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INHERITED DISEASE RESEARCH ARTICLE

Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis

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The major cause of mortality in patients with cystic fibrosis (CF) is lung disease. Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene product in the airways is a potential treatment. Clinical studies in which the CFTR cDNA was delivered to the respiratory epithelia of CF patients have resulted in modest, transient gene expression. It seems likely that repeated administration of the gene transfer vector will be required for long-term gene expression. We have undertaken a double-blinded study in which multiple doses of a DNA/liposome formulation were delivered to the nasal epithelium of CF patients. Ten subjects received plasmid DNA expressing the CFTR cDNA complexed with DC-Chol/DOPE cationic liposomes, whilst two subjects received placebo. Each subject received three doses, administered 4 weeks apart. There was no evidence of inflammation, toxicity or an immune response towards the DNA/liposomes or the expressed CFTR. Nasal epithelial cells were collected 4 days after each dose for a series of efficacy assays including quantitation of vector-specific DNA and mRNA, immunohistochemistry of CFTR protein, bacterial adherence, and detection of halide efflux ex vivo. Airway ion transport was also assessed in vivo by repeated nasal potential difference (PD) measurements. On average, six of the treated subjects were positive for CFTR gene transfer after each dose. All subjects positive for CFTR function were also positive for plasmid DNA, plasmid-derived mRNA and CFTR protein. The efficacy measures suggest that unlike high doses of recombinant adenoviral vectors, DNA/liposomes can be successfully re-administered without apparent loss of efficacy. Gene Therapy (2000) **7,** 1156–1165.

Keywords: *cystic fibrosis; gene therapy; clinical trial; cationic liposome; multiple dose*

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was identified and the cDNA cloned in 1989.[1] This landmark event led to a greater understanding of the molecular biology of cystic fibrosis (CF), a recessive genetic disease caused by mutations in the *CFTR* gene.[1] CFTR is a cAMP-activated chloride channel in the apical membrane of epithelial cells, and loss of CFTR leads to impaired electrolyte movement in a variety of organs.[2] However, it is the loss of CFTR function in the lung that is now the main cause of morbidity and mortality in CF.[3]

Gene transfer to the airways, to correct the underlying genetic defect, is a potential treatment for CF. Early successes in cultured cells[4] and animal models[5] led to a number of clinical studies which demonstrated proof of concept for the expression of CFTR cDNA in human CF airways. The gene delivery systems investigated to date include recombinant adenovirus, recombinant adeno-associated virus (AAV), and cationic liposomes. All have resulted in relatively low levels of gene expression in the airways and modest correction of the CF chloride transport defect.[6]

The efficiency of recombinant adenoviral gene transfer to the undamaged epithelium is low,[7] and high doses of adenovirus vectors can result in an inflammatory response.[8] Moreover adenoviral gene expression is transient, necessitating repeat administration; clinical studies have shown that adenoviral gene transfer results in loss of efficacy upon repeat administration, due to the production of neutralising antibodies.[9] AAV may prove useful for CFTR airway gene transfer in the future, although currently, insufficient clinical data are available for evaluation of this vector.[10]

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As an alternative to viruses, DNA/liposomes have a number of potential advantages for gene delivery.[6] We and others have reported clinical studies in which a single dose of DNA/liposomes was administered to the nasal[11–14] or lung epithelia.[15] In all of these studies gene transfer was demonstrated, and in some cases, correction of the CF chloride channel defect was observed. No serious adverse reactions have been observed following delivery to the nasal epithelium, although delivery of a gene transfer formulation containing the cationic lipid GL67 to the lung, resulted in mild influenza-like symptoms which resolved within 36 h without further intervention.[15]

We have focused on a DNA/liposome formulation containing the CFTR expression plasmid pTRIAL10CFTR2[16] and the cationic liposome DC-Chol/DOPE.[17] This formulation has transiently corrected the CF chloride channel defect in the nasal[16] and tracheal epithelia of mice after single and multiple doses.[18] We have previously reported the results of a single-dose clinical study in the nasal epithelium of CF patients.[12] In that study, the correction observed with our formulation was also transient, confirming the need for repeated administration. We have therefore undertaken a double-blinded clinical study to evaluate the safety and efficacy of multiple doses of our DC-Chol/DOPE formulation to the nasal epithelium.

Results

Study design and clinical evaluation

Twelve CF patients were enrolled in the study: 10 received DNA/liposomes and two received a placebo (Table 1). Each subject attended the clinic as an outpatient on 24 occasions receiving three doses of DNA/liposomes or placebo, administered 4 weeks apart (Table 2). Subject B2 withdrew from the study 2 days after the first dose for reasons unrelated to the study. The remaining 11 subjects completed the study and all procedures were well tolerated.

The subjects showed no significant changes in their vital signs, blood chemistry or respiratory function tests (data not shown). Six subjects had symptoms of upper respiratory tract infections (rhinovirus cultured from patient A2), and in five out of the six subjects the symptoms were accompanied by characteristic changes in nasal PD measurements (see below). Subjects continued with their normal treatments, including the use of oral or intravenous antibiotics by subjects A1, A2, B3 and B5. None of the subjects displayed any symptoms that were outside the range expected for adults with CF. No subjects complained of discomfort in their nose following dosing, and nasal epithelia appeared normal by inspection. There were no clinical deteriorations in liver, kidney or bone marrow function (data not shown). One subject, B4, developed distal ileal obstruction syndrome (DIOS) on day 8 of the study, necessitating admission to hospital for treatment, after which his condition improved over the course of 1 week. Consistent with DIOS, the subject showed an increase in serum inflammatory markers (specifically C reactive protein and to a lesser extent, total white cell count) which returned to pre-study levels by the end of the study.

Immunology

A potential complication of gene therapy is autoimmune disease if the host is immunologically intolerant of the gene product. Therefore we looked for evidence of autoimmune disease and specific activation of the immune system against CFTR, before, during and after completion of the study. No evidence of immune activation, specifically autoimmune or cellular immune responses towards CFTR, was observed in any subject. The subjects had normal lymphocyte subset populations throughout the study. IL2R expression on lymphocytes was not increased and serum levels of cytokines (TNFα, IL6) remained constant throughout the study (data not shown). No anti-CFTR auto-antibodies were detected in any of the subjects and there were no *in vitro* lymphocyte proliferative responses towards CFTR (data not shown). There was no evidence of immune activation by DNA/liposomes or CFTR, as IL2R expression was unaltered in peripheral blood cultures and on challenge in cultures (data not shown).

