

Early Clinical Evaluation of an RNA interference (RNAi) Based Therapy for Respiratory Syncytial Virus (RSV) Infection

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Abstract (revised)

Background: RSV is the most common cause of lower respiratory tract disease and hospitalization of US infants. It is also an important pathogen of the elderly and immunocompromised. Existing prevention and treatment strategies are limited. RNAi based therapy via small interfering RNAs (siRNA) utilizes endogenous cellular mechanisms to catalyze the targeted degradation of mRNA in a sequence specific manner. Therapeutic and prophylactic administration of an siRNA against the RSV N-gene (ALN-RSV01) reduces RSV *in vitro* and *in vivo*. Intranasally aspirated single doses of 2mg/kg reduce RSV in mouse lungs by 3-4 logs. Synthetic siRNAs have not been previously evaluated in man except for direct administration to the eye.

Objective: 1. To evaluate the aerosolizability and clinical safety of an siRNA delivered to the respiratory tract. 2. To develop a safe, reproducible and robust human experimental infection model for testing RSV antiviral proof of concept in man.

Design/Methods: 1. ALN-RSV01 was characterized pre, during, and post nebulization (PARI eFlow nebulizer) *in vitro*. Intranasal sprays of ALN-RSV01 were administered in single and multiple rising doses to healthy adults in randomized, double blind, placebo (saline) controlled trials. 2. Increasing quantities of a wild type GMP-produced RSV strain were administered intranasally to cohorts of healthy volunteers. Safety and clinical data and nasal washes were collected.

Results: Anion exchange HPLC chromatograms and *in vitro* functional activities (transfection/RSV infection Vero cell assay) were unchanged after nebulization. At 75.4 and 25.7 mg/mL concentrations, mass median aerodynamic diameters of aerosolized particles ranging from 2.9-3.3 μm (respirable fraction [% < 5μm]) of 95.0-89.8) respectively were generated. Adult breath simulation tests showed mean pulmonary deliveries of 71-62% respectively of the dose placed in the nebulizer. 65 adults receiving nasal sprays of 1.5-150mg (0.02-2mg/kg) of ALN-RSV01 singly and in 5 daily doses exhibited similar rates and types of adverse events (AEs) as did 36 placebo recipients. All AEs were mild and their frequency did not increase with rising doses. No laboratory abnormalities were observed. Aerosolized ALN-RSV01 is currently being evaluated in healthy adults. A pure wild type RSV strain was manufactured under GMP and was used to infect 36 healthy adult volunteers, producing safe reproducible and measurable viral and disease outcomes.

Conclusions: ALN-RSV01 can be aerosolized in a manner predictive of substantial lung deposition while retaining its structure and function. It appears safe in early clinical evaluation when administered intranasally. A robust RSV experimental human infection model has been developed which will now be used to test proof of concept antiviral effect.

ALN-RSV01 Intranasal Phase I Study Design

US Protocol

- ◆ Single-dose, randomized, placebo-controlled double blind
- ◆ Healthy adult male volunteers
- ◆ 34 subjects
 - ❖ 5 single dose groups, (1.5-150 mgs)
 - 5:2 randomization (drug:placebo)
 - 24 drug:10 placebo

European Protocol

- ◆ Single- and multi-dose, randomized, placebo-controlled, double blind
- ◆ Healthy adult male volunteers
- ◆ 67 subjects
 - ❖ 3 single dose groups, (5-150 mgs)
 - 5:4 randomization
 - ❖ 3 multi-dose groups, (qdx5)
 - 9:5 randomization

Figure 1. Phase I Study Results: Most Common Adverse Events

Body System Preferred Term	Placebo N=10	1.5 mg N=5	5 mg N=4	15 mg N=5	50 mg N=5	150 mg N=5
Respiratory, Thoracic, Mediastinal Disorders	9 (90%)	4 (80%)	4 (100%)	4 (80%)	5 (100%)	5 (100%)
Nasal Edema	6 (60%)	2 (40%)	3 (75%)	4 (80%)	4 (80%)	3 (60%)
Nasal Mucosal Disorder (erythema)	3 (30%)	3 (60%)	2 (50%)	0 (0%)	4 (80%)	3 (60%)
Nasal Dryness	2 (20%)	1 (20%)	1 (25%)	0 (0%)	0 (0%)	0 (0%)
Rhinorrhea	2 (20%)	1 (20%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
Nasal Congestion	0 (0%)	1 (20%)	0 (0%)	1 (20%)	1 (20%)	0 (0%)
Epistaxis	1 (10%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pharyngeal Erythema	1 (10%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)
Postnasal Drip	0 (0%)	0 (0%)	0 (0%)	2 (40%)	0 (0%)	0 (0%)
Nasal Discomfort	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
Pharyngolaryngeal Pain	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)

N is the number of subjects exposed per treatment, each recorded event is the number (and percentage) of subjects that experienced the AE per treatment.

