*Article*

# Glucocorticoid Receptor Polymorphism A3669G Is Associated with Airflow Obstruction in Mild-to-Severe Asthma

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**Featured Application: Allele A of A3669G SNP was associated with steroid response to GCs in asthmatics. The heterozygous genotype of A3669G might be related to the type-2 allergic asthma phenotype. A3669G SNP can be used as a predictor of asthma severity and phenotype.**

**Abstract:** Background: Glucocorticoids (GCs) represent the mainstay therapy for asthmatics. A subset of severe asthmatics fails to respond to steroid-based therapies, leading to important healthcare costs. Single nucleotide polymorphisms (SNPs) of glucocorticoid receptor genes were associated with a response to GC. We evaluate the possible relation of BclI and A3669G SNPs to clinical, biological and functional characteristics of asthmatics. Methods: We recruited 172 mild-to-severe asthmatic outpatients referring to the Severe Asthma and Rare Lung Disease Unit at San Luigi University Hospital. Clinical data were obtained at recruitment when spirometry tests and peripheral blood sampling were performed. Patients were genotyped for BclI and A3669G through the pyrosequencing assay results. Results: Patients with the A3669G AG genotype were younger, allergic and had higher IgE levels compared to AA genotype ($p < 0.05$). Moreover, asthmatics with the AA genotype had a lower post-bronchodilator $FEV_1$/FVC ratio than the GG genotype ($p < 0.05$), and a higher RV/TLC ratio than the AG genotype ($p < 0.05$). Conclusions: The A3669G AG genotype might be related to type-2 allergic asthma; in particular, allele A of A3669G SNP was associated with GC response in our asthmatics. In conclusion, this observational cross-sectional study suggests a possible role of A3669G SNP as a predictor of asthma severity and phenotype.

**Keywords:** asthma; glucocorticoid receptor gene; airflow obstruction; air trapping; SNP

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## 1. Introduction

Asthma is a global health problem defined as a heterogeneous and complex condition characterized by variable airflow obstruction and different respiratory symptoms [1].

The interaction between environmental and genetic factors in conjunction with different inflammatory pathways develops several clinical asthma phenotypes. The two main asthma phenotypes are T2-high and T2-low [2], characterized by different molecular mechanisms. Recent evidence reported the key role of gene–environmental interaction in asthma progression [1,3].

The mainstay therapy for asthma is represented by glucocorticoids (GCs) administered through inhaled (ICS) or oral (OCS) formulations. Inhaled corticosteroids, in combination

with bronchodilators (long-acting β2-adrenoceptor agonists, LABA) are able to reach and maintain disease control in a greater number of asthmatic patients [3]. Although, a subset of asthmatic patients, in particular severe asthmatics, fails to respond to steroid-based therapies, leading to important healthcare costs [4,5].

GC treatment resistance is due to complex mechanisms not yet understood and represents an important clinical barrier to severe asthma management.

Some evidence suggests that the reduced response to GCs in asthma and other conditions is due to the decreased expression of glucocorticoids receptor (GR) provoked by single nucleotide polymorphisms (SNPs) in the NR3C1 (nuclear receptor subfamily 3 group C member 1) gene that encodes for the glucocorticoid receptor [6]. Mutations in the NR3C1 gene are described in association with generalized glucocorticoid resistance (GCCR) [7].

The human NR3C1, a gene composing nine exons, is localized on chromosome 5q31.3, and alternative splicing of this gene results in transcript variants encoding either the same or different isoforms [8,9].

Researchers focused their attention on two isoforms produced as a result of alternative splicing near the end of the primary transcript: GRα, the active form, and GRβ, which plays an inhibitory role on GRα [10]. A few SNPs are responsible for the alteration of the structure of GR domains; the most frequently investigated SNPs among the asthmatic population are the BclI (rs41423247) polymorphism that increases the sensitivity to the GCs and the A3669G SNP (rs6198), namely c.*38833A>G, which is related to steroid-resistant asthma [11–13].

BclI polymorphism, namely c.1184+646C>G, is localized in the intron 2 of the NR3C1 gene and was recognized as part of the SNP haplotype that can affect splicing. The A3669G SNP (rs6198) is situated in the 3′ untranslated region of GRβ exon 9; this sequence change increased its mRNA stability and GRβ protein expression, provoking the inhibition of the transcriptional activity of GRα [11,14,15].

Based on the possible distinct actions of these SNPs on the response of asthmatics to glucocorticoid therapy, we intended to evaluate the possible relation of BclI and A3669G to clinical, biological and functional characteristics of mild-to-severe asthmatics.

## 2. Materials and Methods

### *2.1. Patients*

In this observational cross-sectional real-life study, we recruited 172 consecutive mild-to-severe asthmatic outpatients referred to the Severe Asthma and Rare Lung Disease Unit at San Luigi University Hospital (Orbassano, Turin, Italy) from 2014 to 2018. Inclusion criteria were asthma diagnosis, according to GINA (Global Initiative for Asthma) and ERS/ATS Guidelines [16,17], and adult age (≥18 years). Documented asthma diagnoses, including a typical history of respiratory symptoms in relation to variable airflow limitation, as demonstrated by reversibility to bronchodilators and bronchial hyper-responsiveness to methacholine, were collected and entered in chart data. We ruled out patients with other pulmonary diseases, patients with current or recent asthma exacerbation, and self-reported poor adherence (<50%) to treatment or an inadequate inhalation technique. A sample size of 162 asthmatic patients was required for a power calculation of 80% in relation to the genotypic investigation setting an ICS cut-off of 400 µg/day.

All participants provided written informed consent to participate in this study.

### *2.2. Collection of Clinical, Functional and Biological Data*

Clinical data and patient history (age, gender, BMI, smoking habit, pulmonary function, asthma symptoms, asthma medications and comorbidities) were obtained at recruitment when spirometry, post-bronchodilation reversibility tests and peripheral blood sampling were performed. Pulmonary function testing was performed by assessing spirometry and lung volumes before and 15 min after the administration of albuterol (400 µg) using a body plethysmograph (Vmax Encore 62, Carefusion, 97204, Höechberg, Germany). Frac-

tional exhaled nitric oxide ($F_ENO$) was measured at the flow of 50 mL/s with the $F_ENO$+ instrument (Medisoft, Sorinnes, 5503, Dinant, Belgium). Skin prick tests and specific serum total IgE levels were performed to identify allergic patients according to validated criteria [18]; patients sensitized to two or more allergens were considered poly-sensitized [19]. Hematic counts were retrieved from routinely performed hematological examinations. Asthma exacerbations (AEs) were also retrieved and reported, and ≥2 AE in the previous year identified frequent exacerbators (FE) phenotype [20,21].