Histology

Biopsies were taken from the nasal septum 7 days after the final dose. In one case (B4) biopsy was unsuccessful. Nasal biopsies from the two placebo-treated subjects demonstrated mild chronic (A4) and moderate chronic inflammation (B5). In two subjects receiving

Table 1 Subject details and dosing

Subject No.	*Genotype*	*Dose*	*Sex*	*Age (years)*	*FEV1 (litres)*	*Clinical score*
A1	ΔF508/1078delT	CFTR	M	22.7	3.25	85
A2	ΔF508/ΔF508	CFTR	M	22.9	3.4	90
A3	ΔF508/G542X	CFTR	M	28.5	1.75	70
A5	ΔF508/F945L	CFTR	M	30.3	3.05	70
A6	ΔF508/ΔF508	CFTR	M	24.4	1.25	70
B1	ΔF508/ΔF508	CFTR	M	17.6	3.8	90
B3	ΔF508/1898+1(G>A)	CFTR	F	16.9	2.15	80
B4	ΔF508/G551D	CFTR	M	32.8	1.8	55
B6	ΔF508/ΔF508	CFTR	M	18.8	3.5	95
A4	ΔF508/G551D	Placebo	F	16.5	2.65	95
B5	ΔF508/3659delC	Placebo	M	16.4	2.8	85

All patients were pancreatic insufficient. All values shown are baseline values.
FEV1, forced expiratory volume in 1 s; clinical score: Shwachman–Kulczycki clinical score out of a maximum of 100.[35]

Table 2 Study timetable

Dose	*MD1*							*MD2*							*MD3*						
Study day	*0*	*2*	*4*	*7*	*9*	*11*	*24*	*0*	*2*	*4*	*7*	*9*	*11*	*24*	*0*	*2*	*4*	*7*	*9*	*11*	*24*
Dosing	×							×							×						
Clinical examination	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Nasal PD		×	×	×	×	×	×		×	×	×	×	×	×		×	×	×	×	×	×
Blood samples		×			×		×		×			×		×		×			×		×
Nasal brushing			×							×							×				
Nasal biopsy																		×			

Timetable to show the three consecutive dosing cycles (MD1, MD2 and MD3), each lasting 4 weeks. Pre-study measurements (clinical examination, blood tests, nasal PD measurements and nasal brushings) were made in the 2 weeks before administration of MD1. The days on which the following procedures were performed are shown.
Dosing, treatment administered; clinical examination, clinical examination of patient (including respiratory function tests); nasal PD, transepithelial potential difference measurement; blood samples, blood taken for a variety of investigations (see Materials and methods[12]); nasal brushing, nasal cells collected and divided between five assays (see Materials and methods); nasal biopsy, biopsy of nasal epithelium from subject's left nostril was performed only once during the study, after the third dose. Blood samples were also taken 6 months after completion of the study for evaluation of immunology and determination of serum cytokine levels.

DNA/liposomes there was evidence of marked acute (A1) and chronic (B6) inflammation with a large proportion of eosinophils suggesting an allergic component. In the remaining six subjects the biopsies were normal or demonstrated evidence of mild inflammation. All these observations were consistent with the biopsy site chosen and similar to control CF and non-CF biopsies,[12] indicating that levels of inflammation were within normal limits (data not shown).

Plasmid DNA delivery

Cells were collected from the nasal epithelium and divided for assays of plasmid DNA delivery, plasmid-derived mRNA expression, CFTR protein expression by immunocytochemistry, and CFTR protein function by halide efflux and bacterial adherence. Delivery of plasmid DNA to nasal cells was determined using quantitative TaqMan PCR (Figure 1). All nine subjects who received DNA/liposomes were positive for plasmid DNA delivery for one or more doses. Four subjects were positive for two of the three doses and one subject (B4) was positive after all three doses. In total, 60% (15/25) of the samples were positive for plasmid DNA. No plasmid DNA was detected in any sample before delivery of the first dose of DNA/liposomes, or in any sample from subjects receiving placebo. There was no apparent trend in the numbers of samples positive for plasmid DNA to indicate that any dose was more or less effective than any other.

Plasmid DNA-derived mRNA expression

Vector-derived mRNA expressed in nasal cells was quantitated using TaqMan RT-PCR. Of the nine subjects who received DNA/liposomes, seven were positive for plasmid-derived mRNA for one or more doses; two subjects were positive after both the second and third dose, and one subject (B4) was positive after all three doses. In total, 44% (11/25) of the samples were positive for plasmid-derived mRNA (Figure 2). As in the case of plasmid DNA, there was no apparent trend in the numbers of samples positive for plasmid-derived mRNA after each dose. In all cases where it was possible to determine, samples that were positive for plasmid-derived mRNA were also positive for plasmid DNA delivery (Figures 1 and 2). Four patient samples had levels of plasmid-derived mRNA greater than 5% of their endogenous CFTR mRNA levels, the highest (subject A1 after the first dose) at 32%. No plasmid-derived mRNA was detected in any sample before delivery of the first dose of

