ASTHMA

Cross tolerance to salbutamol occurs independently of β_2 adrenoceptor genotype-16 in asthmatic patients receiving regular formoterol or salmeterol

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Background: The development of tolerance following the use of long acting β_2 agonists in asthmatic patients with either the homozygous arginine (Arg-16) or glycine (Gly-16) genotypes is poorly documented, especially in relation to the acute reliever response to salbutamol in constricted airways. A study was undertaken to evaluate the Arg-16 and Gly-16 genotypes for the acute salbutamol response following methacholine bronchial challenge between the first and last doses of formoterol (FM) and salmeterol (SM) combination inhalers.

Methods: Parallel groups of 10 matched homozygous Arg-16 and 10 homozygous Gly-16 patients completed a randomised, double blind, double dummy, crossover study. Following a 1 week washout period, patients received treatment for 2 weeks with either inhaled budesonide (BUD) 200 μg + FM 6 μg (two puffs twice daily) or inhaled fluticasone propionate (FP) 250 μg + SM 50 μg (one puff twice daily). After washouts and randomised treatments (1 hour after the first and last inhalation) a methacholine challenge was performed followed by salbutamol 200 μg, with recovery over 30 minutes (the primary outcome).

Results: Washout values for forced expiratory volume in 1 second (FEV_1), methacholine hyperreactivity, and salbutamol recovery were similar for both treatments and genotypes. Pre-challenge FEV_1 values for both genotypes did not differ significantly between the first and last doses of each treatment. Salbutamol recovery as mean (SE) area under the 30 minute time-response curve was significantly delayed ($p<0.05$) equally in both genotype and treatment groups. There were no differences in salbutamol recovery in either genotype or treatment group.

Conclusion: Acute salbutamol recovery in methacholine constricted airways was significantly delayed to a similar degree in both genotypes due to cross tolerance induced by FM or SM.

The development of tolerance following the use of long acting β_2 agonists in asthmatic patients with either the homozygous arginine (Arg-16) or glycine (Gly-16) genotypes is poorly understood, especially with respect to the acute reliever response to salbutamol in constricted airways. In vitro, using transfected cell lines, the Gly-16 genotype has been shown to be associated with an increased β_2 agonist promoted downregulation of β_2 adrenoceptors[1 2] while, in vivo, this genotype has been shown to be associated with enhanced bronchodilator tolerance to both short and long acting β_2 agonists.[3–5] Other in vivo data have suggested that the Arg-16 genotype is associated with worse outcomes after exposure to regular short acting β_2 agonist in terms of peak expiratory flow (PEF) and asthma exacerbations.[6 7] No previous studies have compared the genotypes in terms of tolerance development between the first and last dose of β_2 agonist.

Long acting β_2 agonists in conjunction with inhaled corticosteroids are advocated in current guidelines to improve asthma control at step 3 in patients with moderate to severe persistent asthma.[8 9] Blunting of the acute response to salbutamol after methacholine challenge occurs following the use of long acting β_2 agonists despite concomitant inhaled corticosteroids.[10 11] However, there are currently no prospective data directly comparing the two homozygous genotypes at position 16, particularly in patients receiving long acting β_2 agonists such as formoterol (FM) and salmeterol (SM) in combination inhalers with inhaled corticosteroids.

We therefore compared matched groups of patients expressing the homozygous Arg-16 and homozygous Gly-16 genotypes in terms of acute salbutamol recovery following methacholine bronchial challenge between the first and last doses of FM and SM combination inhalers.

METHODS

Patients

Twenty patients who were homozygous for either the Arg-16 or Gly-16 genotypes were identified from our database. Eligible patients were non-smoking moderate persistent asthmatics[8] who had been stable for at least 3 months before the study and none had received a course of oral corticosteroids or antibiotics during this period. Patients were required to exhibit hyperreactivity to methacholine on bronchial challenge testing with a provocative dose causing a 20% reduction from baseline FEV_1 (PC_{20}) of less than 4.0 mg/ml. Informed consent was obtained from all patients and the Tayside Committee on Medical Research Ethics approved the study.

