

Asthma exacerbations during long term β agonist use: influence of β_2 adrenoceptor polymorphism

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Abstract

Background—Polymorphisms of the β_2 adrenoceptor influence receptor function in vitro and asthma phenotypes in vivo. However, their importance in determining responses to inhaled β agonist treatment has not been clearly defined.

Methods—In a retrospective analysis of previously published data we have examined relationships between polymorphisms at codons 16 and 27 of the β_2 adrenoceptor and clinical outcomes in a randomised, placebo controlled, crossover trial of regularly scheduled salbutamol and salmeterol in 115 patients with mild to moderate asthma. Genotyping was obtained for positions 16 and 27 in 108 and 107 patients, respectively. For position 16, 17 patients (16%) were homozygous Arg-Arg, 40 (37%) were heterozygous Arg-Gly, and 51 (47%) were homozygous Gly-Gly.

Results—Within the homozygous Arg-16 group major exacerbations were more frequent during salbutamol treatment than with placebo (1.91 (95% CI 1.07 to 3.12) per year versus 0.81 (95% CI 0.28 to 1.66) per year; $p = 0.005$). No significant treatment related differences occurred for heterozygous Arg-Gly patients (salbutamol 0.11 (95% CI 0.01 to 0.40), placebo 0.54 (95% CI 0.26 to 1.00) exacerbations per year) or homozygous Gly-16 patients (salbutamol 0.38 (95% CI 0.17 to 0.73), placebo 0.30 (95% CI 0.12 to 0.61) exacerbations per year). No adverse changes occurred for any position 16 subgroup with salmeterol. There was no significant relationship between position 27 genotypes and treatment related outcomes.

Conclusion—Homozygous Arg-16 patients are susceptible to clinically important increases in asthma exacerbations during chronic dosing with the short acting β_2 agonist salbutamol.

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Keywords: β_2 adrenoceptor; genotype; polymorphism; asthma; salbutamol; salmeterol

Polymorphisms of the human β_2 adrenoceptor are an important determinant of receptor function in vitro and occur commonly in the population (minor allele frequency approximately 0.4-0.5). The two most common receptor variants are characterised by substitution of glycine for arginine at position 16 (Gly-16) and of glutamic acid for glutamine at position 27 (Glu-27) of the extracellular domain of

the receptor respectively.² Cells which are homozygous for Gly-16 demonstrate increased receptor downregulation during exposure to β agonists whereas homozygous Glu-27 cells are relatively resistant.^{1,3}

Relationships between these in vitro findings and asthma phenotypes have been investigated in vivo but findings have been inconsistent.^{4,5} Of more potential relevance to asthma management is the observation that tolerance to inhaled β agonist drugs may be determined by β adrenoceptor polymorphism. In an early study desensitisation to the acute effects of formoterol was found to be significantly greater after chronic dosing in patients who were homozygous for Gly-16.⁶ However, in contrast to these findings and somewhat unexpectedly, Martinez *et al* have reported that the magnitude of the bronchodilator response to single doses of inhaled β agonist in both asthmatic and non-asthmatic children was determined by the presence of the Arg-16 allele,⁷ and Lima *et al* have confirmed this finding⁸: peak responses to oral salbutamol were greater in Arg-Arg subjects than in those harbouring the other position 16 genotypes.⁸

These latter observations raise the possibility that the effects of long term treatment with inhaled β agonist drugs on asthma control may be determined, at least in part, by specific β_2 adrenoceptor genotypes. In an earlier study in which the effects of chronic treatment with inhaled β agonists were stratified by genotype⁹ we reported enhanced airway responsiveness during regular treatment with fenoterol in subjects who were homozygous for Arg-16.⁹ However, no other important genotype related differences were observed, perhaps because the study was of limited size (of 61 patients 12 were homozygous for Arg-16).

To address this issue further we have analysed retrospectively the results of a larger randomised trial¹⁰ which evaluated asthma control during long term treatment with the short and long acting β agonists salbutamol and salmeterol with respect to genotype at positions 16 and 27 of the β_2 adrenoceptor.