### 2.3. DNA Extraction and PCR Amplification

Total genomic DNA was isolated from 450 µL peripheral blood using the Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol.

Briefly, the protocol was divided into four steps:

1. Red blood cell lysis with the cell lysis solution (1350 µL);
2. Nuclei lysis with nuclei lysis solution (450 µL) and protein precipitation with protein precipitation solution (150 µL);
3. DNA precipitation with isopropanol and 70% ethanol (450 µL);
4. DNA rehydration with rehydration solution (40–50 µL) for 1 h at 65 °C or overnight at 4 °C.

Finally, the amount of DNA obtained was quantified by fluorometer Qubit™ 3.0 (Invitrogen, Waltham, MA, USA) through the "Quantifluor dsDNA" kit (Promega, Fitchburg, WI, USA).

PCR amplification of the region of interest was performed using primers (generating an amplimer of 128 bp for BclI and 125 bp for A3669G) designed using the PSQ Assay Design software (Biotage AB, Uppsala, Sweden, now Qiagen, Table 1).

**Table 1.** PCR and pyrosequencing primer.

| NR3C1 SNPs | Forward Primer | Reverse Primer | Pyrosequencing Primer | TA PCR | Amplicon Length |
|---|---|---|---|---|---|
| rs41423247 | 5′-GGTCTTGCTCACAGGGTTCTTG-3′ | Bio 5′-GAACTTGCAGGAA-CATTGAACG-3′ | 5′-AAGTAGACAAGTTATGTCTG-3′ | 58 °C | 128 bp |
| rs6198 | Bio 5′-ATGTCTTTTTACCTACGCAGTGA-3′ | 5′-CAATTCGGTAAAATGTGTGGTT-3′ | 5′-AATACCAGAACAGCAAATT-3′ | 58 °C | 125 bp |

Bio: the 5′ biotin modification of the PCR primer is required for the DNA single-strand DNA capture in the Pyrosequencing assay protocol. TA: annealing temperature for Polymerase Chain Reaction.

The PCR reactions (30 µL) included 20 ng of DNA and amplifying primers at a final concentration of 0.4 mM. The target region was amplified under the following conditions: 95 °C for 5 min, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72°C for 30 s, followed by 72 °C for 7 min. Then, the PCR products were resolved by 1.5% agarose gel electrophoresis to confirm successful amplification.

The NR3C1 SNPs were studied by pyrosequencing assay, and the results obtained were analyzed using the PSQ-96MA 2.0.2 software (Qiagen, Hilden, Germany) [22].

### 2.4. Statistical Analysis

All the statistical analyses were performed with GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA, USA). Data distributions were assessed using the D'Agostino–Pearson test. To compare the differences between groups, according to data distribution, one-way ANOVA (with Tukey post hoc test) or Kruskal–Wallis H test (with Dunn's post hoc test) were used, while to compare the frequencies, chi-squared ($\chi^2$) test (with Fisher's Exact post hoc approach) was applied [23]. In tables, continuous variables

are presented as mean ± SD; incidences are reported as occurrences/exposed cases as percentages. Odds ratios (OR) and 95% confidence intervals (CI) were calculated when applicable. Outliers were identified using ROUT methods and excluded from the analysis [24]. *p*-values ≤ 0.05 were considered significant.

## 3. Results

The genotype frequencies in our populations for both SNPs (Table 2) were in Hardy Weinberg equilibrium and did not differ significantly from those observed among the European subjects as reported in the database gnomAD [25]. The phase of the two SNPs was not considered due to the number of subjects analyzed and the allelic frequencies observed.

**Table 2.** Single nucleotide polymorphisms frequencies in the asthmatic population (N = 172).

| refSNP | Alias | Change at the DNA Level | Sequence Ontology Term | Ref Allele | Alt Allele | Genotype Frequencies (N) | | | Allelic Frequencies | |
|---|---|---|---|---|---|---|---|---|---|---|
| rs41423247 | BclI | c.1184+646C>G | Intron Variant | G | C | GG 0.10 (18) | GC 0.44 (75) | CC 0.46 (79) | G 0.54 | C 0.46 |
| rs6198 | A3669G | c.*3833A>G | 3′ UTR Variant | A | G | GG 0.06 (11) | AG 0.40 (68) | AA 0.54 (93) | G 0.29 | A 0.71 |

The baseline characteristics of the 172 asthmatics enrolled are shown in Table 3. Moderate-to-severe asthmatics (N = 85) were older, had more asthma exacerbations in the last year, and had higher blood eosinophils compared to mild asthmatics. Furthermore, these patients had a frequent exacerbator phenotype, a higher prevalence of blood eosinophils >300 cells/μL; LABA and LAMA medications, Omalizumab and OCS use than mild asthmatics. No significative differences were observed for what concerns the comorbidities.

**Table 3.** Descriptive statistics in the asthmatic population.

| | All Population (N = 172) | Mild Asthma (N = 87) | Moderate-Severe Asthma † (N = 85) |
|---|---|---|---|
| Age (years) | 58.4 ± 14.0 | 55.7 ± 14.4 | 61.1 ± 13.1 * |
| Gender (F) | 109/172 (63.4%) | 54/86 (62.8%) | 55/86 (63.9%) |
| BMI (Kg/m$^2$) | 26.7 ± 5.4 | 26.2 ± 5.8 | 27.3 ± 5.0 |
| Current smokers (≥10 PY) | 42/172 (24.4%) | 17/87 (19.5%) | 25/85 (29.4%) |
| Former smokers (≥10 PY) | 1/172 (0.6%) | 0/86 (0.0%) | 1/85 (1.2%) |
| Atopy | 100/172 (58.1%) | 53/87 (60.9%) | 47/85 (55.3%) |
| Poly-sensitization | 81/100 (81.0%) | 45/53 (84.9%) | 36/47 (76.6%) |
| Asthma exacerbations/year | 1.3 ± 1.5 | 0.7 ± 0.9 | 1.9 ± 1.7 **** |
| FE Phenotype | 54/172 (31.4%) | 12/87 (13.8%) | 42/85 (49.4%) *** |
| Serum IgE (kU/L) | 275.3 ± 435.7 | 194.0 ± 206.8 | 342.4 ± 550.7 |
| Serum IgE >100 kU/L | 78/135 (57.8%) | 35/61 (57.4%) | 43/74 (58.1%) |
| $F_E$NO (ppb) | 38.8 ± 27.2 | 36.5 ± 27.3 | 41.1 ± 27.1 |
| $F_E$NO > 30 ppb | 74/154 (48.0%) | 33/76 (43.4%) | 41/78(52.5%) |
| Blood eosinophils (cells/μL) | 354.2 ± 287.5 | 300.5 ± 234.7 | 408.6 ± 325.0 * |