Study design

A scheme of the study design is shown in fig 1. The study was conducted in a randomised, double blind, double dummy, crossover, parallel group fashion. Patients were required to stop any second line controller treatment such as long acting β_2 agonists (n = 9), leukotriene $CysLT_1$ receptor antagonists (n = 1), and theophylline (n = 1) for a 1 week period before

Abbreviations: BUD, budesonide; FEV_1, forced expiratory volume in 1 second; FM, formoterol; FP, fluticasone propionate; PEF, peak expiratory flow; SM, salmeterol

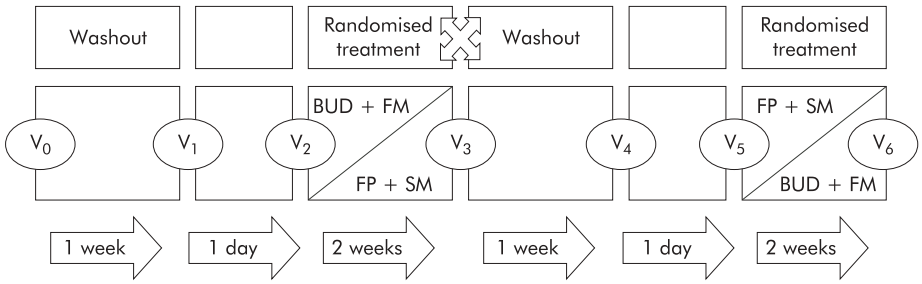

Figure 1 Study design schematic. Methacholine challenges with salbutamol recovery were performed at V_1 and V_4 after each washout, before randomised treatments, and 1 hour after the first and last doses of each randomised treatment at V_2, V_3, V_5 and V_6.

the initial screening visit and for the entire duration of the study. Short acting β_2 agonists were withheld for the duration of the study, with ipratropium bromide 20 µg (Atrovent; Boehringer Ingelheim, Bracknell, UK) being given as reliever therapy instead. There was a 1 week washout period before each randomised treatment during which patients continued to receive their usual inhaled corticosteroid treatment. Patients were randomised to receive for 2 weeks an inhaled combination of either budesonide (BUD) 200 µg + FM 6 µg (Symbicort 200/6 Turbohaler; AstraZeneca, Luton, UK) two puffs twice daily or fluticasone propionate (FP) 250 µg + SM 50 µg (Seretide 250 Accuhaler; Allen and Hanburys, Uxbridge, UK) one puff twice daily. The dose of inhaled corticosteroid as FP in Seretide was chosen to be approximately equipotent to that of BUD in Symbicort, given that FP is twice as potent as BUD, while 50 µg SM is approximately equipotent to 12 µg FM. With each active Turbohaler patients also received a placebo Accuhaler and vice versa. All active and placebo Turbohaler and Accuhaler devices were masked to make them identical in external physical appearance.

Measurements

Spirometry

Spirometric tests were performed according to the American Thoracic Society criteria[12] using a Micro Medical SuperSpiro (Micro Medical Ltd, Rochester, UK). At each of the study visits FEV_1 was measured before and 1 hour after treatment.

Methacholine bronchial challenge

Methacholine challenge was performed following each washout period before each randomised treatment and 1 hour after the first and last doses of each treatment. Methacholine was administered using a standardised breath actuated dosimeter (Mefar; Markos-Mefar SpA, Bovezzo, Italy) at 5 minute intervals in doubling cumulative concentrations from 0.03125 mg/ml to 64.0 mg/ml until a 20% reduction in FEV_1 was recorded. Log linear interpolation was performed using a computer assisted program (Micro Medical Ltd) to calculate the PC_{20} values.

Acute salbutamol recovery

Immediately after methacholine bronchial challenge patients received inhaled salbutamol 200 µg (Ventolin 200 Accuhaler; Allen and Hanburys, UK) and measurements of FEV_1 were recorded at 5 minute intervals for 30 minutes.

Domiciliary PEF

Patients recorded morning domiciliary PEF using a Mini-Wright meter (Clement Clarke International Ltd, Harlow, UK) for the duration of the study. The values for the last 5 days following each washout and randomised treatment were used in the analysis.