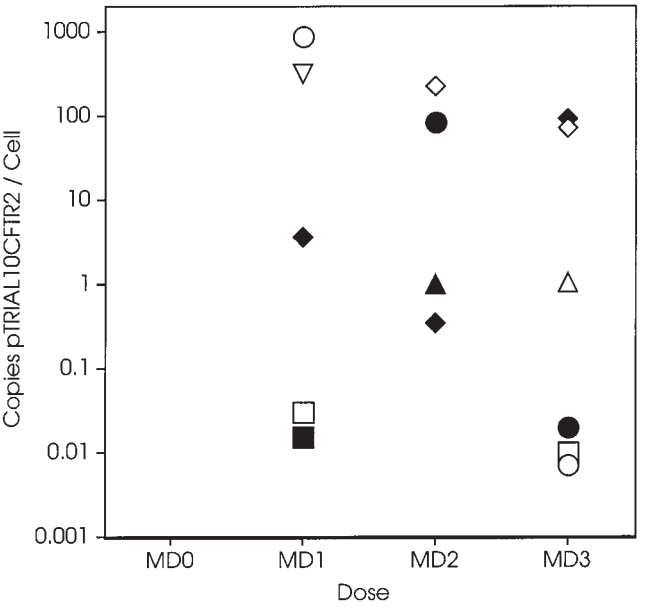

Figure 1 *Plasmid DNA delivery. The number of copies of pTRIAL10CFTR2 plasmid DNA per cell (see Materials and methods) collected from patients by nasal brushing is plotted for each subject after each dose. MD0, MD1, MD2 and MD3 refer to nasal brushings taken before the first dose, and 4 days after the first, second and third doses, respectively. Treated patient data are plotted: A1 (open circle); A2 (filled circle); A3 (open square); A5 (filled square); A6 (open triangle); B1 (filled triangle); B3 (open diamond); B4 (filled diamond); B6 (open inverted triangle). The mean level of plasmid DNA detected in the positive samples was 109 copies per cell, although the level varied between 0.007 and 857 copies per cell. The MD0 sample for patient B3, and the MD2 samples for patients A3 and A5, contained no detectable genomic or plasmid DNA (therefore plasmid DNA levels were not determined). All samples from placebo-treated patients, and all other MD0 samples, contained detectable genomic DNA, but no detectable plasmid DNA (therefore were negative for plasmid DNA).*

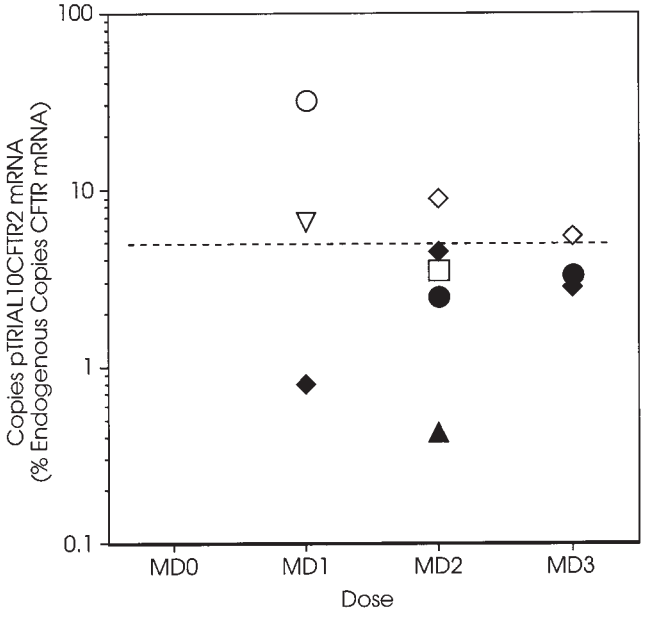

Figure 2 *Plasmid DNA-derived mRNA expression. The number of copies of pTRIAL10CFTR2 derived CFTR mRNA (expressed as a percentage of the number of copies of endogenous CFTR mRNA) in cells isolated by nasal brushing is plotted for each subject after each dose. Details are as in legend to Figure 1. The MD0 sample for patient B3 and the MD2 samples for patients A5 and B6 contained no detectable endogenous CFTR mRNA or plasmid-derived CFTR mRNA (therefore plasmid-derived CFTR mRNA levels were not determined). All samples from placebo-treated patients, and all other MD0 samples, contained detectable endogenous CFTR mRNA but no detectable plasmid-derived CFTR mRNA (therefore were negative for plasmid-derived CFTR mRNA). Samples above the dashed line have transgene expression levels greater than 5% of endogenous CFTR mRNA.*

DNA/liposomes, or in any sample from subjects receiving placebo.

CFTR immunohistochemistry

Plasmid-encoded CFTR protein expressed in ciliated cells after treatment, was detected using anti-CFTR antibody titrated such that the subjects' levels of endogenous mutant CFTR protein remained undetectable.[19] Of the nine subjects who received DNA/liposomes, six were positive for CFTR protein after one or more doses; one subject (A1) was positive for two of the three doses. In total 28% (7/25) of the samples were positive for CFTR protein (Figure 3 and Table 3). Six of the seven samples that were positive for CFTR protein were also positive for plasmid-derived mRNA (Figures 2 and 3). In positive samples, the percentage of ciliated epithelial cells expressing CFTR protein varied from 2 to 15%; cells from non-CF individuals were appproximately 80% positive (Figure 3 and data not shown). No CFTR protein was detected in any sample before delivery of the first dose of DNA/liposomes or in any sample from subjects receiving placebo.

Evaluation of CFTR function by halide efflux (SPQ analysis)

Of the nine subjects who received DNA/liposomes, five were positive after one or more doses; one subject (B3) was positive after the second and third doses. In all, 22% (6/27) of the samples were positive for CFTR function (Figure 4). There was no apparent trend in the numbers of positive samples after each dose (2/9, 2/7 and 2/9 for the first, second and third doses, respectively). No cAMP-dependent halide efflux was detected in any sample before the delivery of the first dose of DNA/liposomes. In all cases where it was possible to determine, samples that were positive for CFTR function were also positive for plasmid DNA delivery, plasmid-derived mRNA and CFTR protein (Figure 1, 2, 3, 4; 4/6 positive for all assays; 2/6 positive for three assays: one not determined for plasmid DNA delivery, one not determined for CFTR protein). There were no positive samples before dosing, or from subjects that received placebo.

Bacterial adherence

An additional assay of CFTR function was included in this study, based on the finding that freshly isolated, ciliated airway epithelial cells from CF patients show increased binding of *Pseudomonas aeruginosa*, which is dependent on the absence of normal CFTR.[20] Figure 5 shows that adherence of *P. aeruginosa* to ciliated cells from control CF and non-CF individuals was significantly different ($P = 0.004$, Mann–Whitney U test). However in this study the bacterial adherence assay was not robust due to high losses of epithelial cells, such that only 60% (16/27) post-dose samples were processed successfully. Although the number of samples processed successfully after each dose was small, there was an apparent trend towards the non-CF range in samples taken after the third dose (Figure 5).