**Table 3.** *Cont.*

| | All Population (N = 172) | Mild Asthma (N = 87) | Moderate-Severe Asthma † (N = 85) |
|---|---|---|---|
| Blood eosinophils >300 cells/μL | 70/163 (42.9%) | 29/82 (35.4%) | 41/81 (50.6%) * |
| ICS/day (μg BDP-HFA) | 330.2 ± 210.3 | 160.9 ± 81.2 | 503.5 ± 152.3 *** |
| LABA | 127/172 (73.8%) | 44/87 (50.6%) | 83/85 (97.6%) *** |
| LAMA | 23/172 (13.4%) | 0/87 (0.0%) | 23/85 (27.0%) *** |
| OCS (≥6 months/year) | 5/173 (2.9%) | 0/87 (0.0%) | 5/85 (5.9%) * |
| Omalizumab | 17/172 (9.9%) | 2/87 (2.3%) | 15/85 (17.6%) ** |
| Mepolizumab | 4/172 (2.3%) | 1/87 (1.1%) | 3/85 (3.5%) |
| Osteoporosis | 10/172 (5.8%) | 3/87 (3.4%) | 7/85 (8.2%) |
| Obesity | 42/172 (24.4%) | 18/87 (20.7%) | 24/85 (28.2%) |
| GERD | 47/172 (27.3%) | 20/87 (23.0%) | 23/85 (27.1%) |
| AERD | 34/172 (19.8%) | 14/87 (16.1%) | 20/85 (23.5%) |
| Persistent Rhinitis | 145/172 (84.3%) | 75/87 (86.2%) | 70/85 (82.3%) |
| CRSsNP | 36/172 (20.9%) | 17/87 (19.5%) | 19/85 (22.3%) |
| CRSwNP | 25/172 (14.5%) | 8/87 (9.2%) | 17/85 (20.0%) |
| Cardiopathy | 14/172 (8.1%) | 4/88 (4.6%) | 10/85 (11.8%) |

† Use of BDP-HFA ≥ 400 μg/day plus LABA; AERD: aspirin-exacerbated respiratory disease; BDP-HFA: beclomethasone dipropionate hydrofluoroalkane; CRSsNP: chronic rhinosinusitis without nasal polyposis; CRSwNP: chronic rhinosinusitis with nasal polyposis; FE phenotypes: frequent exacerbator phenotype ≥2 severe asthma exacerbations per year with OCS burst; GERD: gastro–esophageal reflux disease; LABA: long-acting beta-agonists; LAMA: long-acting muscarinic antagonists; ICS: inhaled corticosteroids; OCS: oral corticosteroids; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and *** $p < 0.0001$ vs. mild asthma.

The BclI and A3669G SNPs genotype and allele frequencies in our asthmatics are reported in Tables 4 and 5, respectively.

**Table 4.** Descriptive statistics in the asthmatic population stratified for the rs41423247 polymorphism.

| | CC (N = 79) | CG (N = 75) | GG (N = 18) |
|---|---|---|---|
| Mild Asthma | 40/79 (50.6%) | 41/75 (54.7%) | 6/18 (33.3%) |
| Moderate-to-severe asthma | 39/79 (49.4%) | 34/75 (45.3%) | 12/18 (66.7%) |
| Age (years) | 58.4 ± 14.1 | 56.9 ± 14.4 | 64.3 ± 10.3 |
| Gender (F) | 47/79 (59.5%) | 53/75 (70.7%) | 9/18 (50.0%) |
| BMI (Kg/m$^2$) | 26.7 ± 5.3 | 26.4 ± 5.7 | 28.2 ± 5.0 |
| Current smokers (≥10 PY) | 24/79 (30.4%) | 14/75 (18.7%) | 4/18 (22.2%) |
| Former smokers (≥10 PY) | 1/79 (1.3%) | 0/75 (0.0%) | 0/18 (0.0%) |
| Atopy | 50/79 (63.3%) | 43/75 (57.3%) | 7/18 (61.1%) |
| Poly-sensitization | 43/50 (86.0%) | 34/43 (79.1%) | 4/7 (57.1%) |
| Asthma exacerbations per year | 1.3 ± 1.3 | 1.2 ± 1.6 | 1.8 ± 1.9 |
| FE phenotype | 26/79 (32.9%) | 20/75 (26.7%) | 8/18 (44.4%) |
| Serum IgE (kU/L) | 327.0 ± 515.0 | 252.9 ± 373.9 | 111.7 ± 96.3 |
| Serum IgE >100 kU/L | 42/64 (65.6%) | 32/59 (54.2%) | 3/12 (25.0%) * |
| $F_E$NO (ppb) | 35.9 ± 26.6 | 40.4 ± 27.0 | 42.5 ± 31.4 |

