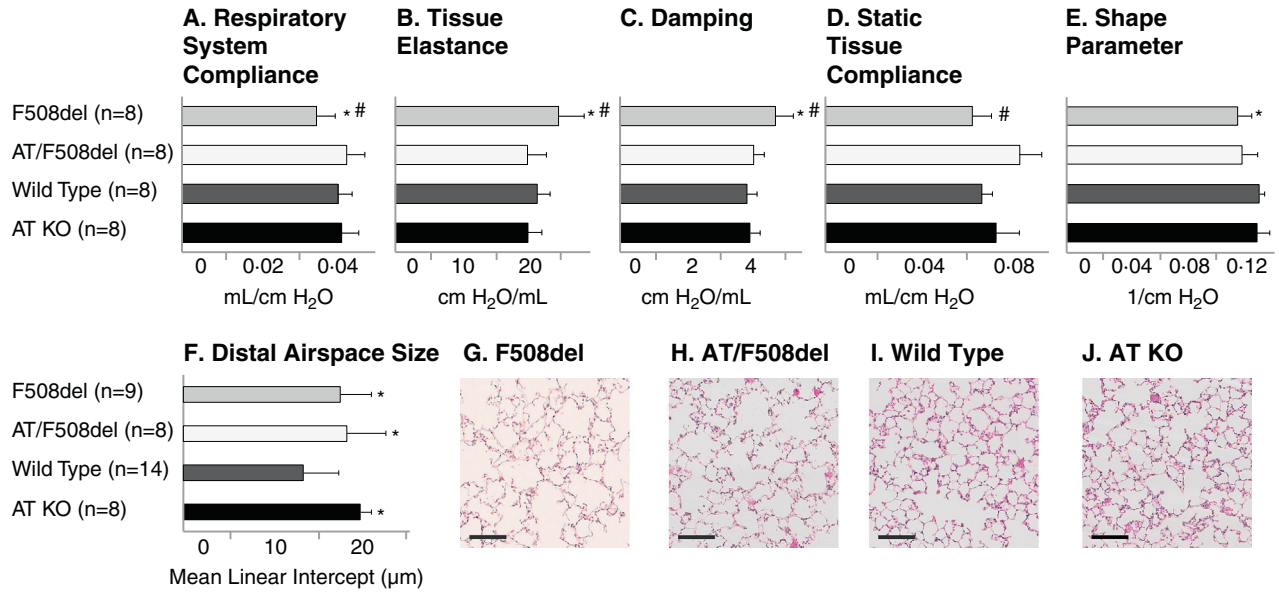


Fig. 1. An inactivating deletion in *AGTR2* prevents aspects of pulmonary dysfunction in F508del CF mice. (A-E) Lung function and (F-J) distal airspace size were quantified in F508del CF mice, F508del CF mice with the genetic deletion of the *AGTR2* gene (double knockout: AT/F508del), wild type C57bl/6 mice and *AGTR2* knockout mice with wild type *CFTR* (AT KO). (G-J) Representative histologic images are displayed (size bars indicate 100 μm). Data are presented as means, with error bars denoting standard deviations. **P* < 0.05 compared to wild type; #*P* < 0.05 compared to AT/F508del.

locus may be acting as a modifier of pulmonary function in CF. Further, the replication of the association in younger individuals suggests the modifying effects may be occurring early in the disease process. Although the *AGTR2* gene is located on the X-chromosome, males and females were combined in this analysis because there is no evidence of differential expression of the *AGTR2* gene in males and females [18]. There are

important limitations to any genetic association study including difficulties controlling for socioeconomic status, environmental factors and other non-genetic modifiers of CF lung disease. This X-chromosome region has been examined in CF related diabetes (CFRD) and was not found to be associated, but its potential association with other aspects of CF pathophysiology are unknown [19]. Future studies will include an examination

