

Clinical Study

Polymorphism of Beta2-Adrenoceptor and Regular Use of Formoterol in Asthma: Preliminary Results

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Polymorphism at codon 16 of the beta2-adrenoceptor (beta2-AR) affects the responsiveness to salmeterol in asthmatics. Data concerning formoterol are more controversial in the literature. The aim of this study was to verify whether homozygous for arginine-16 (ArgArg16) and homozygous for glycine-16 (GlyGly16) genotypes differently influence the long-term responsiveness to formoterol. Twenty-nine patients with mild-to-moderate asthma, in stable clinical conditions, underwent genotyping at codon 16 of the beta2-AR by RFLP-PCR assay. The effects of a 4-week monotherapy with formoterol (12 μ g BID) were tested on the peak expiratory flow (PEF) variability and the forced expiratory volume in 1 sec (FEV1) slope of the dose-response curve to salbutamol. Variability in PEF significantly increased during the 4-week treatment period in 14 patients with GlyGly16, but not in 15 patients with ArgArg16 and ArgGly16 ($P = 0.032$). The FEV1 slope of the dose-response curve to salbutamol decreased after the 4-week treatment period in GlyGly16, but not in pooled ArgArg16 and ArgGly16 patients. This study provides preliminary evidence that tolerance to formoterol develops more frequently in asthmatics with GlyGly16 genotype. If confirmed in a larger population, this finding might be useful in choosing the bronchodilator therapy on the basis of genetic polymorphism of the beta2-AR.

1. Introduction

Genetic factors, mainly the polymorphism at codons 16 and 27 of the beta2-adrenoceptor (beta2-AR) on chromosome 5q31, are known to modulate the bronchodilatory effects of beta2-agonists [1, 2]. In vitro studies performed on peripheral lymphocytes have shown that asthmatics homozygous for glycine-16 (GlyGly16) are more prone to beta2-agonist-induced downregulation than either homozygous for arginine-16 (ArgArg16) or heterozygous (ArgGly16) [3]. Consistent with in vitro findings are the effects of the acute exposure of asthmatics to short-acting beta2-agonists (SABAs) [4, 5]. However, chronic exposure to SABAs or to the long-acting beta2-agonist (LABA) salmeterol resulted in greater desensitisation among ArgArg16 asthmatics [2, 6–9] and these patients are also exposed to an increased risk

of exacerbations [10]. A study carried out in children with severe asthma exacerbation hospitalized in intensive care unit showed that patients with genotype GlyGly16 had a more rapid and intense response to therapy with inhaled salbutamol compared with patients with other genotypes [11]. Furthermore, GlyGly16 genotype protected from increase in responsiveness to methacholine during regular treatment with the SABA fenoterol [12].

Further complicating the issue are findings pertaining to formoterol: asthmatics carrying the GlyGly16 but not the ArgArg16 genotype had a greater risk of desensitisation [13, 14]. Similarly, a study of Korean asthmatics evaluating the response to combination treatment of formoterol and budesonide showed a better response in the group with ArgArg16 genotype [15]. However, other studies did not show any influence of genotype on receptor desensitization in

patients who were on chronic treatment with formoterol plus budesonide or salmeterol plus fluticasone [16].

These contrasting results might reflect differences in study design, duration of the exposition to the beta2-agonist, and main outcome. Furthermore, in order to comply with current therapeutic guidelines for asthma, in some studies inhaled corticosteroids (ICS) and LABA have been coadministered [13, 14, 16]. This choice makes results barely interpretable because steroids and LABA interact with each other and this interaction can affect biologic and clinical effects of both [17].

We report the preliminary results of a study designed to test the hypothesis that GlyGly16 asthmatics, who are otherwise more responsive to chronically used salmeterol, were more prone than ArgArg16 and/or ArgGly16 asthmatics to develop tolerance to formoterol in an experimental design free from the confounding effect of concurrent steroids administration.

2. Materials and Methods

2.1. Patients. We genotyped 29 Caucasian patients, 10 males and 19 females, with mild-to-moderate asthma. None had experience of athletics but 8 of them reported to perform a moderate weekly physical activity. None were smokers and all were in stable clinical condition at the time of recruitment. According to the Global Initiative for Asthma (GINA) guidelines, they had no chronic symptoms, no limitations on activities, and minimal need for “as needed” use of beta2-agonists [18]. Moreover they had no emergency visits, had not been treated with oral steroids, nor had a recent respiratory tract infection or exacerbation within the past 3 months. Eight patients were regularly taking ICS (fluticasone propionate or budesonide) with LABA (formoterol or salmeterol), 4 chromones (nedocromil sodium) alone or associated with formoterol, 6 patients had used ICS in the past, and 11 were occasionally using salbutamol as needed. The patients underwent spirometry and a bronchodilator test. A methacholine challenge test was performed in patients with a forced expiratory volume in 1 sec (FEV1) to forced vital capacity (FVC) ratio $\geq 70\%$.