**Table 4.** *Cont.*

| | CC (N = 79) | CG (N = 75) | GG (N = 18) |
|---|---|---|---|
| $F_ENO > 30$ ppb | 32/70 (45.7%) | 32/66 (48.5%) | 10/12 (83.3%) #* |
| Blood eosinophils (cells/µL) | 359.5 ± 270.9 | 356.0 ± 302.3 | 325.8 ± 307.0 |
| Blood eosinophils > 300 cells/µL | 32/73 (43.8%) | 32/72 (44.4%) | 6/18 (33.3%) |
| ICS/day (µg BDP-HFA) | 325.3 ± 207.2 | 329.3 ± 222.9 | 355.6 ± 175.6 |
| LABA | 58/79 (75.0%) | 54/75 (72.0%) | 14/18 (77.8%) |
| LAMA | 15/79 (19.0%) | 6/75 (8.0%) | 2/18 (11.1%) |
| OCS (≥6 months/year) | 3/79 (3.8%) | 1/75 (1.3%) | 1/18 (5.6%) |
| Omalizumab | 10/79 (12.6%) | 5/75 (6.7%) | 2/18 (11.1%) |
| Mepolizumab | 1/79 (1.3%) | 3/75 (4.0%) | 0/18 (0.0%) |
| Osteoporosis | 6/79 (7.6%) | 3/75 (4.0%) | 1/18 (5.6%) |
| GERD | 16/79 (20.2%) | 21/75 (28.0%) | 6/18 (33.3%) |
| AERD | 15/79 (20.0%) | 16/75 (21.3%) | 3/18 (16.7%) |
| Persistent Rhinitis | 67/79 (84.8%) | 64/75 (44.1%) | 14/18 (77.8%) |
| CRSsNP | 14/79 (17.7%) | 17/75 (22.7%) | 5/18 (27.8%) |
| CRSwNP | 10/79 (12.6%) | 13/75 (17.3%) | 2/18 (11.1%) |
| Cardiopathy | 7/79 (8.9%) | 5/75 (6.7%) | 2/18 (11.1%) |

AERD: aspirin-exacerbated respiratory disease; BDP-HFA: beclomethasone dipropionate hydrofluoroalkane; OCS: oral corticosteroids; CRSsNP: chronic rhinosinusitis without nasal polyposis; CRSwNP: chronic rhinosinusitis with nasal polyposis; FE phenotypes: frequent exacerbator phenotype ≥2 severe asthma exacerbations per year with OCS burst; GERD: gastro–esophageal reflux disease; LABA: long-acting beta-agonists; LAMA: long-acting muscarinic antagonists; ICS: inhaled cortico-steroids; OCS: oral corticosteroids; * $p < 0.05$ vs. CC; # $p < 0.05$ vs. CG.

**Table 5.** Descriptive statistics in the asthmatic population stratified for the A3669G polymorphism.

| | GG (N = 11) | AG (N = 68) | AA (N = 93) |
|---|---|---|---|
| Mild asthma | 7/11 (63.6%) | 35/68 (51.5%) | 45/93 (48.4%) |
| Moderate-to-severe asthma | 4/11 (36.4%) | 33/68 (48.5%) | 48/93 (51.6%) |
| Age (years) | 61.9 ± 11.0 | 54.4 ± 14.6 § | 60.8 ± 13.3 |
| Gender (F) | 9/11 (81.8%) | 41/68 (60.3%) | 59/93 (66.4%) |
| BMI (kg/m$^2$) | 29.9 ± 7.7 | 26.0 ± 4.7 * | 26.7 ± 5.1 |
| Current smokers (≥10 PY) | 2/11 (18.2%) | 12/68 (17.6%) | 28/93 (30.1%) |
| Former smokers (≥10 PY) | 0/11 (0.0%) | 1/68 (1.5%) | 0/93 (0.0%) |
| Atopy | 5/11 (45.4%) | 49/68 (72.1%) §§ | 46/93 (49.5%) |
| Poly-sensitization | 5/5 (100%) | 41/49 (83.7%) | 35/46 (76.1%) |
| Asthma exacerbations/year | 0.9 ± 1.3 | 1.2 ± 1.3 | 1.4 ± 1.6 |
| FE Phenotype | 3/11 (27.3%) | 20/68 (29.4%) | 31/93 (33.3%) |
| Serum IgE (kU/L) | 273.3 ± 524.6 | 361.9 ± 519.4 § | 204.0 ± 328.9 |
| Serum IgE > 100 kU/L | 5/9 (55.6%) | 41/57 (71.9%) §§ | 32/69 (46.4%) |
| FeNO ppb | 31.2 ± 20.0 | 37.3 ± 27.3 | 40.8 ± 27.9 |
| FeNO > 30 ppb | 4/10 (40.0%) | 26/60 (43.3%) | 44/84 (52.4%) |
| Blood eosinophils (cells/µL) | 312.4 ± 268.1 | 359.4 ± 279.1 | 355.1 ± 298.2 |

**Table 5.** *Cont.*

| | GG (N = 11) | AG (N = 68) | AA (N = 93) |
|---|---|---|---|
| Blood eosinophils > 300 cells/μL | 3/10 (30.0%) | 29/65 (44.6%) | 38/88 (43.2%) |
| ICS/day (μg BDP-HFA) | 300.0 ± 279.3 | 322.1 ± 201.4 | 339.8 ± 209.6 |
| LABA | 7/11 (63.6%) | 54/68 (79.4%) | 66/93 (71.0%) |
| LAMA | 1/11 (9.1%) | 12/68 (17.6%) | 10/93 (10.8%) |
| OCS (≥6 months/year) | 0/11 (0.0%) | 1/68 (1.5%) | 4/93 (4.3%) |
| Omalizumab | 1/11 (9.1%) | 7/68 (10.3%) | 9/93 (9.7%) |
| Mepolizumab | 0/11 (0.0% | 1/68 (1.5%) | 3/93 (3.2%) |
| Osteoporosis | 1/11 (9.1%) | 3/68 (4.4%) | 6/93 (6.4%) |
| GERD | 4/11 (36.7%) | 11/68 (16.3%) | 28/93 (30.1%) |
| AERD | 2/11 (18.2%) | 15/68 (22.1%) | 17/93 (18.3%) |
| Persistent Rhinitis | 10/11 (90.9%) | 57/68 (83.8%) | 78/93 (83.9%) |
| CRSsNP | 2/11 (18.2%) | 15/68 (22.1%) | 19/93 (20.4%) |
| CRSwNP | 1/11 (9.1%) | 9/68 (13.2%) | 14/93 (15.1%) |
| Cardiopathy | 2/11 (18.2%) | 2/68 (2.9%) | 10/93 (10.8%) |

AERD: aspirin-exacerbated respiratory disease; BDP-HFA: beclomethasone dipropionate hydrofluoroalkane; CRSsNP: chronic rhinosinusitis without nasal polyposis; CRSwNP: chronic rhinosinusitis with nasal polyposis; FE phenotypes: frequent exacerbator phenotype ≥2 severe asthma exacerbations per year with OCS burst; GERD: gastro–esophageal reflux disease; LABA: long-acting beta-agonists; LAMA: long-acting muscarinic antagonists; ICS: inhaled cortico-steroids; OCS: oral corticosteroids; * $p < 0.05$ vs. GG; § $p < 0.05$; §§ $p < 0.01$ vs. AA.