B European Protocol (single dose)

Body System Preferred Team	Placebo N=15	5 mg N=9	25 mg N=9	150 mg N=9
Respiratory, Thoracic, Mediastinal Disorders	15 (100%)	9 (100%)	8 (89%)	9 (100%)
Rhinorrhoea	9 (60%)	8 (89%)	2 (22%)	5 (56%)
Epistaxis	6 (40%)	4 (44%)	4 (44%)	3 (33%)
Nasal Mucosal Discolouration	6 (40%)	7 (78%)	2 (22%)	3 (33%)
Nasal Oedema	6 (40%)	8 (89%)	5 (56%)	8 (89%)
Nasal Disorder	4 (28%)	3 (33%)	0 (0%)	4 (44%)
Pharyngolaryngeal Pain	4 (28%)	0 (0%)	0 (0%)	2 (22%)
Intranasal Numbness	1 (7%)	0 (0%)	0 (0%)	0 (0%)
Nasal Septum Ulceration	1 (7%)	0 (0%)	0 (0%)	0 (0%)

N is the number of subjects exposed per treatment, each recorded event is the number (and percentage) of subjects that experienced the AE per treatment.

C European Protocol (multi dose)

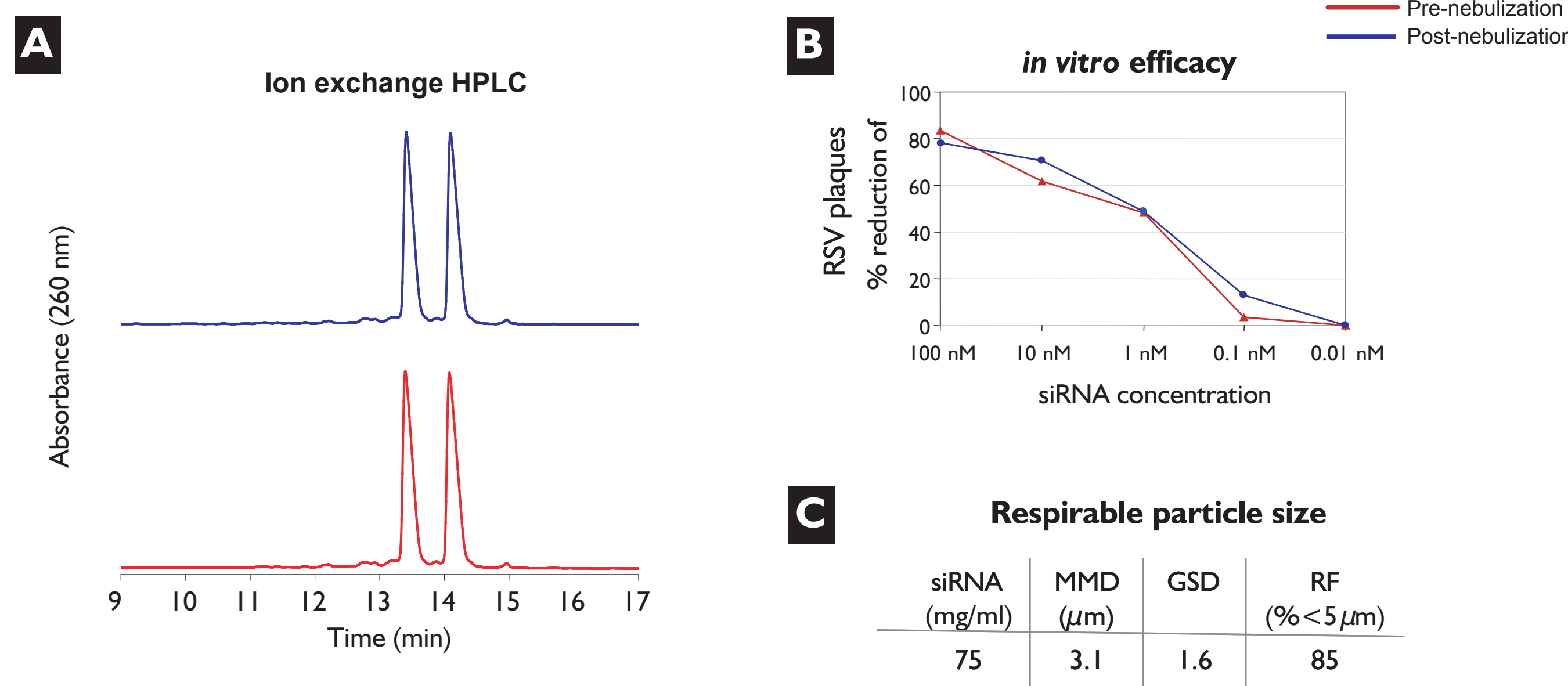
Body System Preferred Team	Placebo N=11	5 mg N=5	25 mg N=4	150 mg N=5
Respiratory, Thoracic, Mediastinal Disorders	11 (100%)	5 (100%)	4 (100%)	5 (100%)
Nasal Oedema	8 (73%)	0 (0%)	3 (75%)	2 (40%)
Rhinorrhoea	6 (55%)	0 (0%)	1 (25%)	4 (80%)
Nasal Mucosal Discolouration	5 (46%)	1 (20%)	1 (25%)	1 (20%)
Epistaxis	4 (36%)	3 (60%)	4 (100%)	1 (20%)
Cough	2 (18%)	2 (40%)	0 (0%)	0 (0%)
Nasal Disorder	2 (18%)	1 (20%)	1 (25%)	0 (0%)
Nasal Congestion	1 (9%)	1 (20%)	1 (25%)	0 (0%)
Nasal Discomfort	1 (9%)	1 (20%)	0 (0%)	1 (20%)
Throat Irritation	1 (9%)	0 (0%)	0 (0%)	0 (0%)