All patients gave a written informed consent for the study which was in agreement with the guidelines approved by the local ethical committee.

2.2. Study Protocol. Each patient underwent the following procedures (Figure 1): an initial run-in period of 4 weeks during which all drugs were withdrawn with the exception of salbutamol as needed; a baseline functional evaluation with spirometry and a dose-response curve to salbutamol at the end of the run-in period; a treatment period of 4 weeks with inhaled formoterol (12 μg BID) during which the peak expiratory flow (PEF) was recorded twice daily; a final functional evaluation with spirometry and a dose-response curve to salbutamol at the end of the treatment period. Patients were further visited after 1 week to control their clinical conditions. Physicians performing respiratory function tests were unaware of the patients' genotypes.

2.3. Respiratory Function Measurements

2.3.1. Spirometry and Bronchodilator Test. Spirometry was performed according to the American Thoracic Society criteria [19] by using the computerized system Vmax 229 (SensorMedics, CA, USA). The subjects performed at least three forced expiratory manoeuvres and the best test was recorded. FVC, FEV1, and forced midexpiratory flow (FEF₂₅₋₇₅) were measured. After spirometry the patients inhaled salbutamol 200 μg to perform the bronchodilator test. A forced expiratory manoeuvre was repeated 20 min after the inhalation and the percentage changes of FEV1 with respect to basal values were recorded.

2.3.2. Methacholine Challenge Test. Bronchial reactivity to inhaled methacholine was measured in patients with a FEV1/FVC ratio $\geq 70\%$, using a dosimeter providing a calibrated output of 9.0 μL per puff [20]. The subjects inhaled an aerosol of diluent followed by doubling concentrations of methacholine from 0.031 to 16 mg/mL. FEV1 was measured after each inhalation. The linear interpolation was used to calculate the provocative concentration of methacholine causing 20% fall in FEV1 (PC20FEV1).

2.3.3. Dose-Response Curve to Salbutamol. This test was used to verify the acute response to a bronchodilator before and after the chronic treatment with formoterol, independently from the presence or not of bronchial obstruction at baseline. Cumulative doses of 200, 400, and 800 μg of salbutamol were consecutively inhaled. The time interval between each delivered dose was of at least 1 hour and a forced expiratory manoeuvre was performed 20 min after each inhalation. The percentage changes of FEV1 after each dose with respect to basal value measured before the first dose were recorded. The results were expressed as slope of the dose-response curve to salbutamol, derived from the beta-coefficient of the linear regression analysis between FEV1 changes after each dose and the corresponding doses of salbutamol inhaled.

2.3.4. PEF Monitoring. PEF was monitored during the 4-week treatment period by using a mini-Wright peak-flow meter (Clement Clarke Int. Ltd, UK). Home measurements of PEF were performed twice daily: the patients were instructed to record their morning and evening peak flow prior to taking each dose of formoterol, recording the best of three readings. The results were expressed as daily variability as follows: $[(\text{higher-PEF} - \text{lower-PEF})/\text{higher-PEF}] \times 100$. The weekly variability was then calculated as an average from the daily variability of each week of treatment.

2.4. Genotypic Analysis. Genomic DNA was extracted from peripheral blood leukocytes using the standard salting-out procedure. For beta2-AR ArgGly16 genotyping, DNA was amplified by PCR in a final reaction volume of 25 μL using the primers described by Martinez et al. [4]. A PCR product of the size of 168 bp was obtained after 30 cycles with the annealing temperature of 64°C. Ten microliters of PCR products were digested with 3U of the restriction enzyme

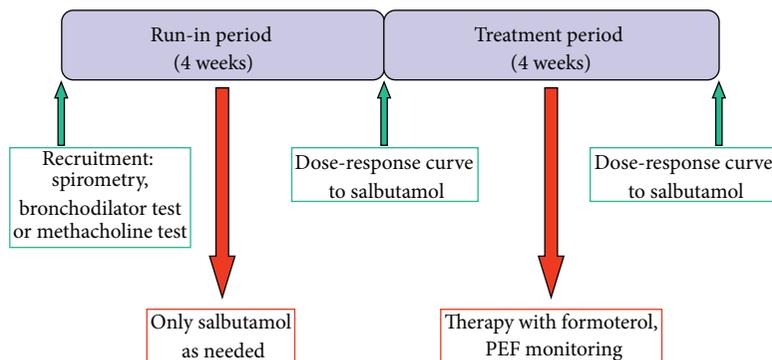


FIGURE 1: Study design.