Methods

STUDY DESIGN

The study design of the clinical trial of regular inhaled β agonist treatment has been reported previously.¹⁰ Briefly, 157 patients with mild to moderate asthma completed a double blind, double dummy, placebo controlled, three way, crossover study. The treatments were salbutamol 400 μ g four times a day and salmeterol 50 μ g twice a day given by Diskhaler (Glaxo

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Table 1 Criteria for the determination of exacerbations¹⁰

Definition	Asthma score	Duration
Minor exacerbation	2	2 or more days
Major exacerbation	3	2 or more days OR 1 day within the context of a minor exacerbation
Major exacerbation/ medical emergency	4	1 or more days
Conclusion of exacerbation	Returned to 0 or 1	3 or more days, otherwise exacerbation was deemed to be continuing

Wellcome, Greenford, UK); the matching placebo Diskhalers contained lactose. After an initial run in of 14 days each of the three randomised treatment periods was of 24 weeks duration and each was followed by a washout interval of four weeks. Most of the subjects (92%) were receiving maintenance inhaled corticosteroid treatment.

Asthma control was assessed in individual patients by recording peak flow rates, asthma symptoms, and "rescue" bronchodilator use twice daily in a diary card, and thereafter by using changes in these end points to calculate a composite daily asthma score.¹⁰ Scores 0, 1, 2, and 3 represented days on which asthma control was stable, mildly unstable, mildly deteriorated, or significantly deteriorated, respectively. These scores were assigned with reference to "best" values obtained during the run in period. Score 4 was allocated in the event of a medical emergency caused by asthma. From these data exacerbation rates were calculated. The criteria used to determine the severity and duration of each exacerbation are shown in table 1. Exacerbations were managed according to individual patients' personalised action plans and comprised increased doses of "rescue" bronchodilator together with either a doubling of inhaled corticosteroid dose or a course of oral prednisone, depending on severity.

Two years after completion of the study each of the 157 patients who completed the principal study was contacted by letter and/or telephone to invite them to participate in this follow up investigation. After obtaining written informed consent, subjects provided a 10 ml venous blood sample for β_2 adrenoceptor genotyping. Ethical approval was obtained from the Otago and Canterbury ethics committees.

IDENTIFICATION OF POLYMORPHISMS

Deoxyribonucleic acid (DNA) was extracted from the citrated blood using a commercial kit (QIAamp; Qiagen, Hilden, Germany) within 48 hours of obtaining the sample. DNA specimens were kept frozen until transfer to the Brigham and Women's Hospital, Boston for genotyping.

The amplification-refractory mutation system (ARMS) used for the detection of mutations at amino acid 16 of the human β_2 adrenergic receptor gene was carried out as described previously.^{11 12} The primers used for detecting the mutation A→G at nucleotide 1633 (Genbank accession no. M15169), corresponding to the amino acid change Arg→Gly at position 16, were: Arg-specific forward primer A1 (5'-GCCTTCTTGC TGGCACCAA AA-3') corresponding to nucleotides

1612–1633, except for the penultimate base at the 3' end (underlined) which was changed from T to A; Gly-specific forward primer A2 (5'-GCCTTCTTGC TGGCACCCAA AG-3'), which differs from the Arg-specific primer at the last nucleotide at the 3' end (shown in bold); and reverse primer Rev2 (5'-AAA GGG CAC CAC TGC CAG-3') corresponding to nucleotides 1837–1854 on the complementary strand.

The primers used to detect the mutation C→G at nucleotide 1666 (Genbank accession no. M15169), corresponding to the amino acid change Gln→Glu at position 27 (Q→E) were: Gln-specific forward primer B1 (5'-CCGGACCACG ACGTCACGCA AC-3') corresponding to nucleotides 1645–1666, except for the penultimate base at the 3' end (underlined) which was changed from G to A; Glu-specific forward primer B2 (5'-CCGGACCACG ACGTCACGCA AG-3') which differs from the wild type primer at the last nucleotide at the 3' end (shown in bold); and reverse primer Rev2.

Amplification by polymerase chain reaction (PCR) of the genomic DNA of each sample included two reactions at each locus—one with primers for wild type and the other with primers for mutation type. After amplification the reaction products were resolved by electrophoresis on 2.0% agarose gel and stained with ethidium bromide for analysis. A second PCR reaction was performed using fluorescent tagged primers and resolved on an ABI automated sequencer (ABI Prism 377, Applied Biosystems, Perkin Elmer Corporation, Foster City, CA, USA). An allele assignment was made when both methods yielded the same genotypic assignments.

STATISTICAL ANALYSIS

The primary end points of this study were total, minor, and major asthma exacerbations and morning peak flows. The use of supplementary corticosteroid (inhaled and oral) was also analysed.