Statistical analysis

The study was powered at 80% with the α error set at 0.05 (two tailed) and the β error set at 0.2, in order to detect a 50% difference in acute salbutamol recovery (the primary outcome variable) for within patient changes between the first and last doses of each treatment, calculated as the area under the 30 minute time-response curve for the percentage change from pre-challenge (1 hour after treatment inhalation) baseline FEV_1. As a secondary outcome, the salbutamol response at 30 minutes was also evaluated, calculated as the percentage change from pre-challenge FEV_1. Tolerance was defined as the difference in salbutamol recovery between the first and last doses of each treatment. An overall analysis of variance followed by multiple range testing with Bonferroni correction set at 95% confidence interval (CI) was performed and a p value of <0.05 (two tailed) was considered significant. Comparisons were made between genotypes and randomised treatments. To normalise distribution, data for methacholine PC_{20} were logarithmically transformed and analyses were performed using Statgraphics statistical software package (STSC Software Publishing Group, Rockville, USA).

RESULTS

Patients

The demographic details of the study patients are shown in table 1. The 10 patients from each homozygous genotype-16 group had similar sex distribution and comparable mean age, FEV_1, and inhaled corticosteroid dose. One Gly-16 patient who had completed the FP + SM limb of the study had an exacerbation during the second washout period before BUD + FM and was withdrawn, although data for the patient were included on an intention to treat basis for completed visits.

Washout values

Values following the washout periods were similar for pre-challenge FEV_1, methacholine PC_{20}, and salbutamol recovery for both genotypes and treatment groups (table 2).

Methacholine challenge

The pre-challenge FEV_1 values (1 hour after inhalation) for each genotype were not significantly different between the first and last doses of each treatment (table 3). There were also no significant differences in methacholine PC_{20} values for either genotype between the first and last doses of either treatment (table 3).

Salbutamol recovery

Following methacholine challenge the mean percentage fall in FEV_1 from the pre-challenge value (1 hour after treatment) was not significantly different for either genotype or treatment group between the first and last doses of BUD + FM and FP + SM (table 4, fig 2). Salbutamol recovery as area under the 30 minute time-response curve (%.min) was significantly delayed in both genotype and treatment groups

Table 1 Demographic characteristics of study patients

Subject	Sex	Age (years)	Genotype-27	FEV_1 (% predicted)	MCh PC_{20} (mg/ml)	ICS	Dose (μg)	Second line
Arg-16								
1	M	27	Gln-Gln	76	0.8	BDP	400	
2	F	50	Gln-Gln	83	2.7	FP	500	SM
3	M	54	Gln-Gln	67	1.4	FP	500	SM, TH, ML
4	F	49	Gln-Glu	83	2.3	BDP	400	SM
5	M	63	Gln-Gln	69	1.3	BDP	100	
6	M	36	Gln-Gln	88	3.1	BDP	400	
7	F	49	Gln-Glu	82	1.3	FP	500	SM
8	F	53	Gln-Gln	90	0.5	BDP	100	
9	F	29	Gln-Gln	80	0.7	BUD	400	
10	F	19	Gln-Gln	82	2.5	FP	500	
Mean		43		80	1.7		380	
Gly-16								
11	F	34	Glu-Glu	91	2.6	FP	500	SM
12	F	69	Gln-Glu	94	2.9	BUD	200	FM
13	F	50	Gln-Glu	75	1.0	FP	500	SM
14	M	49	Glu-Glu	70	0.4	BDP	400	
15	M	46	Glu-Glu	69	0.5	BUD	200	
16	F	52	Gln-Glu	73	0.1	BDP	200	
17	F	51	Glu-Glu	77	0.7	FP	1000	SM
18	M	59	Gln-Glu	67	2.1	BUD	400	
19	F	27	Glu-Glu	94	1.7	FP	200	SM
20	F	27	Gln-Glu	69	3.6	BDP	400	
Mean		46		78	1.6		400	

MCh = methacholine; ICS = inhaled corticosteroids; BDP = beclomethasone dipropionate; FP = fluticasone propionate; BUD = budesonide; SM = salmeterol; TH = theophylline; ML = montelukast; FM = formoterol.

between the first and last doses of each treatment ($p<0.05$; table 4 and fig 3). The mean percentage fall in FEV_1 from the pre-challenge value 30 minutes after administration of salbutamol was significantly greater in both genotype and treatment groups between the first and last doses of BUD + FM and FP + SM ($p<0.05$; table 4 and fig 4).