Patients with the GG genotype of BclI had a higher percentage of patients with $F_ENO$ > 30 ppb than patients with CC and CG genotypes and less percentage of patients with IgE > 100 kU/L than asthmatic patients with the CC genotype. No other relevant differences were observed between genotypes of BclI (Table 4).

Moreover, we analyzed the risk of carrying a specific genotype of BclI for what concerns higher levels of IgE and $F_ENO$. The homozygote CC asthmatics had a major risk of having IgE > 100 kU/L (OR = 5.7; 95%CI = 1.6–20.7; $p = 0.012$) than homozygote GG asthmatics, while the BclI CC genotype (OR = 0.2, 95%CI = 0.04–0.7; $p = 0.026$) and BclI CG genotypes (OR = 0.2, 95%CI = 0.04–0.8; $p = 0.031$) are associated with a lower risk of having $F_ENO$ levels more than 30 ppb than BclI GG genotype (Figure 1A). Examining the A3669G SNP, we observed that patients with the AG genotype were younger, allergic and had higher levels of serum total IgE more percentage of patients with IgE >100 kU/L compared to the AA genotype ($p < 0.05$). Furthermore, patients with AG genotypes were thinner than the GG genotype ($p < 0.05$) (Table 5).

In order to identify whether distinct A3669G genotypes may predispose to specific asthma phenotypes, we performed odds ratio analyses for each factor characterizing our population. Patients with heterozygous AG had a greater probability to had atopic asthma (OR = 3.0; 95%CI = 1.6–5.9; $p = 0.001$) and had IgE >100 kU/L (OR = 3.0; 95%CI = 1.4–6.2; $p = 0.006$) than AA genotype (Figure 1A). No significant OR was observed when evaluating the possible association of A3669G SNP and obesity (BMI ≥ 30 $kg/m^2$).

Finally, we observed that asthmatics carrying the BclI CC genotype had a greater risk of having IgE > 100 kU/L (OR = 2.2; 95%CI = 1.1–4.3; $p = 0.036$) than asthmatics carrying the A3669G AA genotype; moreover, patients with the A3669G AG genotype had a higher probability of being atopic (OR = 4.1; 95%CI = 1.4–11.1; $p = 0.012$) than asthmatics with GG BclI genotype (Figure 1B).

Examining the lung function in the A3669G SNP, we found that asthmatics with the AA genotype had a lower post-bronchodilator $FEV_1/FVC$ ratio than the GG genotype ($p < 0.05$) and a higher RV/TLC ratio than the AG genotype ($p < 0.05$, Figure 2). No differences were reported for what concerns the BclI SNP (Figure 3).

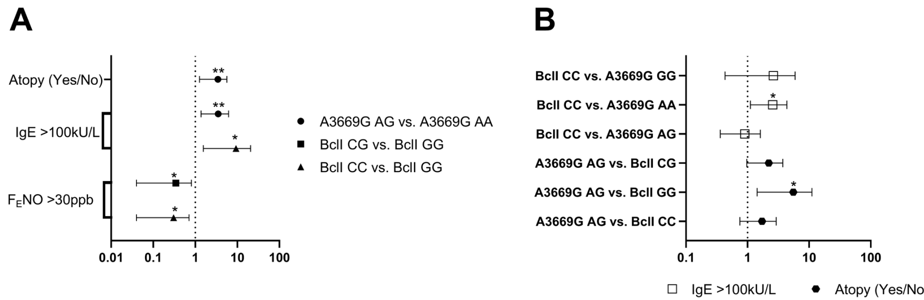


**Figure 1.** Forest plot representing odds ratio. The points represent the confidence interval (95%); X axis is expressed in a logarithmic scale. Graphs representing OR for atopy, IgE and FENO considering the genotype of BclI and A3369G (A). Graphs representing OR for IgE>100kU/L and atopy between BclI CC and all A3669G variants and between A3369 AG and all BclI variants (B). *: $p < 0.05$; **: $p < 0.01$.

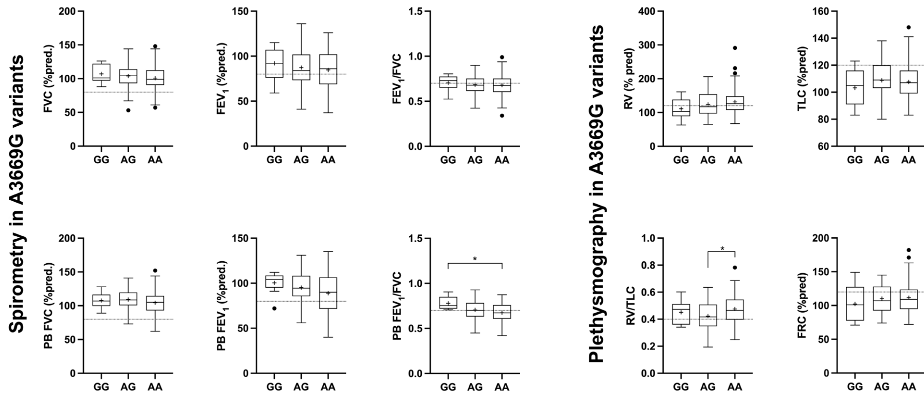


**Figure 2.** Spirometry and plethysmography in asthmatic patients stratified according to A3669G polymorphism variations. FVC: forced vital capacity; FEV1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity; FRC: functional residual capacity; PB: post-bronchodilation. Boxplots represent data from the first-to-third quartile; whiskers are drawn according to the Tukey method; "+" indicates the mean value; the dotted line represents the pathological threshold; * = $p < 0.05$.