Analyses of numbers of events (such as exacerbations) were performed using the General Estimating Equations (GEEs) of the Proc Genmod program of SAS. This is a means of extending the generalised linear model to deal with correlated data such as repeated measures. A Poisson error distribution and log link function were used. The number of days on each treatment for each subject was used as an offset to adjust for the different treatment exposures. An independent working correlation matrix was created. This adjusted the standard errors of the estimates for the fact that each subject contributed up to three numbers of events for the three different treatments. Where appropriate, homozygous "wild type" group values during placebo treatment were used as reference.

For continuous normally distributed end points, such as morning peak flows, between-treatment and between-genotype comparisons were carried out by mixed model analysis of variance, controlling for period and randomisation sequence. Treatment/genotype

Table 2 Number of subjects with polymorphisms at positions 16 and 27

Position 27 (n=107)	Position 16 (n=108)			Total
	Arg/Arg	Arg/Gly	Gly/Gly	
Gln/Gln	12	14	9	+2=37
Gln/Glu	4	25	28	+1=58
Glu/Glu	0	1	11	12
Total	+1=17	40	+3=51	

Genotyping was unsuccessful in four subjects for position 16 and in three subjects for position 27. Hardy-Weinberg χ^2 for position 16 = 3.41; $p = 0.064$; Hardy-Weinberg χ^2 for position 27 = 2.31; $p = 0.130$.

Table 3 Baseline characteristics by genotype at position 16 (means with ranges)

	Arg/Arg (n=17)	Arg/Gly (n=40)	Gly/Gly (n=51)
Age (years)	39.5 (20–60)	40.3 (18–64)	40.8 (22–64)
Sex (M/F)	9/8	13/27	28/23
Non-smokers	15	39	42
Ex-smokers	2	1	9
FEV ₁ (% predicted)	81.1 (47.6–107.0)	80.9 (41.5–117.2)	76.0 (30.5–101.1)
PC ₂₀ methacholine (GM, mg/ml)	1.18 (0.16–4.46)	0.87 (0.06–7.28)	0.90 (0.06–7.30)
Regular inhaled corticosteroids (μ g/day)			
1–400	5	14	30
401–1000	6	16	15
>1000	6	10	6

interactions were included in the model. Comparisons for morning peak flow rates were adjusted for mean pretreatment run in values (14 days). No adjustments were made for multiple testing.

Results

One hundred and fifteen of the 157 patients (73%) who completed the principal study¹⁰ were able to be contacted and gave consent to provide a blood sample. The β_2 adrenoceptor genotype could not be determined for either position 16 or position 27 in four subjects, for position 16 in a further three subjects, and for position 27 in a further four subjects. Thus, analysis was restricted to data from 108 and 107 subjects for positions 16 and 27, respectively. The distribution of the polymorphisms is shown in table 2. The allelic frequency was 65.7% for Gly-16 (142/216) and 38.2% for Glu-27 (82/214). The distribution of genotypes at each locus was in accordance with that predicted by Hardy-Weinberg equilibrium (table 2). As expected, there was highly significant linkage disequilibrium between genotypes ($\chi^2 = 24.52$, d.f. = 4, $p < 0.001$).

The baseline characteristics of the patient population by position 16 genotype are shown in table 3. Data for position 27 subgroups have not been shown but similar baseline results were obtained. Analyses for each of the principal end points were conducted for both position 16 and position 27 genotypes. No important pattern of differences emerged for any of the position 27 comparisons. For the sake of clarity they are not reported further.

ASTHMA EXACERBATIONS

Exacerbation rates during each treatment period, expressed as an annual rate per patient per annum, are shown in fig 1 and table 4. The rate for major exacerbations was significantly greater during treatment with salbutamol among homozygous Arg-16 patients than placebo (1.91 (95% CI 1.07 to 3.12) and 0.81 (95% CI 0.28 to 1.66) major exacerbations/year, respectively, $p = 0.005$) but not for the other genotype subgroups. In addition, the total exacerbation rate (major plus minor) was significantly greater during treatment with salbutamol for homozygous Arg-16 patients (3.57 (95% CI 2.37 to 5.16) per year) than for homozygous Gly-16 and heterozygous subjects (1.19 (95% CI 0.79 to 1.72) and 0.72 (95% CI 0.38 to 1.24) per year, $p = 0.003$ and $p = 0.003$, respectively).

