

SUPPLEMENTARY MATERIAL

METHODS

Study Design

This is an epidemiological observational case-control study in which subjects were eligible for patient or control group according to strict criteria of inclusion with a long period of recruitment because of the difficulty of obtaining large series of patients with immediate hypersensitivity reactions to beta-lactam and controls phenotypically well characterized.

Setting

This study was developed in the Allergy Department of the University Hospital of Salamanca. Data were collected by allergists in the outpatient clinic. The study was approved by the Clinical Research of Ethics Committee of University Hospital of Salamanca (Reference PI120914). The methods were carried out following the EMQN good practice standards and in a laboratory with ISO 15189 accreditation. All of individuals gave an informed written consent prior to be studied.

Participants

This study included 232 individuals that attended the Immunoallergy Service of the University Hospital of Salamanca for suspected immediate reactions to BL during a nine-year period. Since 1994 we have been systematically performing the equivalent to ENDA's short algorithm [1] for evaluating patients with suspected immediate beta-

lactam reactions, including two identical procedures separated by a three-week period (Supplementary Fig 1).

To be included patients should have had symptoms within 1 hour after the administration of one BL, suggesting an IgE-mediated reaction. A structured clinical history, specific IgE, skin tests with BL reagents (including the suspected drug) and controlled provocation test (CPT) with the suspected BL were performed to all patients. Exclusion criteria were pregnancy, β -blocker use, and severe cardiovascular, renal, or respiratory disease.

Skin tests

STs were performed on the volar forearm as previously described [2], with PPL and MDM (Allergopen, Allergopharma-JGKG, Reinbek, Germany) until 2006, and with BP-OL (0.04 mg/ml) and DM (0.5 mg/ml) (Bencylpenicilloyl-octa-L-lysine and bencylpenilloate, DAP-Diater Madrid, Spain) afterwards; bencylpenicillin 10.000 UI (BP); and amoxicillin 20 mg/ml (AMX). For other BL, concentrations were those referred in the bibliography [3].

All reagents were firstly tested by prick; results were considered positive when a wheal larger than 3 mm in diameter with surrounding erythema was present 20 minutes later. If negative, intradermal tests were performed. An increase larger than 3 mm in the initial wheal diameter with erythema after 20 minutes was considered positive. A positive result with one or more reagents was considered diagnostic.

Specific IgE

Specific IgE to penicilloyl G, penicylloyl V, ampicilloyl or amoxicilloyl was performed (ImmunoCAP System, Phadia-AB, Uppsala, Sweden). A value > 0.35 KU/L was considered positive.

Controlled provocation tests

When STs were negative a CPT with the suspected BL was performed as a single blind, placebo-controlled challenge. When the patient did not remember it, a CPT with phenoxymethylpenicillin was performed. Doses were administered at 45-minute intervals in one day (1/8-1/4-1/2-1 of a full dose).

Re-evaluation of patients with negative STs results

Patients with negative STs tolerating therapeutic doses of the eliciting antibiotic underwent a repetition of the study protocol 3 weeks later. The diagnostic algorithm is shown in supplementary figure 1.

Finally, individuals were divided in those with a positive result and those with a negative result that did tolerate BL.

Patients were considered atopic if they had at least a positive skin test from a locally adapted battery of common aeroallergens as previously described [2].

Variables Data sources

A total of 110 variables were analyzed in this study, including epidemiological data, comorbidities and family history as well as variables related to the BL sensitization studies and variables related to cytokine genotyping. Age and IgE levels

were the only quantitative variables included in the study. Distribution was assessed to apply parametric or non-parametric tests accordingly.

Epidemiological and data related to clinic were collected by the allergist in the outpatient clinic.

All individuals underwent a diagnostic protocol of hypersensitivity to antibiotics BL performed by an allergist of the Allergy Service. The study followed the diagnostic criteria and the algorithm recommended by the ENDA group [4,5]. Specific IgE to BL was performed using the CAP System (Thermo Fisher Scientific, Waltham, MA USA).

Skin prick tests were performed in both patient and control groups following the recommendations of the Subcommittee on allergen standardization and skin tests of the EAACI [2] with a standard battery of common airborne allergens adapted to our environment. Atopy was considered when a patient had any positive result to any of the aeroallergens of the battery. Subjects also underwent a blood extraction by venipuncture to determine total IgE levels (ImmunoCAP, Thermo Fisher Scientific Inc, Waltham, MA USA) and to DNA extraction.

Genotyping data were recorded in the Molecular Laboratory of the Immunology Department of the University Hospital of Salamanca according to the protocol described below.

Bias

Special efforts were done to address potential sources of bias from the beginning of the design, specifically for atopy as a risk factor, since this was one of the objectives of the study. Sex and age were considered as potential confounders between cases and control groups so statistical control of these variants were applied.

Study Size

For the population size calculation, we used the platform <http://statpages.org/proppowr.html> statistical power (SP) [6] setting the minimum number of cases and controls needed for the study. Two different calculations were performed, one at the beginning of the study to calculate sample size to start with. A second calculation was developed in the middle of the study to adjust this sample size. The sample size was calculated to obtain a minimum of 80% of statistical power for a 0.05 alpha error. We want to highlight the difficulty of obtaining so well characterized groups of BL allergy.

Molecular Analysis

DNA extraction from whole blood was performed using the MagnaPure Compact (Roche Applied Science, Mannheim, Germany). Performance was evaluated by a BioPhotometer spectrophotometer (Eppendorf AG, Hamburg, Germany).