Pulmonary function

FEV_1 values both before and 1 hour after treatment (pre-challenge) were not significantly different for either genotype or treatment group between the first and last doses of BUD + FM and FP + SM (table 3). Domiciliary morning PEF values (l/min) were not significantly different for Arg-16 *v* Gly-16 in both treatment groups (BUD + FM: mean (SE) washout values 452 (33) *v* 412 (20) (95% CI −48 to 128), randomised treatment values 479 (29) *v* 450 (14) (95% CI −41 to 100); FP + SM: washout values 465 (28) *v* 422 (15) (95% CI −25 to 111), randomised treatment values 482 (31) *v* 450 (23) (95% CI −50 to 115). PEF values were significantly higher ($p<0.05$) after treatment in both genotypes. The following PEF values were obtained when washout values were compared with randomised treatment values for BUD + FM (Arg-16: 452 (33) *v* 479 (29) (95% CI 8 to 47); Gly-16: 412 (20) *v* 450 (14) (95% CI 22 to 57)) and for FP + SM (Arg-16: 465 (28) *v* 482 (31) (95% CI 11 to 47); Gly-16: 422 (15) *v* 450 (23) (95% CI 10 to 45)).

DISCUSSION

Our results show that acute salbutamol recovery in constricted airways following methacholine challenge is significantly delayed to a similar degree in both Arg-16 and Gly-16 genotypes following treatment with either BUD + FM or FP + SM. The cross tolerance for the salbutamol response was evident in terms of the difference between the first and last doses of each combination inhaler.

The values for FEV_1, methacholine PC_{20}, and salbutamol recovery after washout for both genotypes were not significantly different before BUD + FM and FP + SM, indicating that there were no carryover effects between the randomised treatment limbs. Furthermore, the values for pre-challenge FEV_1 (1 hour after inhalation) and post-challenge FEV_1 (after methacholine) were also not significantly different for either genotype or treatment, suggesting that the altered salbutamol response could not be accounted for by alterations in airway calibre. Moreover, there were no differences in methacholine PC_{20} values for either genotype when the first and last doses of each randomised treatment were compared, suggesting that the delay in salbutamol recovery was independent of the dose of inhaled methacholine.

We observed a non-significant trend towards reduced protection—as indicated by a lower PC_{20} threshold value—after the last doses compared with the first doses of each

Table 2 Mean (SD) washout values before randomised treatments for pre-challenge FEV_1 (% predicted), methacholine PC_{20} (mg/ml), and salbutamol recovery (%.min)

	Pre-challenge FEV_1			Methacholine PC_{20}			Salbutamol recovery		
Genotype	BUD + FM	FP + SM	95% CI	BUD + FM	FP + SM	95% CI	BUD + FM	FP + SM	95% CI
Arg-16	80 (12)	82 (12)	(−13 to 10)	1.5 (0.2)	1.4 (0.3)	(−0.8 to 0.8)	282 (29)	214 (54)	(−122 to 259)
Gly-16	74 (13)	74 (14)	(−13 to 14)	1.3 (0.6)	1.4 (0.4)	(−1.6 to 1.5)	150 (89)	181 (34)	(−200 to 121)
95% CI	(−7 to 18)	(−5 to 20)		(−1.3 to 1.5)	(−0.9 to 1.1)		(−72 to 337)	(−103 to 169)	

BUD = budesonide; FM = formoterol, FP = fluticasone propionate; SM = salmeterol.
95% CI values for methacholine PC_{20} are expressed as doubling dilution difference.

Table 3 Mean (SE) values at first and last doses of treatments for pretreatment % predicted FEV_1, % predicted FEV_1 1 hour after treatment (pre-challenge), and methacholine PC_{20} (mg/ml)