NcoI at 37°C overnight in a reaction volume of 30 μ L. *NcoI* cuts the Gly16 allele generating a fragment of 146 bp and leaving the Arg16 allele uncut. Genotypes were analysed by electrophoresis on a 3.5% standard agarose gel.

2.5. Statistical Analysis. Differences among genotypes in terms of clinical and respiratory function parameters at baseline were analyzed by analysis of variance and unpaired *t*-test or nonparametric Kruskal-Wallis and Mann-Whitney tests, as appropriate. Differences among genotypes in changes of recorded variables during the treatment period were assessed by the analysis of variance for repeated measures having the group membership as the grouping factor. A *P* value ≤ 0.05 was considered significant.

3. Results

According to the genotypic analysis, 14 patients were GlyGly16, 5 were ArgArg16, and 10 were ArgGly16. All the patients remained clinically stable during the run-in period and only 5 of them occasionally used salbutamol as needed. Moreover they did not report significant respiratory symptoms during the treatment period and at the last visit performed 1 week after the end of the study protocol.

According to the working hypothesis, a comparison was made between the GlyGly16 and pooled ArgArg16 and ArgGly16 patients. No significant difference in baseline clinical and functional data was found between the two groups (Table 1).

The results derived from the dose-response curve to salbutamol performed before and after the 4-week treatment with formoterol are reported in Figure 2. The GlyGly16 patients showed a trend towards a lower FEV1 slope after treatment in comparison with baseline, indicating a lower response to the acute effect of salbutamol. On the contrary, the slope improved after the treatment period in pooled ArgArg16 and ArgGly16 patients, although the difference between the two groups did not reach the statistical significance.

In GlyGly16 patients, PEF variability remained substantially unchanged during the first 3 weeks, but it dramatically increased in the last week of treatment (Figure 3). On the

TABLE 1: Comparison of baseline clinical and functional data between the two groups of patients.

	GlyGly16	ArgArg16 + ArgGly16	<i>P</i> value
Number	14	15	
Age (years)	34.28 \pm 14.97	32.73 \pm 8.34	0.73
Duration of disease (years)	16.50 \pm 8.10	15.87 \pm 9.98	0.92
FVC (% pred)	111.36 \pm 15.30	105.93 \pm 14.78	0.34
FEV1 (% pred)	100.27 \pm 13.07	91.93 \pm 14.19	0.11
FEV1/FVC (%)	76.87 \pm 9.46	75.07 \pm 8.34	0.59
FEF ₂₅₋₇₅ (% pred)	72.14 \pm 23.14	61.80 \pm 23.67	0.24
FEV1 changes after salbutamol (%)	9.43 \pm 5.40	9.73 \pm 7.63	0.90
PC20FEV1 (mg/mL)*	1.93 \pm 1.10	1.54 \pm 1.26	0.53

Data are expressed as mean \pm SD.

*The methacholine challenge test was performed in 11 patients of the GlyGly16 group and in 10 of the ArgArg16 + ArgGly16 group.

FVC: forced vital capacity; FEV1: forced expiratory volume in 1 sec; FEF₂₅₋₇₅: forced midexpiratory flow; PC20FEV1: provocative concentration of methacholine causing 20% fall in FEV1.

other hand, the pooled ArgArg16 and ArgGly16 patients showed a slow but evident trend towards a decrease in PEF variability during the whole treatment period. The difference between groups was significant (*P* = 0.032), indicating a worsened clinical control of the disease in the GlyGly16 patients, especially in the last week of treatment with formoterol.

4. Discussion

We found that the GlyGly16 genotype is associated with a well evident liability to develop tolerance during chronic treatment with the LABA formoterol. This finding confirms those of Tan et al. and Aziz et al, but does not suffer from the confounding effect due to concurrent steroid administration [13, 14].