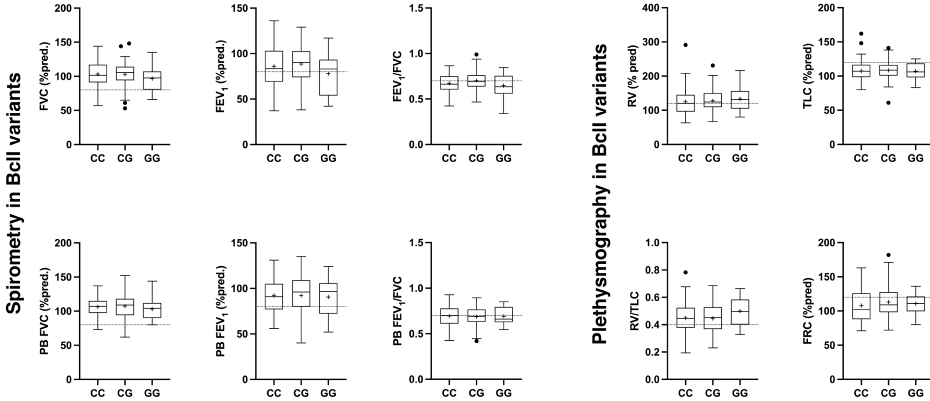


**Figure 3.** Spirometry and plethysmography in asthmatic patients stratified according to BclI polymorphism variations. FVC: forced vital capacity; FEV1: forced expiratory volume in 1 s (FEV1); RV: residual volume; TLC: total lung capacity; FRC: functional residual capacity; PB: post bronchodilation. Boxplots represent data from the first-to-third quartile; whiskers are drawn according to the Tukey method; "+" indicates the mean value; the dotted line represents the pathological threshold.

## 4. Discussion

In this single-center study, we evaluated the association between clinical, biological and functional characteristics of mild-to-severe asthmatics with the different genotypes of BclI and A3669G polymorphisms. In this study, when dividing the population based on the dose of ICS (beclomethasone HFA $\geq$ 400 µg/day), we observed that patients with moderate-to-severe asthma seem to have a T2-high phenotype, although blood eosinophils of more than 300 cells/µL were the unique T2-high biomarker statistically different from the mild asthmatics. No difference was found between the groups regarding atopy and serum total IgE.

In our asthmatic population, a few differences were reported when subdividing the population based on the SNP genotypes studied. Patients characterized by homozygous GG genotype for BclI had a higher percentage of asthmatics with greater $F_ENO$ levels (>30 ppb) and a lower prevalence of asthmatics with elevated total serum IgE concentrations (>100 kU/L). Considering the A3669G genotypes, we found that patients with heterozygous AG genotypes were younger, characterized by atopy and higher IgE levels. Moreover, the AA genotype of A3669G was associated with lower BMI than the GG genotype.

In the past years, different pharmacogenomics and genome-wide association studies focused their attention on the gene-encoding glucocorticoid receptor due to its central role in the GC-signaling pathway [26]. The NR3C1 gene is related to asthma inflammation, and the occurrence of polymorphisms can lead to resistance to glucocorticoids in asthmatics [27], causing difficult management of severe asthma patients [28]. Several studies analyzed the possible interaction between SNPs present within the NR3C1 gene and asthma, although the specific role of BclI and A3369G SNPs in asthma is still under study.

Considerable evidence indicates the involvement of GCs in regulating adipose tissue [15]. The work of Syed and colleagues evaluated the possible association of the A3369G variant with obesity and revealed that the variant of this SNP did not correlate with obesity status, but with the waist-to-hip ratio and with waist circumference in a population of European women [15]. Here, we observed a possible contribution of the 3669G alleles with the obesity status in our asthmatic patients but the prevalence of BMI $\geq$30 is not associated with female asthmatics probably due to the smaller sample size of the GG group. For what concerns the BclI SNP in our asthmatic population, we did not find significant differences in BMI even though the G-allele seems to be related to increased BMI, as reported by previous several studies [29].

In the literature, data regarding the interaction between BclI SNP and response to GC was described in a study in which it was related to OCS response due to $FEV_1$-predicted levels changing in adults [30]. Recently, another study suggested that in asthmatic children, the BclI GG genotype was associated with ICS response due to an improvement of $FEV_1$ after 4 h upon high fluticasone propionate dose [31]. Here, no significant differences were observed between BclI genotypes and pulmonary function levels, assuming that an association between BclI SNP and GC sensitivity did not exist in our population.

Considering the other SNP, for the first time, we describe an association between the AA genotype of A3669G SNP and worse lung function. Asthmatic patients carrying AA, after administration of 400 µg albuterol, did not improve their post-bronchodilation $FEV_1$/FVC ratio (airflow obstruction), implying a potential relationship between the allele A of A3669G SNP and the scarce response to GCs in our asthmatics.

In the past, several studies connected the NR3C1 gene with asthma phenotypes suggesting a possible involvement of this gene in the identification of specific asthma phenotypes [32]. Recent work exploring the genetic susceptibility profile of moderate-to-severe asthma through an integrative bioinformatics platform revealed that the SNPs on the N3RC1 gene were related to inflammation processes in asthmatic airways and severe asthma phenotype [33]. This evidence supports our result as patients with allele A of A3669G SNP had air trapping (higher RV/TLC) and airflow obstruction (lower $FEV_1$/FVC ratio), indicating the possible involvement of this SNP in asthma severity.

The literature reported that the NR3C1 gene polymorphism can also provoke the overexpression of T2-related cytokines (such as IL-4) or several key molecules that cause hormone resistance [8]. This evidence was in part supported by our analysis; patients with AG genotype of A3669G were characterized by higher total serum IgE levels (typically modulated by IL-4) [34] and a higher prevalence of atopy, suggesting a possible interaction between this SNP and type-2 allergic asthmatic phenotype. Furthermore, through the OR analyses, we established a significant association between A3669G SNP and atopic asthma; thus, the A3669G AG genotype could regulate, along with other SNP [35], the immune response in asthma patients.

In our analyses, no differences were observed for what concerns the prevalence of the comorbidities and BclI and A3669G polymorphisms. These results were in contrast with the literature that highlighted a relationship between genetic variants of BclI and cardiovascular comorbidities, hypertension and obesity [36].

The main limitation of this study is represented by the small number of patients (N = 11) carrying the rarer GG genotype of A3669G SNP and (N = 18) carrying the homozygous GG genotype of BclI SNP, which reduces the statistical power of our analysis.