During treatment with salmeterol the total number of exacerbations (major plus minor) was significantly reduced for homozygous Gly-16 subjects compared with placebo (0.34 (95% CI 0.15 to 0.67) versus 1.49 (95% CI 1.04 to 2.07); $p < 0.003$) and heterozygous subjects (0.49 (95% CI 0.22 to 0.93) versus 1.41 (95% CI 0.92 to 2.06); $p < 0.04$), but not for homozygous Arg-16 subjects (0.64 (95% CI 0.21 to 1.49) versus 1.91 (95% CI 1.07 to 3.15); $p = 0.16$).

PEAK FLOWS

Treatment related changes in morning peak flows compared with mean run in values are shown in fig 2. There was a highly significant difference between treatments ($p < 0.0001$), but no significant differences between genotypes. There were no significant treatment/genotype interactions. During treatment with

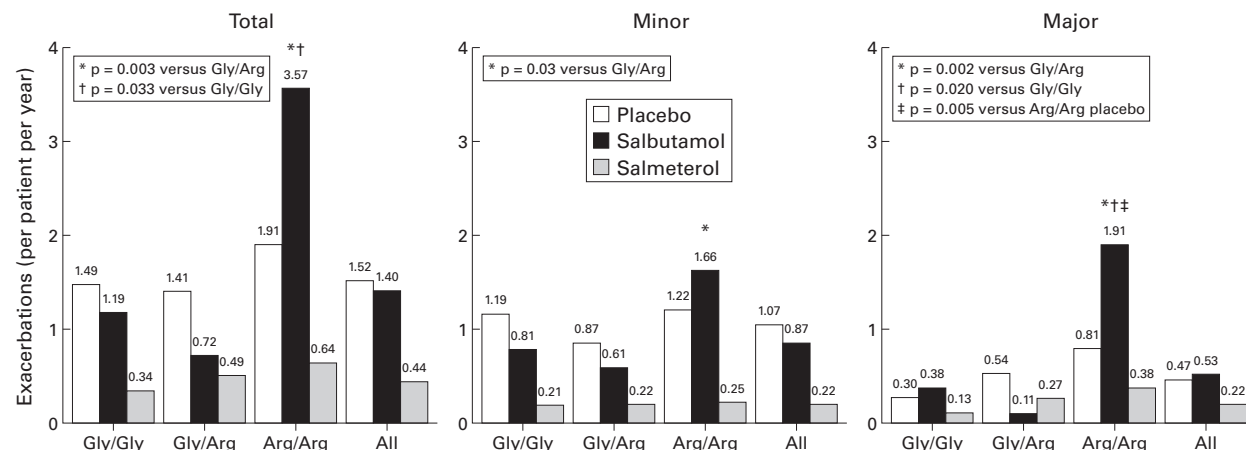


Figure 1 Total, minor, and major exacerbation rates expressed as number per patient per year. Initial comparisons were made between genotype subgroups using Arg-16 homozygous subjects ("wild type") during placebo treatment as the reference group.

Table 4 Exacerbation data by genotype at position 16

	Placebo			Salbutamol			Salmeterol		
	Arg/Arg (n=17)	Arg/Gly (n=40)	Gly/Gly (n=51)	Arg/Arg (n=17)	Arg/Gly (n=39)	Gly/Gly (n=51)	Arg/Arg (n=17)	Arg/Gly (n=40)	Gly/Gly (n=51)
No. (%) patients with exacerbations	4 (23.5)	11 (27.5)	16 (31.4)	7 (41.2)	9 (23.1)	18 (35.3)	3 (17.6)	6 (15.0)	7 (13.7)
Minor exacerbations (n)	9	16	28	11	11	19	2	4	5
OR	1.0	0.69	0.98	1.41	0.49	0.70	0.22	0.18**	0.17**
95% CI		0.18 to 2.71	0.30 to 3.19	0.55 to 3.60	0.14 to 1.70	0.21 to 2.29	0.02 to 2.29	0.04 to 0.83	0.05 to 0.65
Major exacerbations (n)	6	10	7	15	2	9	3	5	3
OR	1.0	0.65	0.37	2.44*	0.13**	0.49	0.49	0.33	0.16**
95% CI		0.14 to 2.94	0.07 to 1.85	1.31 to 4.53	0.02 to 0.86	0.11 to 2.14	0.14 to 1.65	0.06 to 1.68	0.03 to 0.86
Total exacerbations (n)	15	26	35	26	13	28	5	9	8
OR	1.0	0.68	0.73	1.82	0.35	0.62	0.32	0.24**	0.17**
95% CI		0.22 to 2.12	0.24 to 2.20	0.86 to 3.84	0.11 to 1.07	0.22 to 1.74	0.01 to 1.54	0.06 to 0.89	0.05 to 0.54

Odds ratios for exacerbations by treatment by genotype have been calculated using the Arg/Arg group during treatment with placebo as reference.