PD analysis

Measurements of basal potential difference and the response to amiloride were taken as indicators of basal sodium movement.[21] Overall, there were no statistical differences in basal PD between representative CF values and measurements made before the study (MD0) and after each of the doses (MD1, MD2, MD3) ($P = 0.22$, Kruskal–Wallis; Figure 6). Basal PD was significantly reduced in 11 individual measurements in five subjects with symptoms of upper respiratory tract infections; these were predominantly in the MD3 measurement period explaining, in part, the apparent trend in MD3 values towards the non-CF range (Figure 6). Similarly, there were no significant changes in the response to amiloride (data not shown). Perfusion of a low chloride solution was used to assess the chloride secretory capacity of the airway epithelium. Although several individual post-dose measurements were in the range of representative non-CF values, overall there were no statistical differences in low chloride PD between MD0, MD1, MD2, MD3 measurements and representative CF values ($P = 0.38$, Kruskal–Wallis; Figure 7).

Discussion

Gene expression following the administration of DNA/liposomes to the respiratory epithelium of CF patients is transient.[11–13] Therefore, repeated administration is likely to be required for long-term gene expression. We have previously shown that repeated administration of DNA/liposomes can correct the CF ion transport defect in mice[18] and here we describe a clinical study to evaluate repeated administration of DNA/liposomes in CF patients.

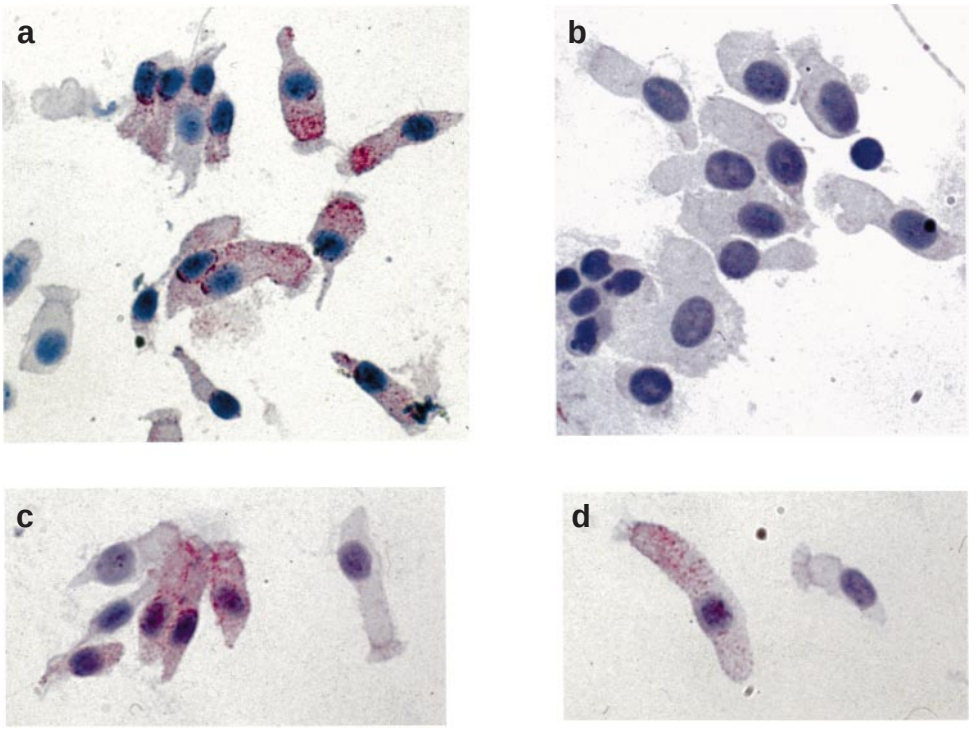

Figure 3 *Immunohistochemistry. Representative images of CFTR immunohistochemical staining of nasal brushing cells are shown. (a) Typical sample from a non-CF individual; (b) typical sample from a CF subject (A1) before the first dose; (c and d) representative post-dose samples from subjects receiving DNA/liposomes (A1 and B3) showing positive CFTR immunoreactivity (punctate pink/red staining) in some cells. All micrographs are approximately ×300.*

Table 3 Summary of efficacy assays

Assay	*MD1*				*MD2*				*MD3*			
	D	*R*	*P*	*S*	*D*	*R*	*P*	*S*	*D*	*R*	*P*	*S*
A1	+	+	+	+			+		+			
A2					+	+			+	+	+	+
A3	+				n	+	+	+	+			
A5	+				n	n						
A6									+			
B1					+	+	+					
B3					+	+	n	+	+	+	+	+
B4	+	+			+	+			+	+		
B6	+	+	+	+		n	n					

Positive samples for four independent assays of efficacy for each patient after each dose are shown (+).
D, detection of plasmid DNA; R, detection of vector specific mRNA; P, detection of CFTR protein by immunocytochemistry; S, increase in halide efflux (SPQ); n, result was not determined for this sample.
No sample taken before the first dose, or taken from subjects receiving placebo (A4 and B5) were positive for these assays.

The safety and efficacy of repeated administration of DC-Chol/DOPE liposome-mediated CFTR gene transfer, was assessed using the respiratory epithelium of the nasal cavity as a surrogate tissue for the lung. This was the first clinical study to evaluate repeat dosing of DNA/liposomes for any genetic disease. As in our previous single dose study,[12] no serious adverse effects were observed and there were no significant changes in any of the clinical parameters measured. It is possible that the development of an abnormal immune response towards expressed products following gene transfer could have serious long-term consequences for CF gene therapy. Individuals with CF may have immune tolerance towards normal CFTR, depending on their specific mutation(s). In this study, expression of *CFTR* could have resulted in an immune response against the gene product if the CF subjects had no immune tolerance. However, after three doses of *CFTR* DNA/liposomes, no autoantibodies or cellular immune responses against CFTR were detected.