	BUD + FM			FP + SM		
Genotype	**First dose**	**Last dose**	**95% CI**	**First dose**	**Last dose**	**95% CI**
Pretreatment FEV_1						
Arg-16	82 (3)	86 (4)	(−14 to 7)	82 (4)	86 (5)	(−17 to 9)
Gly-16	75 (5)	81 (5)	(−21 to 8)	74 (5)	79 (5)	(−19 to 9)
95% CI	(−5 to 20)	(−8 to 17)		(−5 to 22)	(−7 to 21)	
FEV_1 1 hour after treatment (pre-challenge)						
Arg-16	90 (4)	92 (4)	(−14 to 9)	89 (4)	90 (4)	(−14 to 11)
Gly-16	86 (5)	87 (5)	(−14 to 14)	81 (5)	83 (4)	(−16 to 12)
95% CI	(−9 to 16)	(−8 to 18)		(−6 to 23)	(−5 to 20)	
Methacholine PC_{20}						
Arg-16	12.2 (2.9)	9.1 (2.6)	(−0.7 to 1.5)	6.2 (2.5)	4.1 (1.6)	(−1.0 to 2.2)
Gly-16	13.2 (4.2)	11.8 (2.4)	(−1.0 to 1.3)	10.7 (3.1)	6.4 (2.3)	(−0.6 to 2.1)
95% CI	(−1.3 to 1.1)	(−1.5 to 0.7)		(−2.2 to 0.7)	(−2.2 to 0.9)	

BUD = budesonide; FM = formoterol, FP = fluticasone propionate; SM = salmeterol.
95% CI values for methacholine PC_{20} are expressed as doubling dilution difference.

randomised treatment with both genotypes, as seen in previous studies.[13 14] We did not feel it was necessary to compare the first and last dose salbutamol recovery with that after each washout as the dose of methacholine was much higher after BUD + FM and FP + SM. The higher dose of methacholine after the first and last doses of each randomised treatment would thus have resulted in delayed recovery on its own, irrespective of any effects on β_2 adrenoceptor function by the long acting β_2 agonists.

Irrespective of genotype-16, salbutamol recovery exhibited cross tolerance in patients exposed to BUD + FM or FP + SM.

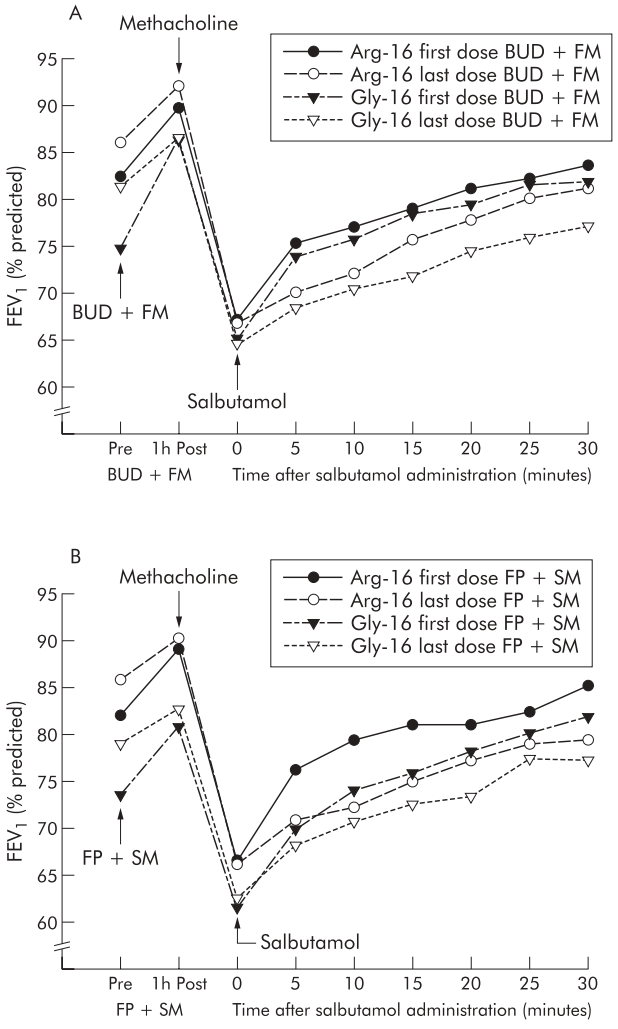

Figure 2 Mean FEV_1 values (% predicted) before and 1 hour after first and last doses, methacholine challenge, and salbutamol recovery over 30 minutes for (A) BUD + FM and (B) FP + SM.