*Significantly greater than reference group.

**Significantly less than reference group.

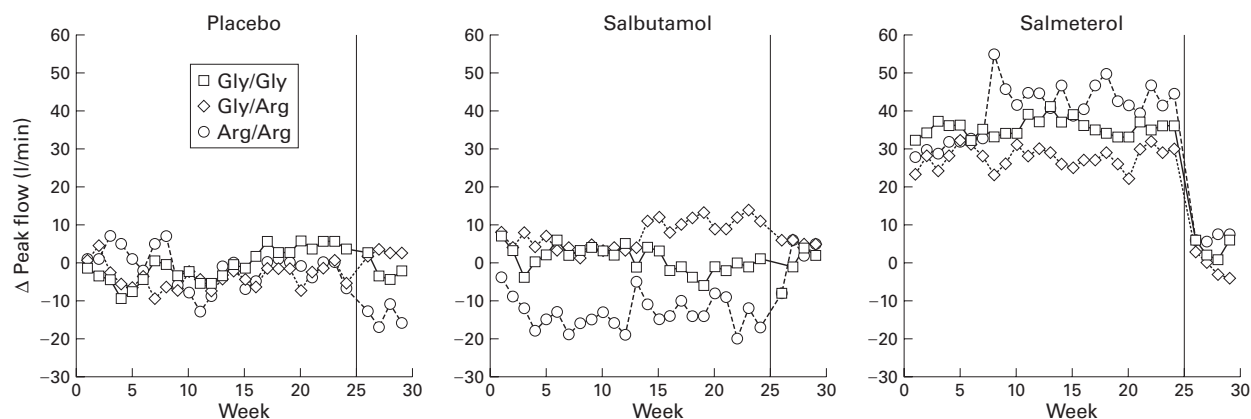


Figure 2 Mean change in morning peak flows compared with run in values obtained immediately before each treatment for each of the three position 16 genotype subgroups. Active treatment was concluded after 24 weeks followed by a four week washout interval.

salbutamol, although there was an apparent fall for homozygous Arg-16 subjects compared with pretreatment run in values (-7.5 (95% CI +8.2 to -27.2) l/min), this was not statistically significant.

SUPPLEMENTARY CORTICOSTEROIDS

The use of prednisone was significantly greater in homozygous Arg-16 subjects during treatment with salbutamol than with placebo (OR 1.71 (95% CI 1.08 to 2.91), p = 0.02). Similar comparisons for Arg-Gly and homozygous Gly-16 subjects did not reveal any significant differences. There were no between-genotype differences in the use of supplementary inhaled corticosteroids during any of the treatment periods (data not shown).

Discussion

In this study we have retrospectively examined asthma control during long term treatment with the β_2 agonists salbutamol and salmeterol by stratifying our results in relation to the two most frequently occurring β_2 adrenoceptor polymorphisms. The principal finding was that, among patients who were homozygous for the Arg-16 polymorphism, the frequency of major exacerbations more than doubled during treatment with salbutamol compared with placebo, in contrast to patients with the other position 16 genotypes in whom no specific adverse trends occurred. Furthermore, the major exacerbation rate during treatment with salbutamol was five times greater for homo-

zygous Arg-16 than for homozygous Gly-16 patients. These results were statistically significant despite the small number of homozygous Arg-16 subjects studied (16% of the study population) and suggest that this particular genotype is associated with an adverse effect of salbutamol treatment in the long term. Indeed, no adverse outcomes were noted in patients harbouring the homozygous Gly-16 genotype, even though there were three times as many subjects in that group. Furthermore, none of these outcomes would have been apparent if salbutamol/placebo comparisons had been limited to all subjects.