In the absence of a definitive, surrogate, clinical endpoint marker for CF, a series of efficacy assays was performed to assess CFTR gene transfer after repeated administration. A summary of the efficacy data is shown

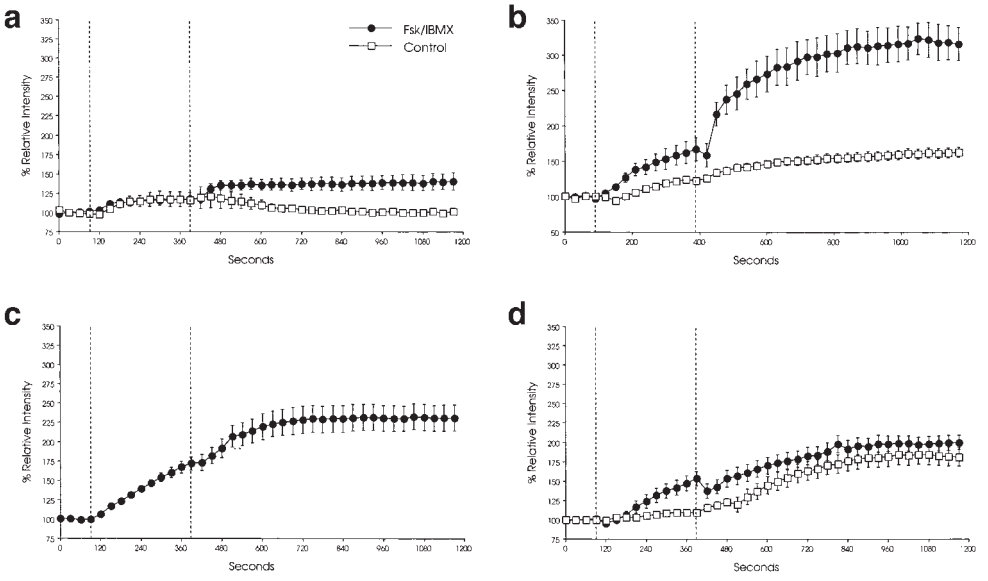

Figure 4 *Evaluation of CFTR function by halide efflux (SPQ analysis). Representative SPQ analysis from nasal brushing cells for one patient (B6) are shown. (a) MD0; (b) MD1; (c) MD2; and (d) MD3. Patient B6 was selected to illustrate a typical CF response in an MD0 sample (a); typical post-treatment positive response (b, MD1); typical post-treatment negative responses (c, MD2 and d, MD3). Where possible, a comparison of the response to cAMP agonists (forskolin/IBMX, filled circles) with control treatment (open squares) is shown; in the event of insufficient cell harvest (c) the response to cAMP agonists only was investigated. Vertical dashed lines at 90 and 390 s indicate changes from sodium iodide to sodium nitrate buffer systems (see Materials and methods) and sodium nitrate to sodium nitrate ± cAMP agonists, respectively. Percentage relative intensity of fluorescence (see Materials and methods) is plotted against time (s). Error bars represent s.e.m.*

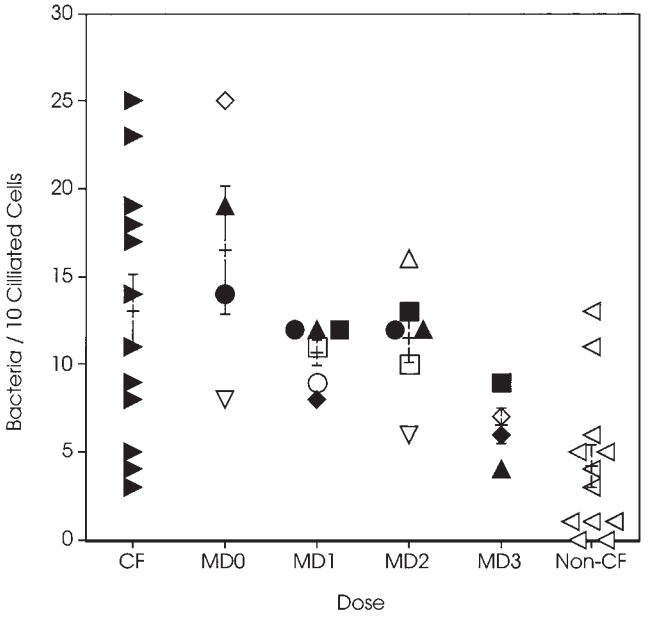

Figure 5 *Bacterial adherence. The number of bacteria adhering to ciliated epithelial cells is plotted for each subject after each dose. Details are as in legend to Figure 1. Representative data from CF (n = 12) and non-CF individuals (n = 12) are also shown. In total, 16/36 samples were not determined. Means (+) and error bars (s.e.m.) are shown.*

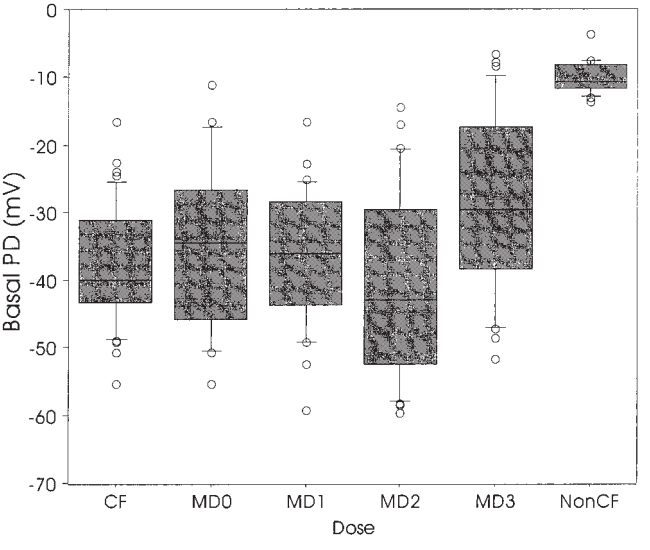

Figure 6 *Basal in vivo nasal potential difference measurements. Basal nasal potential difference measurements are plotted as box-plots (indicating 10th, 25th, 50th, 75th and 90th centile) plus outliers (open circles) after each dose. In this figure, MD0 refers to all measurements for each individual taken before the first dose. MD1, MD2 and MD3 refers to the mean of measurements taken 2, 4 and 7 days after the first, second and third doses respectively (Table 2). Representative data from CF (n = 43) and non-CF individuals (n = 22) are also shown. There were no statistical differences in basal PD between MD0, MD1, MD2, MD3 measurements and representative CF values (P = 0.22, Kruskal–Wallis). Within group comparisons of pre- (the two most recent preceding measurements) and post- (2, 4 and 7 days after) dose measurements also showed no statistical differences (comparisons of MD0 with MD1 P = 0.61; pre-MD2 with MD2 P = 0.95 and pre-MD3 with MD3 P = 0.21, Wilcoxon signed rank).*