## 5. Conclusions

In conclusion, our study suggests a possible role of the studied SNPs in asthma. Our findings highlight the association of allele A of A3669G SNP with asthma severity and poor response to glucocorticoids. Furthermore, the presence of the heterozygous genotype could be useful as a predictor of type-2 allergic asthma. Moreover, the BclI SNP seems to be involved in the recognition of patients characterized by IgE more than 100 kU/L, indicating the possible application of this SNP in determining the omalizumab treatment eligibility.

The findings of our study focused on the promising role of A3669G and BclI genotyping as predictors of asthma severity and specific asthma phenotype; thus, further analyses involving a larger number of mild-to-severe asthmatics were needed to replicate and confirm their role in asthma.

Finally, this work shed light on the possible usefulness of A3669G and BclI, together with other biomarkers, as tools for asthma characterization.

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**Data Availability Statement:** Data are available on request from the authors.

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## References

1. Ricciardolo, F.L.M.; Guida, G.; Bertolini, F.; Di Stefano, A.; Carriero, V. Phenotype overlap in the natural history of asthma. *Eur. Respir. Rev.* **2023**, *32*, 220201. [CrossRef] [PubMed]
2. Ricciardolo, F.L.M.; Sprio, A.E.; Baroso, A.; Gallo, F.; Riccardi, E.; Bertolini, F.; Carriero, V.; Arrigo, E.; Ciprandi, G. Characterization of T2-Low and T2-High Asthma Phenotypes in Real-Life. *Biomedicines* **2021**, *9*, 1684. [CrossRef]
3. Wadhwa, R.; Dua, K.; Adcock, I.M.; Horvat, J.C.; Kim, R.Y.; Hansbro, P.M. Cellular mechanisms underlying steroid-resistant asthma. *Eur. Respir. Rev.* **2019**, *28*, 190096. [CrossRef] [PubMed]
4. Barnes, P.J.; Adcock, I.M. Glucocorticoid resistance in inflammatory diseases. *Lancet* **2009**, *373*, 1905–1917. [CrossRef] [PubMed]

5. Hansbro, P.M.; Kim, R.Y.; Starkey, M.R.; Donovan, C.; Dua, K.; Mayall, J.R.; Liu, G.; Hansbro, N.G.; Simpson, J.L.; Wood, L.G.; et al. Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma. *Immunol. Rev.* **2017**, *278*, 41–62. [CrossRef]
6. Sivapalan, P.; Borresen, S.W.; Eklof, J.; Klose, M.; Holm, F.S.; Feldt-Rasmussen, U.; Rossing, M.; Jørgensen, N.R.; Marvig, R.L.; Saeed, M.I.; et al. Adrenal suppression in patients with chronic obstructive pulmonary disease treated with glucocorticoids: Role of specific glucocorticoid receptor polymorphisms. *PLoS ONE* **2022**, *17*, e0262898. [CrossRef]
7. Online Mendelian Inheritance in Man, OMIM®; MIM Number: {#615962}; Johns Hopkins University: Baltimore, MD, USA. 2022. Available online: https://omim.org/ (accessed on 23 August 2022).
8. Fu, G.; Fu, L.; Cai, Y.; Zhao, H.; Fu, W. Association between polymorphisms of glucocorticoid receptor genes and asthma: A meta-analysis. *Cell. Mol. Biol.* **2018**, *64*, 13–23. [CrossRef]
9. Sevilla, L.M.; Jiménez-Panizo, A.; Alegre-Martí, A.; Estébanez-Perpiñá, E.; Caelles, C.; Pérez, P. Glucocorticoid Resistance: Interference between the Glucocorticoid Receptor and the MAPK Signalling Pathways. *Int. J. Mol. Sci.* **2021**, *22*, 10049. [CrossRef]
10. Ramos-Ramírez, P.; Tliba, O. Glucocorticoid Receptor β (GRβ): Beyond Its Dominant-Negative Function. *Int. J. Mol. Sci.* **2021**, *22*, 3649. [CrossRef]
11. Stevens, A.; Ray, D.W.; Zeggini, E.; John, S.; Richards, H.L.; Griffiths, C.E.; Donn, R. Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 892–897. [CrossRef]
12. Huang, H.; Wang, W. Molecular mechanisms of glucocorticoid resistance. *Eur. J. Clin. Investig.* **2023**, *53*, e13901. [CrossRef] [PubMed]
13. Russo, P.; Tomino, C.; Santoro, A.; Prinzi, G.; Proietti, S.; Kisialiou, A.; Cardaci, V.; Fini, M.; Magnani, M.; Collacchi, F.; et al. FKBP5 rs4713916: A Potential Genetic Predictor of Interindividual Different Response to Inhaled Corticosteroids in Patients with Chronic Obstructive Pulmonary Disease in a Real-Life Setting. *Int. J. Mol. Sci.* **2019**, *20*, 2024. [CrossRef]
14. Derijk, R.H.; Schaaf, M.J.; Turner, G.; Datson, N.A.; Vreugdenhil, E.; Cidlowski, J.; de Kloet, E.R.; Emery, P.; Sternberg, E.M.; Detera-Wadleigh, S.D. A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J. Rheumatol.* **2001**, *28*, 2383–2388. [PubMed]
15. Syed, A.A.; Irving, J.A.; Redfern, C.P.; Hall, A.G.; Unwin, N.C.; White, M.; Bhopal, R.S.; Weaver, J.U. Association of glucocorticoid receptor polymorphism A3669G in exon 9beta with reduced central adiposity in women. *Obesity* **2006**, *14*, 759–764. [CrossRef]
16. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention. 2020. Available online: www.ginasthmaorg (accessed on 15 March 2023).
17. Chung, K.F.; Wenzel, S.E.; Brozek, J.L.; Bush, A.; Castro, M.; Sterk, P.J.; Adcock, I.M.; Bateman, E.D.; Bel, E.H.; Bleecker, E.R.; et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* **2014**, *43*, 343–373. [CrossRef]
18. Muraro, A.; Roberts, G.; Halken, S.; Agache, I.; Angier, E.; Fernandez-Rivas, M.; Gerth van Wijk, R.; Jutel, M.; Lau, S.; Pajno, G.; et al. EAACI guidelines on allergen immunotherapy: Executive statement. *Allergy* **2018**, *73*, 739–743. [CrossRef] [PubMed]
19. Hellings, P.W.; Fokkens, W.J.; Bachert, C.; Akdis, C.A.; Bieber, T.; Agache, I.; Bernal-Sprekelsen, M.; Canonica, G.W.; Gevaert, P.; Joos, G.; et al. Positioning the principles of precision medicine in care pathways for allergic rhinitis and chronic rhinosinusitis: A EUFOREA-ARIA-EPOS-AIRWAYS ICP statement. *Allergy* **2017**, *72*, 1297–1305. [CrossRef]
20. Carriero, V.; Bertolini, F.; Sprio, A.E.; Bullone, M.; Ciprandi, G.; Ricciardolo, F.L.M. High levels of plasma fibrinogen could predict frequent asthma exacerbations. *J. Allergy Clin. Immunol. Pract.* **2020**, *8*, 2392–2395.e7. [CrossRef]
21. Sprio, A.E.; Carriero, V.; Levra, S.; Botto, C.; Bertolini, F.; Di Stefano, A.; Maniscalco, M.; Ciprandi, G.; Ricciardolo, F.L.M. Clinical characterization of the frequent exacerbator phenotype in asthma. *J. Clin. Med.* **2020**, *9*, 2226. [CrossRef]
22. Reimondo, G.; Chiodini, I.; Puglisi, S.; Pia, A.; Morelli, V.; Kastelan, D.; Cannavo, S.; Berchialla, P.; Giachino, D.; Perotti, P.; et al. Analysis of BCLI, N363S and ER22/23EK Polymorphisms of the Glucocorticoid Receptor Gene in Adrenal Incidentalomas. *PLoS ONE* **2016**, *11*, e0162437. [CrossRef]
23. Shan, G.; Gerstenberger, S. Fisher's exact approach for post hoc analysis of a chi-squared test. *PLoS ONE* **2017**, *12*, e0188709. [CrossRef] [PubMed]
24. Motulsky, H.J.; Brown, R.E. Detecting outliers when fitting data with nonlinear regression: A new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinform.* **2006**, *7*, 123. [CrossRef]
25. The Genome Aggregation Database. GnomAD. Available online: https://gnomad.broadinstitute.org (accessed on 14 April 2023).
26. Oakley, R.H.; Cidlowski, J.A. The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease. *J. Allergy Clin. Immunol.* **2013**, *132*, 1033–1044. [CrossRef] [PubMed]
27. Panek, M.; Pietras, T.; Antczak, A.; Fabijan, A.; Przemęcka, M.; Górski, P.; Kuna, P.; Szemraj, J. The N363S and I559N single nucleotide polymorphisms of the h-GR/NR3C1 gene in patients with bronchial asthma. *Int. J. Mol. Med.* **2012**, *30*, 142–150. [PubMed]
28. Mohamed, N.A.; Abdel-Rehim, A.S.; Farres, M.N.; Muhammed, H.S. Influence of glucocorticoid receptor gene NR3C1 646 C>G polymorphism on glucocorticoid resistance in asthmatics: A preliminary study. *Cent. Eur. J. Immunol.* **2015**, *40*, 325–330. [CrossRef]
29. Van Rossum, E.F.; Koper, J.W.; van den Beld, A.W.; Uitterlinden, A.G.; Arp, P.; Ester, W.; Janssen, J.A.; Brinkmann, A.O.; de Jong, F.H.; Grobbee, D.E.; et al. Identification of the Bcl I polymorphism in the glucocorticoid receptor gene: Association with sensitivity to glucocorticoids in vivo and body mass index. *Clin. Endocrinol.* **2003**, *59*, 585–592. [CrossRef]