As noted previously, conflicting results are reported in the literature about the relationship between different genotypes and chronic use of different LABAs alone or added to ICS. In the LARGE study, both ArgArg16 and ArgGly16 patients

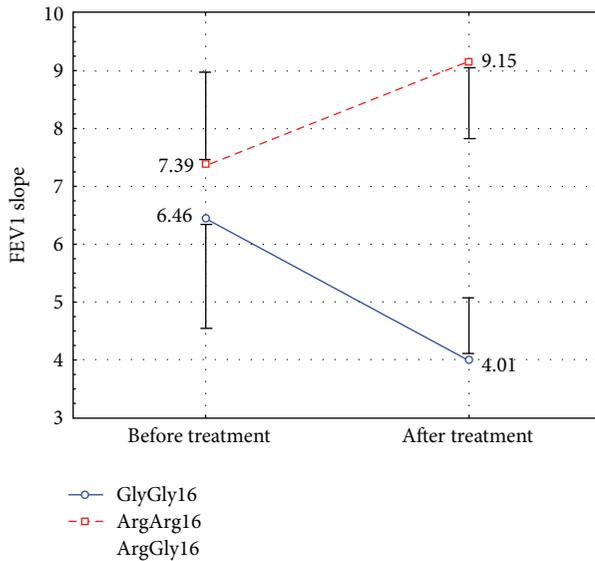


FIGURE 2: FEV1 slope of the dose-response curve to salbutamol before and after the treatment period in the GlyGly16 (continuous line) and in pooled ArgArg16 and ArgGly16 patients (dashed line). Values are expressed as means with error bars. Between-groups differences: $F = 1.02$; $P = 0.327$.

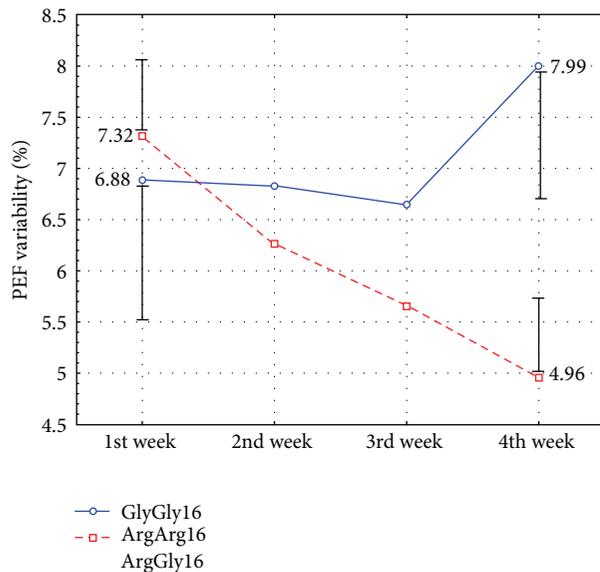


FIGURE 3: PEF variability during the 4-week treatment period in the GlyGly16 (continuous line) and in pooled ArgArg16 and ArgGly16 patients (dashed line). Values are expressed as means with error bars. Between-groups differences: $F = 3.18$; $P = 0.032$.

experienced an improved airway function with salmeterol added to moderate dose of ICS [21]. On the contrary, methacholine PC20FEV1 doubled when salmeterol was added to ICS in GlyGly16 patients, but remained unchanged in ArgArg16 subjects [21]. Other studies failed to demonstrate a pharmacogenetic effect of beta2-AR variation at codon 16 on response to salmeterol alone or in combination with

fluticasone propionate in patients with asthma [22, 23]. Lipworth et al. found no difference in clinical efficacy and protection against methacholine-induced bronchoconstriction between salmeterol and formoterol chronically used in GlyGly16 asthmatics [24]. Analogously, Lee et al. showed that tolerance to salbutamol developed independently from the genotype and the type of LABA (formoterol versus salmeterol) chronically used [25]. Finally, a recent study on patients with chronic obstructive pulmonary disease (COPD) showed that the therapeutic response and the tolerability to long-term treatment with formoterol alone or in combination with budesonide were not modified by beta2-AR genotype at codon 16 [26].

On purely pharmacological ground, salmeterol and formoterol have different structures, mechanism of action, and pharmacodynamic properties [14, 17], and this might support different profiles of activity with regard to genetic patterns. On the other hand, the structural analogy between salbutamol, the parent compound, and salmeterol likely account for the comparable profiles of activity of these related drugs [27]. The LABAs salmeterol and formoterol are considered almost equivalent drugs, except for a faster onset of action for formoterol [28]. Indeed, these drugs have comparable half life, duration of action, and nonbronchodilator effects such as anti-inflammatory properties. However, formoterol is less lipophilic than salmeterol and, *in vitro*, has greater bronchodilating and anti-inflammatory potency [17]. Furthermore, formoterol and salmeterol behave as a full and partial agonist of the beta2-AR, respectively [29]. This bulk of evidence shows that salmeterol and formoterol cannot be considered interchangeable LABAs. However, it cannot explain the observed differences in the onset of tolerance to their bronchodilating effect because the respective profile of tolerance seems either drug-specific or patient-specific [17]. Accordingly, the patient-drug interaction rather than the drug *per se* seems relevant to explain the interindividual variability in the onset of tolerance. Thus, a given genotype, the ArgArg16, seems to protect from tolerance to formoterol while predisposing to tolerance to salmeterol and *vice versa* for the GlyGly16 genotype [6–9, 13, 14].