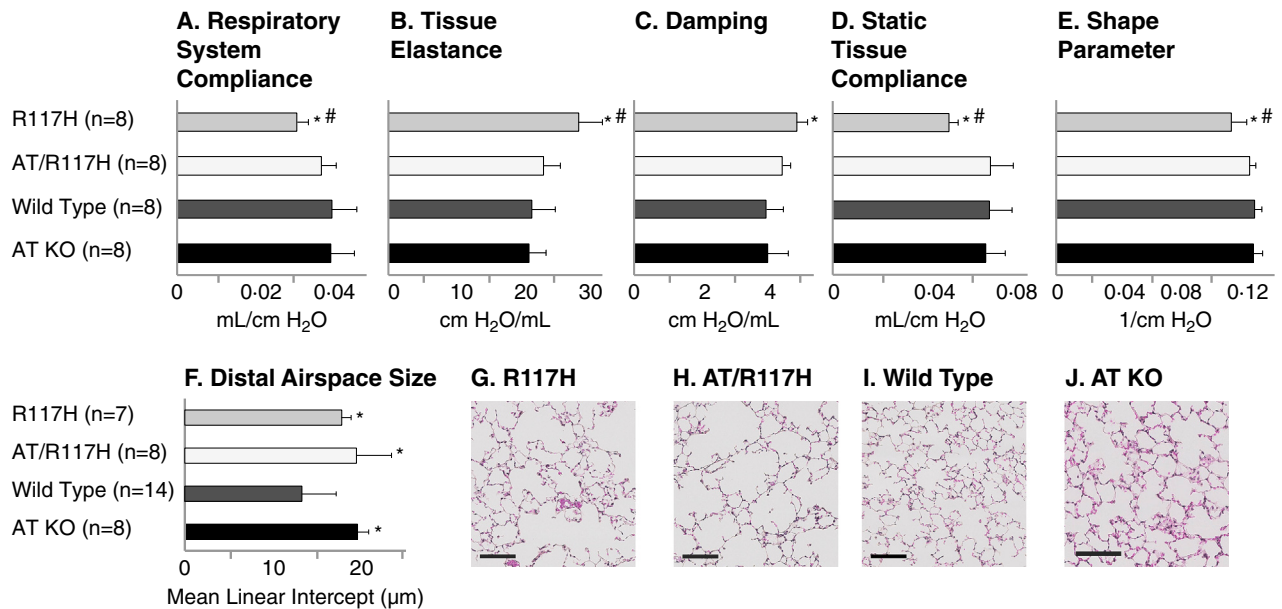


Fig. 2. An inactivating deletion in *AGTR2* aspects of pulmonary dysfunction in R117H CF mice. (A-E) Lung function and (F-J) distal airspace size were also quantified in CF mice homozygous for the R117H *CFTR* mutation (R117H), and compared to CF mice with the genetic deletion of the *AGTR2* gene (double knockout: AT/R117H), wild type C57bl/6 mice and *AGTR2* knockout mice with wild type *CFTR* (AT KO). (G-J) Representative histologic images are displayed (size bars indicate 100 μm). Data are presented as means, with error bars denoting standard deviations. **P* < 0.05 compared to wild type; #*P* < 0.05 compared to AT/F508del.

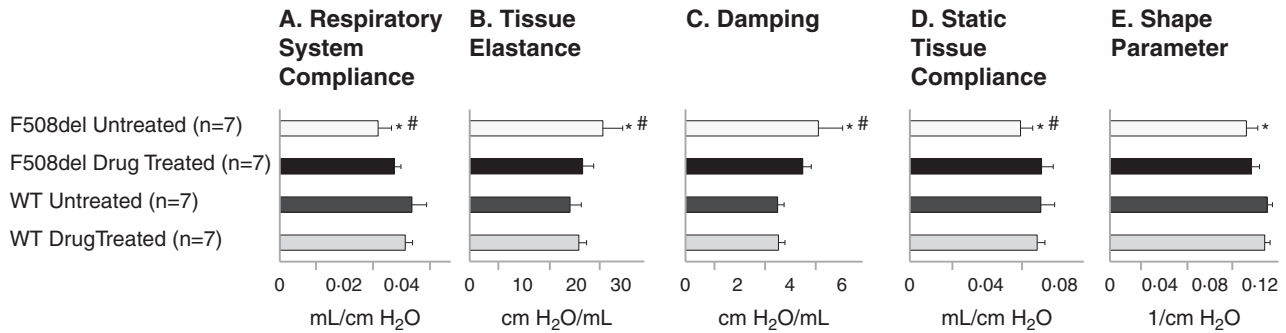


Fig. 3. Pharmacologic antagonism of AGTR2 prevents the development of CF-related pulmonary dysfunction. (A–E) Lung function was quantified in drug treated F508del CF mice and compared to untreated CF mice and wild type C57bl/6 mice (both treated and untreated). * $P < 0.05$ compared to wild type; # $P < 0.05$ compared to AT/F508del.

of the additional GWAS-identified modifying regions in the cohort, as well as other aspects of CF-related phenotypes such as pseudomonas colonization.

While GWAS analyses are hypothesis-generating, genetic association does not prove a gene's involvement as a genetic modifier of the examined phenotype. The region on the X-chromosome associating with CF lung disease was located in a non-coding region 3' of the *AGTR2* gene. This suggests that the modifying effect is likely due to variation in gene regulation rather than a difference in protein coding sequence.

Further, the genetic analyses do not indicate whether increasing or decreasing expression of a candidate gene like *AGTR2* would be beneficial. We determined that an inactivating deletion of *Agtr2* in two independent CF mouse models improved a CF-specific distal lung compartment phenotype of decreased respiratory system compliance, increased tissue elastance and damping, and reduced static tissue compliance. Importantly, there is high homology (95.3%) between mouse and human *AGTR2*, underscoring a potential relevance to human disease. Taken together, these findings established that *AGTR2* is a modifier of lung function in CF mice independent of *CFTR* mutation type, and more importantly, determined that reduced *AGTR2* was beneficial.