N is the number of subjects exposed per treatment, each recorded event is the number (and percentage) of subjects that experienced the AE per treatment.

Summary of Phase I Intranasal Studies

- ◆ ALN-RSV01 appears safe when administered in relevant doses to human respiratory epithelium
 - ❖ Adverse event profile comparable to placebo
 - ❖ No drop outs
 - ❖ No serious adverse events
 - ❖ No laboratory or EKG abnormalities
- ◆ Pharmacokinetics consistent with little systemic exposure
- ◆ Cumulative clinical experience with 65 exposed subjects is encouraging

Figure 2. Characterization of aerosolized ALN-RSV01

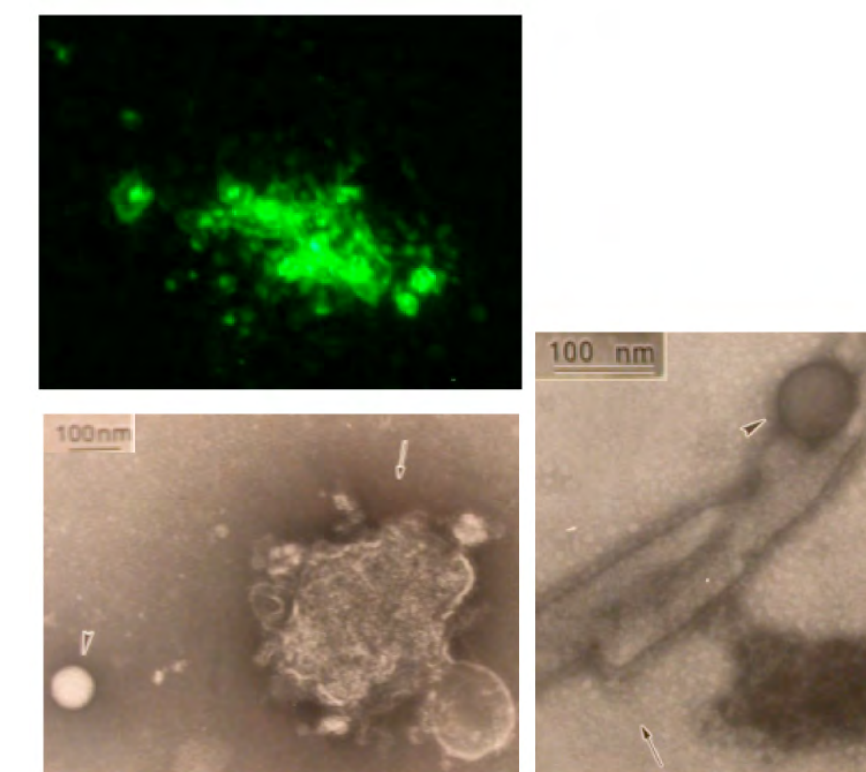


ALN-RSV01 was collected from the nebulizer bowl before nebulization and collected in a rain-out chamber after nebulization. Aerosolization using jet nebulizer and Pari eFlow nebulizers produced similar results. Both collections were (A) analyzed by ion exchange HPLC (B) analyzed *in vitro* in RSV plaque reduction assay by transfection into Vero cells subsequently infected with RSV A2 (C) The aerosol particle size distribution was determined by both laser diffraction and cascade impactation methods (MMD: mass median diameter, GSD: geometric standard deviation, RF: respirable fraction)

Plan for Clinical Proof of Concept

Develop and manufacture a safe, low passage, wild type RSV strain

- ◆ Original nasal aspirate from hospitalized infant with known high RSV load (RSV infection identified by FDA approved method)