30. Keskin, O.; Farzan, N.; Birben, E.; Akel, H.; Karaaslan, C.; Maitland-van der Zee, A.H.; Wechsler, M.E.; Vijverberg, S.J.; Kalayci, O. Genetic associations of the response to inhaled corticosteroids in asthma: A systematic review. *Clin. Transl. Allergy* **2019**, *9*, 2. [CrossRef]
31. Keskin, O.; Uluca, U.; Birben, E.; Coşkun, Y.; Ozkars, M.Y.; Keskin, M.; Kucukosmanoglu, E.; Kalayci, O. Genetic associations of the response to inhaled corticosteroids in children during an asthma exacerbation. *Pediatr. Allergy Immunol.* **2016**, *27*, 507–513. [CrossRef]
32. Hawkins, G.A.; Amelung, P.J.; Smith, R.S.; Jongepier, H.; Howard, T.D.; Koppelman, G.H.; Meyers, D.A.; Bleecker, E.R.; Postma, D.S. Identification of Polymorphisms in the Human Glucocorticoid Receptor Gene (NR3C1) in a Multi-racial Asthma Case and Control Screening Panel. *DNA Seq.* **2004**, *15*, 167–173. [CrossRef]
33. Zayed, H. Novel Comprehensive Bioinformatics Approaches to Determine the Molecular Genetic Susceptibility Profile of Moderate and Severe Asthma. *Int. J. Mol. Sci.* **2020**, *21*, 4022. [CrossRef]
34. Ricciardolo, F.L.; Sorbello, V.; Silvestri, M.; Giacomelli, M.; Debenedetti, V.M.; Malerba, M.; Ciprandi, G.; Rossi, G.A.; Rossi, A.; Bontempelli, M. TNF-alpha, IL-4R-alpha and IL-4 polymorphisms in mild to severe asthma from Italian Caucasians. *Int. J. Immunopathol. Pharmacol.* **2013**, *26*, 75–84. [CrossRef] [PubMed]
35. Salhi, M.; Lahmar, O.; Salah, M.O.; Banić, I.; Binghao, B.; Malik, W.; Hamzaoui, K.; Turkalj, M.; Hamzaoui, A. GLCCI1 and STIP1 variants are associated with asthma susceptibility and inhaled corticosteroid response in a Tunisian population. *J. Asthma* **2021**, *58*, 197–206. [CrossRef] [PubMed]
36. Bertalan, R.; Patocs, A.; Vasarhelyi, B.; Treszl, A.; Varga, I.; Szabo, E.; Tamas, J.; Toke, J.; Boyle, B.; Nobilis, A.; et al. Association between birth weight in preterm neonates and the BclI polymorphism of the glucocorticoid receptor gene. *J. Steroid Biochem. Mol. Biol.* **2008**, *111*, 91–94. [CrossRef] [PubMed]

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