Our and most other previous studies on the genetic determinants of tolerance to beta2-agonists have focused on the Arg-Gly polymorphic site at codon 16 which seems to have a more important role in beta2-AR desensitisation, compared to the other polymorphic sites at codon 27 (Gln-Glu) [11, 13, 30]. However a study of asthmatics children with and without nocturnal asthma highlighted an association between beta2-AR 27 polymorphism and response to terbutaline nebulizer [31], whereas Carroll et al. in a pooled cohort of 104 children admitted to the intensive care unit (ICU) with a severe asthma exacerbation found that those with the ArgGly16-GlnGln27 haplotype of the beta2-AR were four times more likely to be intubated and mechanically ventilated so showing that also genetic variation at codon 27 can influence the development of a more severe asthma phenotype during acute exacerbations [32]. Thus, both additional polymorphisms and variability in the signalling pathways of the beta2-AR might affect the response to beta2-agonists [33, 34]. Accordingly, interpreting the response to LABAs on

the basis of the site 16 dependent beta2-AR genotype and the type of LABA might be an oversimplification. Nevertheless the strength of the evidence is in favour of this dual mechanism as are data by Palmer et al. who retrospectively analysed the relationship between genotype and frequency of exacerbations in 546 children and young adults with asthma [35]. They found that the ArgArg16 status was associated with more exacerbations in those on regular salmeterol and inhaled steroids, but, interestingly, polymorphism at site 27 was unrelated to frequency of exacerbations in any treatment group. Unfortunately, no patient was given formoterol, and, thus, the hypothesis at the basis of our study could not be tested.

Prevalence of the ArgArg16 and GlyGly16 patterns in our patients was 17% and 48%, respectively. These figures are comparable to those reported by most of previous studies of Caucasian populations for ArgArg16 pattern, while the prevalence of GlyGly pattern was slightly higher in our sample than in other studies [3, 4, 7, 23, 33, 35, 36], but similar to that reported by Hancox et al. [12]. Thus, more than half of asthmatics, but in selected studies even a greater percentage [6, 37], carry a genotype predisposing them to selective sensitivity to individual LABAs. Accordingly, typifying asthmatics with regard to the genotype at site 16 might improve therapy and, by reducing the frequency of exacerbations, curtail health care expenditures. This interesting perspective deserves to be confirmed by a properly designed trial.

This study has important limitations. First, the sample size was small. However, the fact that a significant association between GlyGly16 genotype and tolerance to formoterol emerged in a small sample is consistent with this association being biologically plausible. Second, a 4-week period might not be long enough to disclose genotype-dependent differences in tolerance to formoterol. However, it would be technically difficult and ethically unsound to maintain on LABA therapy selected subjects likely to benefit from concurrent inhaled steroids. Third, the boundary between onset of tolerance and mild exacerbation is not so straightforward, and this might result in overestimating the incidence of tolerance. This limitation affects any study aimed at providing an unbiased assessment of the risk of beta2-AR desensitisation during any pharmacological therapy in asthmatics. Eventually, asthma is a heterogeneous disease involving several pathogenetic mechanisms. Accordingly, any putative pharmacogenomic association can be variously confounded by simultaneous genetic factors and nongenetic host-related and environmental factors [38]. Only randomised placebo-controlled trials conducted on very large and well-characterized populations would have the potential for assessing pharmacogenomic-related variability. Our study and the vast majority of the clinical trials so far conducted should hopefully herald such trials.

5. Conclusions

Our results support the hypothesis that the GlyGly16 genotype, which protects from tolerance to salmeterol and SABAs, qualifies as a risk factor for tolerance to formoterol. This preliminary finding needs to be confirmed in properly sized

placebo-controlled studies including also the analysis of codon 27. If confirmed, it would affect asthma management and would be the rationale for synthesizing other LABAs with different molecular structures in order to enlarge the spectrum of LABA-beta2-AR interactions and, thus, tailoring the choice of LABAs to individual genotypes.

Conflict of Interests

No financial or any other potential conflict of interests exists for each author.

Acknowledgment

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