The CF mouse exhibits a complex pulmonary phenotype characterized by changes in lung mechanics, increased respiratory rate, histological differences including distal airspace enlargement, exaggerated inflammatory cell recruitment and altered responses to infection and other pathologic stimuli [7, 9, 16, 17, 20–22]. However, the mouse model has relevant limitations. For example, although CF mice do develop spontaneous pulmonary infections [23], the involved microorganisms are not those typically observed in CF patients. Further, the changes in lung mechanics observed in CF mice are mild compared to human adult CF lung disease [9], and the CF mouse does not naturally progress to more severe disease pathology, including bronchiectasis and mucus plugging of the airways. However, it has been suggested that the pulmonary phenotype observed in the CF mouse may serve as an animal model of early CF lung disease, which is now recognized as an important time period for clinical intervention [24–31]. The association of the genetic region studied with small airways

disease (decreased FEF_{25-75}) is consistent with this perspective. Decreased respiratory system compliance in the CF mouse indicates that additional work will be required to exchange a given volume of air during breathing. Increased tissue elastance and tissue damping localizes the phenotype to the peripheral lung compartment [9]. A similar phenotype has been reported in CF patients with spirometry within normal limits [32–34].

Complete genetic ablation of the *Agtr2* gene was beneficial in a CF mouse, but this approach is not translationally viable. The data presented here indicate that the *AGTR2* antagonist administered beginning at a young age results in similar improvements in pulmonary function compared to the genetic models. Furthermore, this pharmacologic approach to treatment may potentially benefit all CF patients, regardless of *CFTR* genotype, as our genetic results indicated improvements in CF mouse models with two independent *CFTR* mutations.

Pharmacologic agents targeting the renin-angiotensin signaling pathway exist, and therefore translation of our findings to clinical benefit may be a possibility. Though there is not currently an FDA-approved antagonist for *AGTR2*, ACE inhibitors would likely result in reduced *AGTR2* activity, as they reduce the amount of ligand available. In contrast, angiotensin receptor blockers (ARBs) selectively antagonize *AGTR1* and thus may result in increased angiotensin II binding to *AGTR2*, raising the possibility that they could even worsen lung function in CF patients. Although there is still a great deal to be understood about this pathway in relationship to CF lung disease, clinicians might exercise caution when selecting agents to treat hypertension in CF patients. Careful clinical trials will be required to evaluate the efficacy of *AGTR2* antagonism in humans, and to determine optimal dose, timing, threshold for initiation, and synergy with other medications for either ACE inhibitors, or clinical application of *AGTR2* blockers as therapies for CF lung disease.

While the results reported here provide an exciting opportunity for potential therapeutic development, the mechanism of these benefits remains unknown. The renin-angiotensin signaling pathway is involved in tissue growth, inflammation, apoptosis, tissue repair, and vascular responsiveness. *AGTR2* expression has been localized in the lung, where it is found in bronchi [35] and airway epithelial cells, mucous glands,

vascular endothelial cells, fibroblasts, chondrocytes, and macrophages [36]. Increased AGTR2 signaling has been shown to be detrimental in other pulmonary diseases. AGTR2 antagonism attenuates lung fibrosis [37] and decreases migration and proliferation of fibrotic fibroblasts [38] in bleomycin-induced murine lung fibrosis. The expression of both angiotensin II receptors (AGTR1 and AGTR2) is increased in the lungs of human adults with chronic obstructive pulmonary disease (COPD) compared to both healthy controls and non-COPD smokers [36]. The lung tissue from individuals with idiopathic pulmonary fibrosis also had elevated amounts of both receptor types, with a relatively greater increase in AGTR2 [38]. Further, hypoxia-induced collagen synthesis of human lung fibroblasts is attenuated by the antagonism of AGTR2 [39]. A meta-analysis of asthma susceptibility genes in the Chinese population identified an insertion/deletion polymorphism in ACE as one of seven SNPs significantly associated with the risk of asthma [40]. Studies in rats have identified activation of the p42/44 signaling pathway by angiotensin as a part of the hyper-responsiveness of the bronchial smooth muscle [41]. While other data also support that AGTR2 signaling contributes to the pathogenesis of lung disease [36, 38, 42, 43], the mechanism behind these contributions remains elusive. Here we show reduction of AGTR2 signaling may be effective for the treatment of CF lung disease, though the exact mechanism of this association will be the focus of future studies.

In conclusion, our data indicate that impeding AGTR2 signaling is beneficial to CF lung disease in a mouse model. These studies are an exciting example of validation of a novel therapeutic target identified by GWAS. Successfully determining the direction of effect and establishing the feasibility of pharmacologic manipulation of the signaling pathway in a pre-clinical model set the stage for first-in-human trials of AGTR2 blockade for treatment and prevention of CF lung disease.

Conflicts of interest

Authors Darrah, Jacono, Mitchell and Drumm are named on a patent application for the use of AGTR2 inhibition as a therapy for CF lung disease. The authors declare no other relevant conflicts of interest.

Author contributions

RJD, FJJ, ALM and MLD designed the research studies. NJ and JF acquired human samples. CKC, PL, JF and ESB conducted experiments. AS, DEN and LJS provided statistical guidance and analysis. CAH provided mouse models. RJD, FJJ and MLD interpreted results. All authors contributed to writing the manuscript.

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