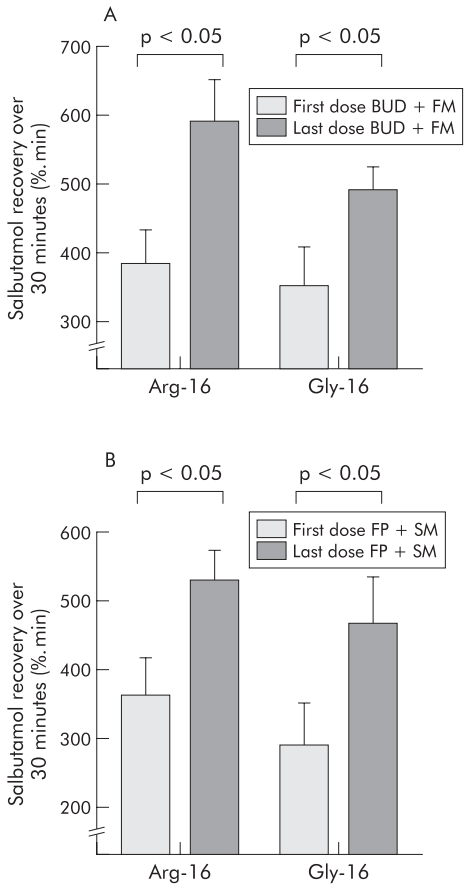

Figure 3 Salbutamol recovery after methacholine challenge as the area under the 30 minute time-response curve for the percentage change in baseline FEV_1 (%.min) from the pre-challenge value (1 hour after treatment) for each genotype after the first and last doses of (A) BUD + FM and (B) FP + SM. A larger value for the area under the 30 minute time-response curve indicates a greater degree of delayed recovery.

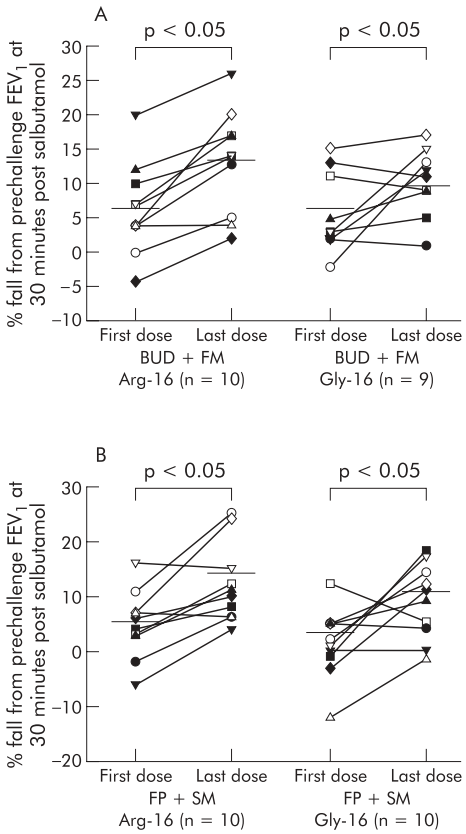

Figure 4 Individual data for percentage fall in FEV_1 from the pre-challenge value (1 hour after treatment) 30 minutes after administration of salbutamol for (A) BUD + FM and (B) FP + SM. Data points for each individual are joined together, with mean values depicted as horizontal lines. Values are shown for each genotype after the first and last doses of each randomised treatment.

Long acting β_2 agonists have been shown to downregulate and uncouple β_2 receptors leading to tolerance of response.[15–17] Moreover, prolonged β_2 receptor occupancy by long acting β_2 agonists may result in attenuation of the salbutamol response, aside from tolerance.[18 19] Similar negative interactions between long acting β_2 agonists and salbutamol have been shown previously in genotype unselected patients in the presence of methacholine constricted airways.[10 11 13 14]

We expected to see differences in salbutamol recovery between the genotypes at position 16 on the basis of previous in vitro data which have suggested development of enhanced downregulation of β_2 adrenoceptors and tolerance in the homozygous Gly-16 genotype.[1 2] Our data would therefore question the clinical relevance of β_2 adrenoceptor genotype-16 in determining the potential for interaction between short and long acting β_2 agonists. Nevertheless, we recognise that, in retrospect, we should have performed two separate power calculations for differences between treatments and for differences between genotypes, and as such our study may have had low power to detect the latter. We also acknowledge that comparisons between the first and last doses of treatment were uncontrolled in the strictest statistical sense as there were no study limbs evaluating before and after placebo effects.