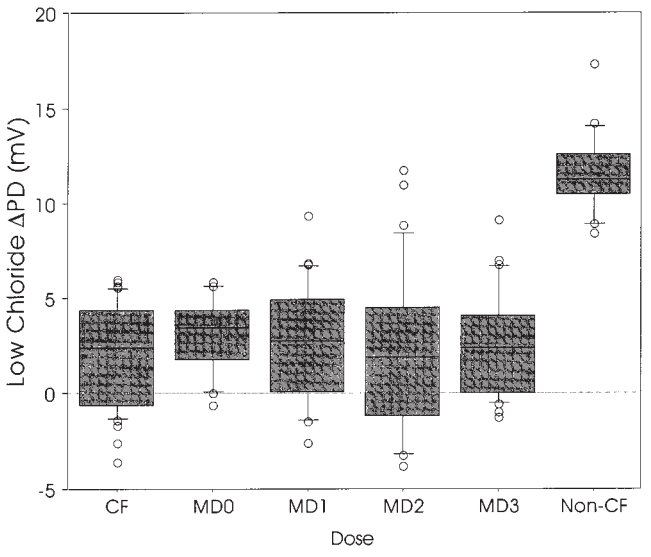

Figure 7 *Low chloride in vivo nasal potential difference measurements. Low chloride nasal potential difference measurements are plotted as boxplots. Details are as in legend to Figure 6. There were no statistical differences in low chloride PD between MD0, MD1, MD2, MD3 measurements and representative CF values (P = 0.38; Kruskal–Wallis). Within group comparisons of pre- and post-dose measurements (as in legend to Figure 6) also showed no statistical differences (comparisons of MD0 with MD1 P = 0.53; pre-MD2 with MD2 P = 0.88 and pre-MD3 with MD3 P = 0.27, Wilcoxon signed rank).*

in Table 3. The technical failure rate of these assays was low (plasmid DNA 3/36, plasmid-derived mRNA 3/36, CFTR protein 2/36 and CFTR function 0/36); indicating that these assays are robust and suited for use in clinical studies. The exception was the bacterial adherence assay, which in our hands was not robust and would need to be significntly improved before inclusion in future clinical work.

As might be expected, the frequency of samples positive for detection of plasmid DNA (15/25) was greater than the frequency of samples positive for plasmid-derived mRNA (11/25), and the latter was more frequent than samples positive for CFTR protein (7/25) or CFTR function (6/27). There was a good correlation between the efficacy assays, such that all samples that were positive for plasmid-derived mRNA, were also positive for plasmid DNA; and all samples positive for CFTR function were also positive for plasmid DNA, plasmid-derived mRNA and CFTR protein. Such correlations have proven difficult to achieve in some clinical studies of CFTR gene transfer. In particular, there has been a poor correlation between the detection of vector-specific DNA and mRNA, and indicators of CFTR function.[11,15] The use of highly sensitive, highly reproducible and truly quantitative TaqMan (RT)-PCR can greatly facilitate the molecular analysis of gene transfer in clinical studies.[22,23] The application of TaqMan PCR technology in our study contributed to the increased consistency observed between the different efficacy assays reported here.

There was no apparent trend in numbers of positive samples for any of the efficacy assays after any of the doses. These data are consistent with DC-Chol/DOPE-mediated plasmid gene transfer efficacy being equivalent after each dose. These data are also consistent with our pre-clinical studies which indicated that a second dose of DC-Chol/DOPE liposomes was as effective as the first dose in restoring the ion transport abnormalities of CF transgenic mice.[18] This is in contrast to recombinant adenovirus, whereupon repeated administration, efficacy was markedly diminished, as anti-adenoviral antibody titre increased.[9] Thus DNA/liposomes offer a distinct advantage over current recombinant adenoviruses, in that efficacy upon repeat administration is possible without the requirement for immunosuppressive interventions.[24]

Molecular evidence of gene delivery and expression (as determined by detection of DNA, mRNA and recombinant protein) was observed more frequently than changes in CFTR-dependent cellular function (halide efflux, bacterial adherence and nasal PD) (Table 3). As in our single-dose study, changes in CFTR function were readily detected *ex vivo* using a halide efflux assay (Figure 4, Table 3). A limited number of low chloride PD measurements in treated subjects were also consistent with a change towards non-CF values (Figure 7). However, a grouped analysis of the treated subjects failed to find any evidence for statistically significant changes in CFTR-related transepithelial ion transport properties after any of the doses. This is in contrast to our single-dose clinical study[12] using an identical formulation (albeit a lower dose administered over a longer period of time), where a small (25%) correction of the CF chloride channel defect was observed. Other clinical studies using viral and nonviral gene transfer formulations have reported similar levels of efficacy with correction of the CF chloride channel defect varying from undetectable[13,25] to approximately 25%.[9,11,12,14,15] In common with all of the reported viral or nonviral clinical studies there was no correction of the CF sodium ion transport defect. Interestingly, treatment of CF mice with a pharmacological agent shown to stimulate epithelial chloride secretion[26] corrects several CF pathological features (including a predisposition for lung inflammation),[27] suggesting that even correction of the CF chloride defect alone following gene transfer, may improve CF prognosis.

Ultimately, the target organ for CF gene therapy is the lung, but for reasons of safety and comparative ease of access, the majority of clinical studies, including this one, have used the nasal epithelium as a surrogate. The nasal epithelium is contiguous with the epithelium lining the lung and has broadly similar cell types and similar CF-associated ion transport abnormalities.[3] However, in a recent clinical study where both the nose and lung were targeted,[15] the administration of DNA/genzyme lipid 67 (GL-67) resulted in a more striking correction of the chloride ion transport defect in the lower airway than in the nasal epithelium. In addition, the study also highlighted important safety issues for the delivery of this formulation to the lung, which were not predicted by earlier clinical studies in the nose.[14] These observations suggest that the lungs represent a critical site for the evaluation of gene transfer formulations in CF.

Initial clinical studies of gene transfer in CF subjects have been encouraging. Both viral and nonviral formulations have been shown to transfer the CFTR cDNA to airway epithelia and result in CFTR expression and CFTR chloride channel activity. This study shows that repeated administration of our DNA/liposome formulation is safe, with no activation of the immune system, and results in gene transfer after each of the three doses. However, to

improve the clinical prognosis of CF patients, current gene transfer formulations will need to be improved, in particular, to increase the level and persistence of transgene expression.