- ◆ Plaque purified on Vero GMP cell line from initial nasal aspirate
- ◆ 5 subsequent passes in Vero GMP cell line
- ◆ RSV identity by sequence, culture, IFA and electron microscopy
- ◆ No other adventitious agents identified
- ◆ PCR negative for other human pathogens
- ◆ Sterility testing completed and passed
- ◆ Manufactured and individually vialled under GMP



Electron Micrographs were produced by evaluating vials of GMP-produced RSV. Vial contents were mixed with known quantity of 108nm latex spheres for viral particle count determination, fixed with buffered glutaraldehyde, washed with ultrapure water, stained with phosphotungstic acid, and examined in a Hitachi HT7000 Transmission Electron Microscope. No adventitious agents, fungi, bacteria, or other viruses were identified. Additional RSV identity testing included partial N-gene sequence, and by FDA approved culture methodology followed by terminal Indirect fluorescent pan-RSV antibody confirmation.

Develop RSV experimental infection

Goal

- ◆ Develop an experimental human RSV infection model in healthy subjects that allows safe and reproducible infection, with measurable and reproducible viral and disease outcomes

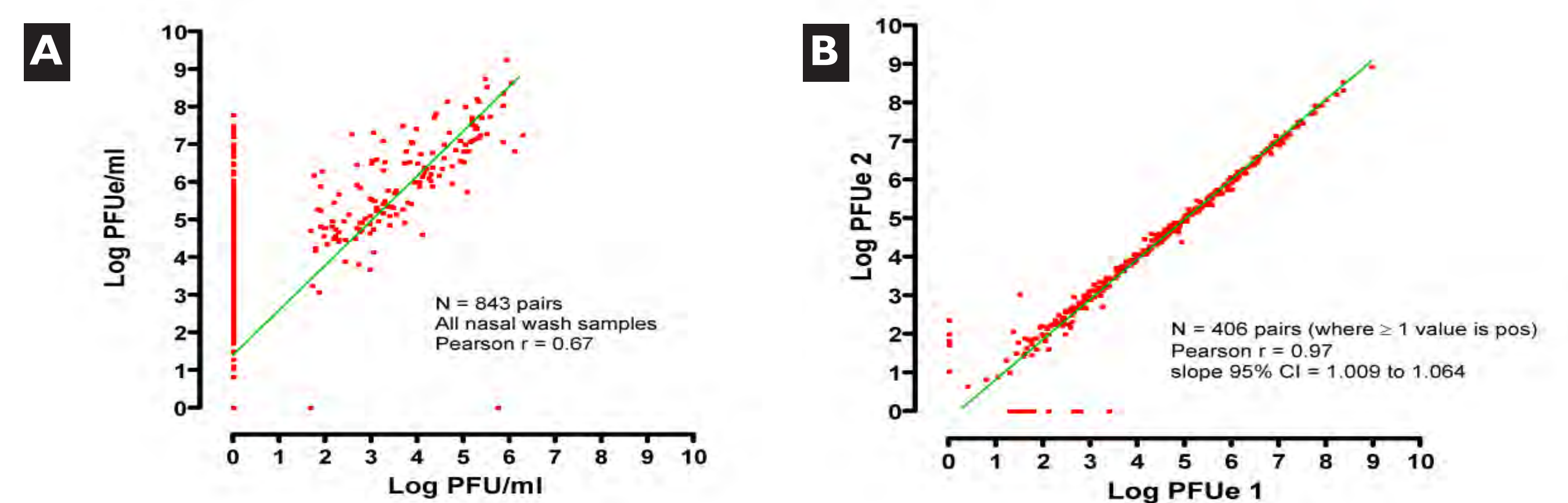
Protocol

- ◆ 36 subjects
- ◆ Cohorts receiving escalating quantity of RSV inoculum
- ◆ Interim safety evaluation after each cohort