We elected to use the methacholine challenge model as this produces a high degree of functional antagonism in terms of uncovering a subtle degree of salbutamol cross tolerance induced by prior exposure to long acting β_2 agonists.[20] Previous data suggesting adverse effects on PEF and asthma exacerbations were associated with regular salbutamol in patients with the Arg-16 genotype,[6 7] which may reflect rebound end of dose effects rather than tolerance between the first and last dose per se. It would be interesting to evaluate retrospectively genotyped data on asthma exacerbations from large multicentre trials where long acting β_2 agonists conferred improvements,[21] as no such analysis has been performed.

There are some retrospective data which suggest that complex β_2 adrenoceptor haplotypes may influence the acute bronchodilator response to salbutamol.[22] However, as many of these haplotypes are relatively uncommon, one can always question their clinical relevance. We selected our patients according to homozygous genotypes at position 16, irrespective of the genotype at position 27. Data for isoproterenol induced venodilatation in the dorsal hand vein are available which show enhanced tolerance of the response in association with the haplotype comprising homozygous Arg-16 in combination with homozygous glutamic acid (Gln-27) compared with the haplotype combination of homozygous Gly-16 with either homozygous Gln-27 or homozygous glutamine (Glu-27).[23] None of our patients had the haplotype

Table 4 Mean (SE) % fall in FEV_1 from pre-challenge value for first and last doses of randomised treatments after methacholine challenge before salbutamol, salbutamol recovery over 30 minutes (%.min), and % fall from pre-challenge value 30 minutes after salbutamol

	BUD + FM			FP + SM		
Genotype	**First dose**	**Last dose**	**95% CI**	**First dose**	**Last dose**	**95% CI**
% fall from pre-challenge FEV_1 after methacholine challenge pre salbutamol						
Arg-16	25 (2)	29 (2)	(−2.3 to 9.7)	26 (2)	27 (1)	(−5.7 to 6.9)
Gly-16	25 (3)	25 (2)	(−5.7 to 5.9)	24 (3)	22 (4)	(−8.7 to 5.9)
95% CI	(−7 to 7)	(−3 to 10)		(−5 to 11)	(−4 to 14)	
Salbutamol recovery						
Arg-16	386 (48)	591 (60)*	(97−314)	363 (53)	529 (43)*	(40 to 292)
Gly-16	352 (56)	491 (34)*	(9−269)	289 (62)	466 (68)*	(36 to 319)
95% CI	(−122 to 189)	(−51 to 250)		(−97 to 246)	(−107 to 233)	
% fall from pre-challenge FEV_1 30 minutes after salbutamol						
Arg-16	6 (2)	13 (2)*	(4 to 10)	5 (2)	12 (2)*	(3 to 11)
Gly-16	6 (2)	10 (2)*	(1 to 9)	2 (2)	10 (2)*	(2 to 14)
95% CI	(−5 to 7)	(−3 to 9)		(−3 to 10)	(−4 to 9)	

BUD = budesonide; FM = formoterol, FP = fluticasone propionate; SM = salmeterol.
*$p<0.05$ first dose *v* last dose of each randomised treatment.
There were no significant differences in either genotype or treatment group for % fall in FEV_1 from the pre-challenge value after methacholine challenge pre salbutamol.

combination of homozygous Gly-16 and homozygous Gln-27, which is uncommon due to linkage disequilibrium between homozygous genotypes at position 16 and 27.[24] For this reason, our small genotype selected sample was inadequate for the purposes of making any meaningful haplotype comparisons.

There were no differences in the effects of BUD + FM and FP + SM on acute salbutamol recovery, even though there are differences in the β_2 adrenoceptor intrinsic activity between FM and SM.[25] The present findings are in keeping with previous data showing no difference between both long acting β_2 agonists in bronchoprotection as add-on therapy to inhaled corticosteroids in Gly-16 selected patients,[26] or in downregulation of peripheral blood lymphocyte β_2 adrenoceptors.[27] One might have expected to see greater cross tolerance of the salbutamol response with a full β_2 agonist such as FM, perhaps due to enhanced uncoupling of the β_2 adrenoceptor adenylate cyclase subunit. Indeed, the present data are also in keeping with our previous results which showed a similar degree of cross tolerance to salbutamol recovery between BUD + FM or FP + SM compared with BUD or FP alone.[10]

We conclude that cross tolerance to the acute salbutamol response occurs in methacholine constricted airways following treatment with FM or SM combination inhalers, irrespective of genotype-16.

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