Materials and methods

Subjects and study design

Twelve CF patients (over 16 years of age) with defined CF genotypes were enrolled in the study (Table 1) and gave written informed consent. The subjects were counselled before enrolment, particularly regarding the length and the nontherapeutic nature of the study. The study was approved by the UK Gene Therapy Advisory Committee, the Medicines Control Agency and the appropriate Hospital Research Ethics Committee. Subjects attended the clinic as out-patients on 24 occasions (Table 2) and continued with their normal treatment throughout. A routine clinical examination was performed on each study day and weekly sputum and nasal lavage samples were taken for culture. The 12 subjects were divided into two groups of six and were randomly assigned a treatment regime at a ratio of 1:5 placebo:DNA/liposome treatment. Both subjects and investigators were blinded to the treatment each subject received, and remained blinded until after data analysis was completed. Subjects received three doses of DNA/liposomes or a placebo administered 4 weeks apart. For the duration of the study, the subjects were examined by the lead clinician (KWS) and any changes were discussed with the attending physician. An independent ombudsperson (JJE) reviewed all clinical results and observed all clinical management decisions.

Gene transfer reagents

Plasmid pTRIAL10CFTR2[16] in which the CFTR cDNA is under the transcriptional control of the Rous sarcoma virus 3′LTR promoter and DC-Chol/DOPE cationic liposomes[17] were used in this study. Plasmid DNA was prepared at the Imperial Cancer Research Fund's Biotherapeutics and Hybridoma Development Unit in London according to Medicines Control Agency approved procedures. The plasmid DNA was supplied sterile and essentially free of endotoxin (<1 EU/mg DNA), in single-use vials at a concentration of 0.667 mg/ml in Kreb's HEPES buffer (pH 9.0). DC-Chol/DOPE cationic liposomes were prepared at the University of Pittsburgh under conditions approved by the US Food and Drug Administration.[17] The DC-Chol/DOPE liposomes (6:4 molar ratio of DC-Chol to DOPE) were made by a microfluidisation procedure, to a final concentration of 2 μmol/ml (equivalent to 1.2 mg/ml of total lipid) in endotoxin-free water[28] and packaged in single-use vials. The transfection efficiency of the liposomes was assayed using HeLa cells and a plasmid expressing the chloramphenicol acetyl transferase reporter gene.[16]

Dosing

The DNA, cationic liposomes and Kreb's HEPES solutions were equilibrated to room temperature before use and mixed, where appropriate, approximately 5 min before patient administration. All patients received identical doses in each nostril. For each nostril, the dose (8 ml containing 400 μg pTRIAL10-CFTR2 plasmid DNA complexed with 4 μmol DC-Chol/DOPE, or 8 ml Kreb's HEPES buffer for placebo) was divided into four aliquots of 2 ml, delivered over 30 min each. After 30 min, the subjects would turn to dose the other nostril, such that the complete dose was administered in just over 4 h. The treatment solutions were instilled directly on to the nasal septum of each nostril using an MS16A syringe driver (Graseby Medical, Watford, UK), via a soft, infant, F, 50 cm feeding catheter (Portex, Hythe, UK) as described.[12]

Nasal biopsy

An epithelial biopsy was taken from the left nostril of each patient 7 days after the third dose (Table 2). The biopsy was taken using local anaesthesia as described previously.[12] The tissue was stored in formalin fixative and the tissue processed and stained with haematoxylin and eosin for histological examination. Adjacent sections were immunostained with peroxidase-labelled monoclonal antibodies against either neutrophil elastase (By87a; anti-neutrophil elastase), T cells (CD3; anti-CD3), B cells (JCB117; anti-CD79a) or macrophages (PGM1 or KP1; anti-CD68) according to standard pathology procedures and examined for evidence of inflammation.

Collection of nasal epithelial cells

Nasal brushings were collected from each patient on three occasions during the study (Table 2), and one to two times during the pre-study assessment period. Nasal epithelial cells were taken from the region between the middle and inferior turbinates by gentle brushing with a 3-mm cytology brush. Both nostrils were brushed and the cells from each nostril combined. A proportion of the resulting cell suspension consisted of ciliated epithelial cells, which we and others have shown to possess properties characteristic of the airway epithelium.[12,15,29] Cells were divided between five efficacy assays, with 30% taken for the molecular biology assays (DNA and RNA), 30% for *ex vivo* electrophysiology, 30% for the bacterial adherence assay and 10% for immunocytochemistry. Thus, at any given time-point, all of the *ex vivo* efficacy assays for each subject were performed on cells from the same sample.

Evaluation of immune response

In addition to weekly routine blood tests, serum samples were collected pre- and post-study, and 6 months after the completion of the study. Samples were tested, as a batch, for immunoglobulins, complement (C3, C4), rheumatoid factor, IgG anti-nuclear, anti-mitochondrial, anti-gastric, anti-liver/kidney/microsomal and anti-double stranded DNA autoantibodies. Serum levels of cytokines (TNFα, IL6) were determined by enzyme-linked immunoassay (Medgenix, Antwerp, Belgium), pre-study, 2 days after each dose and 6 months after completion of the study. Antibodies to CFTR in study subjects were determined by indirect immunofluorescence using murine C127 cells with or without human CFTR.[30] Healthy donor serum was used as negative control and mouse anti-human CFTR antibodies (mAb 13–1 at 1 mg/ml, Genzyme, Cambridge, MA, USA) as a positive control. Lymphocyte subsets (CD3, CD19, CD4, CD8 and NK cells) were analysed by flow cytometry (FACScan, Becton Dickinson, Oxford, UK). Cellular immune responses to CFTR protein were assayed by the culture of lymphocytes with C127 cells with or without human CFTR. The

cells were pulsed with tritiated thymidine, 24 h before being harvested after 5 days of culture. Expression of IL2R was determined using antibody to IL2R and flow cytometry.