Evaluations

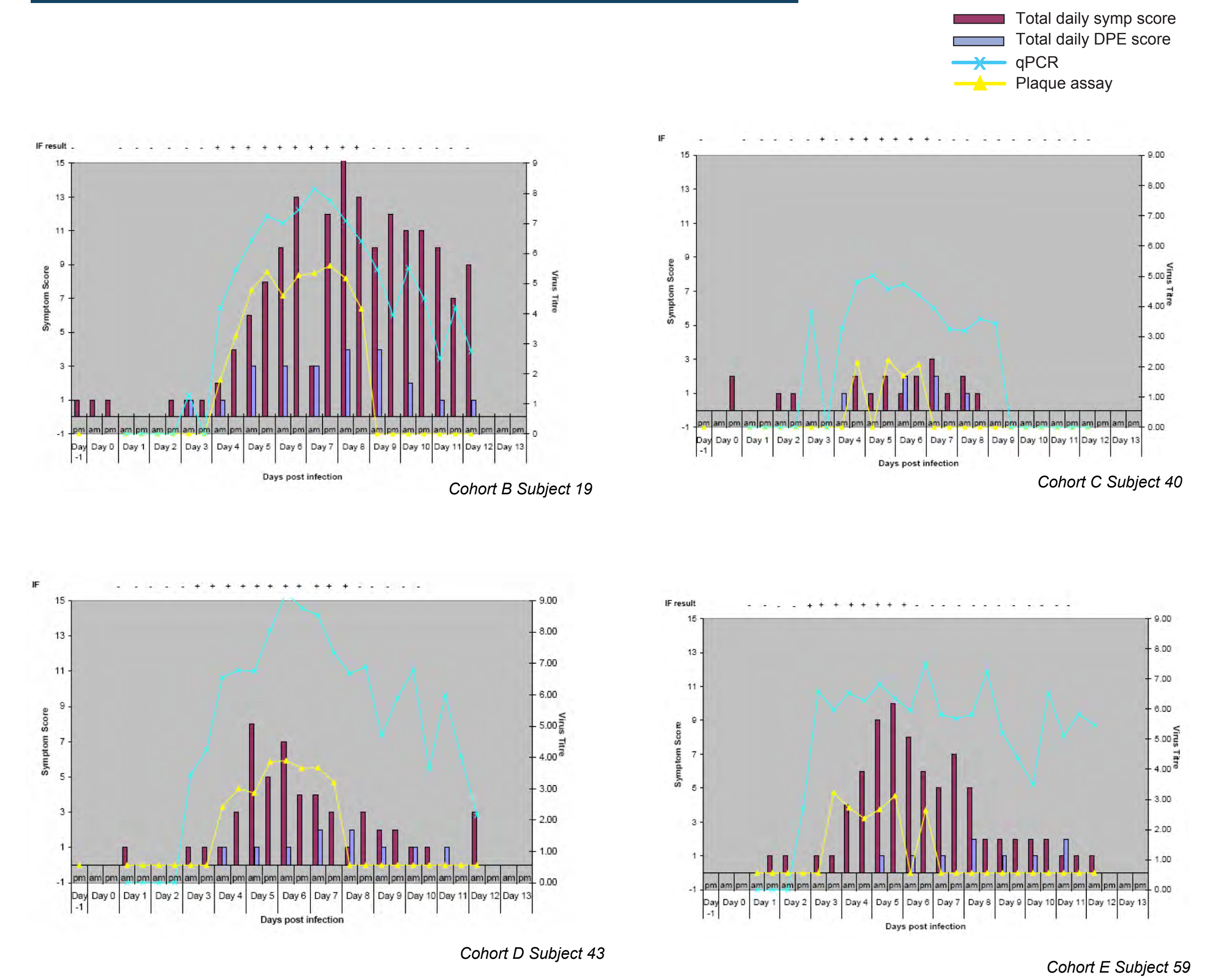
- ◆ Safety evaluations
 - ❖ Laboratory evaluations
 - ❖ Adverse event recordings
- ◆ Viral measurements from nasal wash
 - ❖ Quantitative culture (fresh plaque assay)
 - ❖ Quantitative real time RT-PCR
 - ❖ Spin-enhanced culture
- ◆ Measurements of RSV disease
 - ❖ Self assessed symptom score
 - ❖ Physician exam score
 - ❖ Others