On the basis of in vitro data^{1 3} and the results of an earlier clinical study⁶ it might have been anticipated that adverse outcomes during regularly scheduled salbutamol treatment would have occurred in homozygous Gly-16 subjects. However, any causal link between these in vitro and in vivo findings presupposes that enhanced β adrenoceptor downregulation is a determining factor for asthma control or long term responses to β agonist treatment. This has never been demonstrated. If this were indeed the case, a possible explanation for our findings is that a greater degree of endogenous downregulation in homozygous Gly-16 individuals occurs before treatment and is "protective" against the additional effects of exogenous salbutamol whereas homozygous Arg-16 subjects remain susceptible.¹³ This might also explain why both Martinez *et al*¹ and Lima *et al*⁶ have observed larger bronchodilator responses

in salbutamol-naive subjects harbouring the Arg-16 allele than in other position 16 genotypes. More recently Israel *et al* have reported that morning peak flows declined significantly in Arg-16 homozygotes during treatment with regular salbutamol compared with “as needed” salbutamol in patients harbouring the same genotype, as well as in homozygous Gly-16 subjects receiving regular treatment.¹⁴ The results of the present study are in keeping with each of these observations and suggest that the increased acute response to salbutamol in bronchial smooth muscle in homozygous Arg-16 individuals^{7,8} is also associated with an increased predisposition to adverse effects, perhaps mediated via β_2 receptors in other cell types, during chronic dosing.

A potential criticism of our study is that the system of scoring for exacerbations was somewhat arbitrary. However, the approach used was similar to that taken in another large randomised trial of asthma treatment.¹⁵ Furthermore, since our trial was conducted in a blinded, placebo controlled, crossover fashion, and since supplementary corticosteroid requirements (either inhaled or oral) were not used per se to calculate exacerbation rates, the increased odds ratio for prednisone use (1.70) in homozygous Arg-16 subjects provides a further independent indicator that asthma control was indeed worse with salbutamol in this particular subgroup. It also indicates that the result reported for exacerbations is unlikely to have arisen by chance. Likewise, concerns that patients with the Arg-Arg genotype had more severe asthma before randomisation are not substantiated from our data. There were no differences in baseline percentage predicted forced expiratory volume in one second (FEV₁), concentration of methacholine provoking a fall in FEV₁ of 20% or more (PC₂₀ methacholine), or inhaled corticosteroid requirements between subgroups. Although the exacerbation rates for homozygous Arg-16 patients were numerically greater during placebo treatment than for the other two genotypes, the differences were not significant. It is possible that this last observation marks an adverse response even to “as required” salbutamol (which was permitted) compared with other genotypes, but this is speculative.

Polymorphism at position 16 may not necessarily be the functional polymorphism but rather other mutations, including those at position 27 or the recently identified polymorphism in the leader cistron of the β_2 adrenoceptor,¹⁶ may be the moieties which mediate the observed adverse treatment effects. In human subjects it is impossible to prove causation at an implied genetic locus unequivocally. This is especially true when the phenotype of interest is common and may result from multiple genetic and environmental influences. In this regard, there may be numerous factors affecting the phenotype which we observed—namely, an increased likelihood of asthma exacerbations with regular inhaled salbutamol. Although we cannot

establish causation, our data suggest that an adverse effect of long term use of a short acting β_2 agonist on asthma control can be identified by the Arg-Arg genotype.

Compared with salbutamol the outcomes for salmeterol were largely unaffected by position 16 genotype. This contrast is difficult to explain. Although the benefits of salmeterol appeared to be least in the Arg-Arg subgroup, in whom the rate of major exacerbations was nearly three times that of homozygous Gly-16 subjects, there was a similar pattern in the frequency of major exacerbations across genotypes during the placebo treatment period. Both short and long acting β agonists are associated with similar β adrenoceptor downregulation when given regularly.¹⁷ It therefore seems unlikely that, even though β adrenoceptor polymorphisms alter downregulation in vitro, this will prove to be the mechanism by which genotype differences affect treatment responses or explain apparent differences between salbutamol and salmeterol.

Although current guidelines for the management of chronic asthma indicate that short acting β_2 agonists should be used only “as required” for symptom relief,^{18,19} a number of patients use “reliever” bronchodilator frequently or to excess, particularly during exacerbations.²⁰ Hence the clinical relevance of the present study. In addition, an increase in major exacerbation rates from approximately one to approximately two per annum during treatment with salbutamol is clinically important. The frequency of homozygous Arg-16 subjects (17/108) among our patients was similar to that recorded in other populations.^{21,22} Thus, if the pattern suggested by the present study is a consistent one for even one in six patients with the Arg-Arg genotype, it would make sense to identify such individuals and offer them alternative treatments. It may also explain why withdrawal of β agonist treatment may have a dramatically beneficial effect in some patients but not in others.^{23,24} Before specific recommendations can be made, confirmatory studies in genotype-stratified treatment groups are warranted.

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