Detection of plasmid DNA and mRNA

DNA and total RNA were simultaneously prepared from cells collected by nasal brushing, using a custom supplied kit based on RNeasy and DNeasy spin columns (Qiagen, Hilden, Germany). Plasmid DNA, genomic DNA, endogenous CFTR mRNA and plasmid-specific mRNA were quantitated using a PE Biosystems 7700 real-time sequence detector and TaqMan PCR reagent or TaqMan Gold RT-PCR kits (PE Biosystems, Warrington, UK), as appropriate. Absolute reference standard curves for DNA PCR were prepared from samples of human genomic DNA (Clontech, Basingstoke, UK) or plasmid DNA prepared for the study. Quantitation of DNA concentration before establishing standard curves was performed using PicoGreen (Molecular Probes, Eugene, OR, USA). Copies of pTRIAL10CFTR2 and human DNA were quantified using plasmid-specific primers and primers specific for CFTR exon 24, respectively (Hyde *et al*, manuscript in preparation). The number of cells in any given sample was estimated to be $0.5 \times$ number of CFTR genes. Reference standards, no-template controls and experimental samples were analysed in triplicate, such that coefficients of variance were less than 3%. The sensitivities of the plasmid specific and genomic DNA PCR assays were eight and seven copies respectively, and were linear to at least 1.1×10^5 and 2.8×10^5 copies, respectively. Absolute reference standard curves for mRNA RT-PCR were prepared from synthetic RNA targets prepared by *in vitro* transcription (Ambion, Austin, TX, USA) of Vent polymerase PCR products (New England Biolabs, Hitchin, UK) (Hyde *et al*, manuscript in preparation). Quantitation of RNA concentration before the construction of standard curves was performed using RiboGreen (Molecular Probes). Copies of pTRIAL10CFTR2 specific and endogenous CFTR mRNA were quantified using primer sets spanning the transgene mRNA polyadenylation site and CFTR exon 24 respectively (Hyde *et al*, manuscript in preparation). Reference standards, no-template controls and experimental samples were analysed ± reverse transcriptase in triplicate and coefficients of variance were typically less than 7%. No copies of pTRIAL10CFTR2 specific mRNA RT-PCR product were detected without the addition of reverse transcriptase. The contribution of genomic DNA to the levels of endogenous CFTR mRNA was <1% in all samples. The sensitivities of the plasmid-specific and endogenous CFTR RT-PCR assays were 261 and 44 copies respectively, and were linear to at least 2.6×10^8 and 4.5×10^6 copies, respectively.

Immunohistochemical detection of CFTR protein

The method was essentially as described,[31] with minor modifications. Approximately 1×10^4 cells were cytocentrifuged on to glass slides, air-dried and stored in foil at –20°C. Once thawed to room temperature, the foil was removed and the cells were fixed in acetone:methanol (1:1 v/v) for 90 s, rinsed, and then incubated in primary anti-human CFTR monoclonal antibody (50 μl per slide; 1 μg/ml Genzyme mAb 13–1) in the presence of protease inhibitors[31] for 90 min. Slides were subsequently incubated with anti-mouse immunoglobulins (DAKO Z259, 1:25 dilution for 30 min) and then in alkaline phosphatase mouse monoclonal anti-alkaline phosphatase (DAKO D651, 1:50 dilution, for 30 min). The intensity of immunoreactivity was enhanced by repeating the last two antibody incubation steps for 10 min each. Slides were stained (Fast Red DAKO K597, 15 min), counterstained with haematoxylin and mounted (Aquamount, DAKO C563) for examination. As controls, nasal epithelial cells from CF and non-CF individuals were included. These conditions were selected such that endogenous mutant CFTR present in CF subjects' cells was not detectable, whereas CFTR from non-CF individuals was detected with an apical staining pattern in approximately 80% cells. All slides were evaluated in a blinded fashion and only ciliated epithelial cells with a pattern of apical membrane staining were scored positive.

Ex vivo electrophysiological measurements (SPQ analysis)

Cells were loaded with the halide-sensitive fluorophore 6-methoxy-N-(3-sulphopropyl)-quinolium (SPQ) from Cambridge Bioscience (Cambridge, UK) by hypotonic shock. Halide efflux stimulated by the addition of 100 μM 3-isobutyl-1-methyl-xanthine (IBMX) and 25 μM forskolin (Fsk) from Sigma Chemical (Poole, UK), was measured as described.[12] In the presence of functioning CFTR protein, the addition of cAMP agonists results in the opening of the CFTR channel and a reduction in the intracellular halide concentration. This reduction can be measured by an increase in SPQ fluorescence. Wherever possible the cell sample was divided and one half used as a negative control in which the cAMP agonists were omitted. Where insufficient cells were obtained for division of the sample, the cells were subjected to the experimental conditions only. Fields of cells (typically 50–500 cells) were visualised at ×100 magnification. Regions of interest were selected from the last image without knowledge of the rate of fluorescence change (between 28 and 118 per sample). The brightest 33% of the selected regions of interest were used to calculate %relative intensity, which is $100 \times (F_t/F_0)$ where F_t is fluorescence intensity at time t and F_0 is the mean of fluorescence intensity for the initial 120 s. A greater than three-fold increase in the rate of change of %relative intensity with time after the addition of cAMP agonists was taken as indicative of CFTR activity.

Bacterial adherence assays

Samples containing approximately 1×10^5 ciliated nasal epithelial cells were mixed with *Pseudomonas aeruginosa* laboratory strain K (ATCC 25102),[32] in a ratio of 250 bacteria per cell. Suspensions were incubated for 1 h at 37°C and centrifuged through a 50% Percoll (Pharmacia, Uppsala, Sweden) gradient (40 min at $16000 \times (g_{av})$ at 4°C). Samples were treated as described,[20] except that cells on coverslips were fixed in 4% glutaraldehyde and stored at +4°C for 48 h before dehydration and critical point drying. Samples were mounted on to aluminium stubs and coated with gold ready for scanning electron microscopy and the number of bacteria on each ciliated cell quantified. A minimum of 10 ciliated cells was examined for each sample.

In vivo electrophysiological measurements (PD analysis)

CFTR function was assessed *in vivo* by measuring the potential difference (PD) across the nasal epithelium on

the floor of the nasal cavity on 18 occasions during the study (Table 2), and two to three times during the pre-study assessment period. Measurements were performed essentially as described,[12] except that the response to isoprenaline in a low chloride solution was omitted, as changes after isoprenaline were not anticipated[12] and changes after perfusion with low chloride buffer have been demonstrated to be the most discriminatory[33] and the response to ATP in a low chloride solution determined.[34] For discussion purposes, increases and decreases in PD refer to absolute magnitude of the PD.

Statistical analysis

Means were compared between multiple groups using Kruskal–Wallis, non-parametric ANOVA. Means were compared between two groups using the Mann–Whitney *U* test. Means were compared within groups using the Wilcoxon signed ranks test. The null hypothesis was rejected at $P < 0.05$.

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