Figure 6. Comparison of Quantitative RSV Assays from All Nasal Washes



(A) Quantitative Culture (Log PFU/ml) vs quantitative PCR (Log PFUe/ml). The units for quantitative PCR are based on viral RNA extracted from aliquots containing known plaque assay quantity of Hep-2 cell cultured RSV-A long standards. These same aliquoted standards were also run in parallel with every plaque assay. The nasal washes were collected quantitatively into cold RSV stabilization media and placed fresh onto HEP-2 cell plates within approximately 20 minutes. Quantitative PCR was performed on parallel aliquots of these nasal washes after freezing at -80°C. (B) Precision of Quantitative PCR assay. QPCR assays were performed using a robotic extractor (Qiagen Biorobot EZ-1) incorporating magnetic silica beads. Real Time Reverse Transcriptase PCR was performed using specific sets of RSV-A primers and probes on an ABI-7900i machine as previously reported and validated in clinical specimens (Perkins, DeVincenzo et al. J Clin Micro 23(5):2356-62, 2005). Nasal wash samples were run in duplicate with each 96 well plate having its own internal standard curve. Only data sets where at least one of the duplicate samples were positive by PCR were included in this graph.

Figure 4. Selected Individual Subject Data Sets



Cohorts of volunteers were inoculated with increasing amounts of GMP RSV on day 0. Twice daily nasal wash collection was performed, and daily directed physical exam (DPE) scores were obtained by a physician assigned to that cohort. Twice daily volunteer's self assessed symptom scores were obtained. Nasal wash fluid was aliquoted and placed fresh onto HEP-2 cell plaque assays. Nasal wash fluid was also placed fresh into spin enhanced cultures followed by immunofluorescent staining for RSV after 48 hours of incubation (see "IF result" for each time point at top of each figure). Quantitative PCR (qPCR) was performed on frozen aliquots. All viral and disease assessments were stopped on study day 12. Safety evaluations continued through day 28.

Figure 5. Experimental Infection summary Table

Total volunteers: 36*
% completing study: 97
% infection (qPCR): 72

	Spin-enhanced	Quantitative Culture	qPCR	Lee et al *: Quant Culture
AUC viral load** (Log ₁₀ PFU-days)	NA	26.6 (+/-12.4)	61.9 (+/-27.8)	10.6 (+/-8.9) (log TCID ₅₀ -days)
Duration of Shedding** (Days)	4.9 (+/-1.4)	3.7 (+/-1.1)	7.0 (+/-2.6)	4.7 (+/-2.4)
Incubation Period (Days)	3.5 (+/-1.7)	3.5 (+/-1.7)	3.2 (+/-1.7)	3.1 (+/-1.0)

*Lee et al. Antiviral Research. 63(2004) 191-6
**calculated on subjects who were infected.

Area Under the Curve (AUC) is calculated as the sum of the rectangles formed by time and log quantity of RSV (by PCR) for each tested time point for the duration of testing. Each subject was tested twice daily for a total of 12 days from the day after inoculation. PFU = Plaque Forming Units. The units for qPCR are in Plaque Forming Unit Equivalents/ml. AUC and duration of shedding are calculated only from those subjects who were infected (who had two consecutive positive viral quantities detected in their nasal washes between days 2 and 8 after inoculation). Numbers in parenthesis represent +/- Standard Deviation. Comparison is made to recently published description of a different RSV infection model using a laboratory strain of RSV which is no longer available.

Conclusions

- ◆ RNA interference (RNAi) is a natural process existing in all cells
- ◆ ALN-RSV01 is an siRNA utilizing this RNAi pathway targeting RSV and achieves ≈4 log reductions in virus *in vivo* with a single 2mg/kg dose delivered topically to the respiratory tract.
- ◆ ALN-RSV01 is the first RNAi based therapy to be tested in humans that targets an infectious disease
- ◆ ALN-RSV01 can be aerosolized into respirable particles while maintaining its structure and function
- ◆ ALN-RSV01 administered intranasally appears safe and well tolerated at doses up to 150mg (≈2mg/kg)
- ◆ Experimental wild type RSV infection model has been established achieving safe and robust viral quantitative detection and percent infection
- ◆ This experimental RSV infection model will be used in a proof of concept study designed to show antiviral effect in man