Twenty-two polymorphisms in 13 cytokine genes [*IFNG* (874 A>T), *IL1A* (-889 C>T), *IL1B* (-511 C>T; 3954 C>T), *IL1R1* (pst1 1970 C>T), *IL1RN* (mspa1 11100 T>C), *IL2* (-330 T>G;166 G>T), *IL4* (-33 C>T; -1098 T>G; -589 C>T), *IL4R* (1902 A>G), *IL6* (-174 G>C;-597 G>A), *IL10* (-592 C>A ; -1082 A>G ; -819 C>T), *IL12B* (-1188 A>C), *TGFB1* (915 G>C;869 T>C) and *TNFA* (-308 G>A; -238 G>A)] were performed using the "Cytokine genotyping Kit" (Invitrogen™, Deerbrook, Trail, USA). This system has been certified by the European Community regarding used requirements for In Vitro Diagnostics 98/79/EC. It is based on the polymerase chain reaction (PCR) system using sequence-specific primers PCR-SSP (sequence specific primers polymerase chain reaction) [7].

Amplification was carried out using thermocycler [TProfessional 96 Thermocycler, Whatman Biometra®, (Goettingen, Alemania)] under the following conditions: initial denaturation at 94°C for 4 minutes followed by 10 cycles of denaturation at 94°C for 30 seconds and annealing and extension at first 65°C for 1 minute; 20 cycles of denaturation at 94°C for 30 seconds, annealing at first 65°C for 50 sec and extension at 72°C for 1 minute. Upon completion of the cycle PCR products were analyzed on a 2% agarose gel.

Results were transcribed to a database coupled performed with SPSS version 18.0 statistical package (IBM, Chicago, IL). To ensure the correct interpretation of the data, a second blinded interpretation was performed. In all steps of the experimental procedure were followed the recommendations of the European Molecular Genetics Quality Network (EMQN) [8].

Statistical Methods

Firstly, a descriptive analysis using SPSS 18.0 was performed by central and dispersion tendency measurements. Secondly, we conducted a bivariate and multivariate analysis was performed; distribution and homogeneity of variance were assessed before selecting the appropriate statistical tests. Subgroups were established according to the presence of atopy and sensitization variables.

When a statistically significant association was found, three statistical controls were applied: (i) calculation of binary logistic regression adjusted for potential confounding variables as sex and age; (ii) the statistical power (SP) by using the online platform [6] to evaluate the appropriate sample size for this comparison; and (iii) the probability of a false positive report probability (FPRP) using the method described by

Wacholder and colleagues [9] to identify potential type I error. In addition, Bonferroni correction was applied when appropriate.

Differences in the distribution of allele and genotype frequencies between controls and patients were established by the SHEsis online platform [10] (<http://analysis.bio-x.cn/myAnalysis.php>). Hardy Weinberg equilibrium was also calculated.

In addition to the analysis of individual polymorphisms, haplotype and diplotype analysis for *IL1B*, *IL2*, *IL4*, *IL6*, *IL10*, *TGFB1* and *TNFA* were studied by using the statistics online platform SHEsis [10] and SPSS 18.0.

Funding

The data that support the findings of this study are available from the corresponding author on reasonable request.

RESULTS

Participants:

A total of 202 were confirmed for eligibility and were included in the study. Considering all the different variables, missing data represented less than 5%. Other important variables to study are the type of reaction and the BL antibiotic involved, in both cases with a missing data of 1%. Another variable would be the genotyping of the 22 polymorphisms in the 13 cytokine genes with a maximum missing value of 3.10% and a minimum of 0%. The statistical power was calculated for the final 202 patients.

Descriptive analysis

From the 202 individuals included in the study, 98 were diagnosed as patients with immediate hypersensitivity to BL and 104 with proven tolerance to BL were considered as controls. No statistically significant differences were observed between patients and controls regarding age, sex, atopy, percentages of mono and polysensitization to aeroallergens or total IgE levels (Supplementary table 1). Reactions and involved antibiotics are presented in supplementary table 2. Amoxicillin was the most frequently involved beta-lactam.

Main Results

By analyzing 22 polymorphisms in patients with BL hypersensitivity compared to controls, statistically significant differences were identified only for the c25 *TGFB1* SNP. We found that the C allele at codon 25 of *TGFB1* gene was more frequent in BL allergic patients (11.8%) versus in BL negative controls (4.5%), $p = 0.029$.

Classifying patients with BL hypersensitivity according to atopy presence, statistically differences were found in both control and patient groups for the following SNPs: pst1 1970 *IL1R*, 874 *IFNG* and -33 *IL4*.

We found that the T allele in position 1970 of *IL1R* gene was more common in patients with atopy compared to non-atopic controls (45.7% vs. 31.1%) with $p = 0.014$; OR: 1.86; 95% CI (1.13 - 3.05). Regarding genotype frequencies, a statistically significant difference ($p = 0.017$) was observed in the TT genotype that was more frequent in atopic patients compared to non-atopic controls (23.9% vs. 7.9%). This finding was confirmed using logistic regression adjusted for age and sex, $p = 0.007$; OR:

5.23; (95% CI 1.59 - 17.21). The statistical power was 88% for an error $\alpha = 0.05$, and FPRP = 1% for a prior probability of 10%.

The A allele in position 874 *IFNG* was also observed more frequently in atopic patients in relation to non-atopic controls (71.4% vs. 50%), $p = 0.027$, OR: 2.50; 95% CI (1.09 - 5.68).

The T allele and TT genotype of SNP -33 of *IL4*, were more frequent in non-atopic controls than in atopic patients, $p = 0.004$; OR: 0.23; 95% CI (0.08 - 0.68). After adjusting for age and sex, this association was confirmed, obtaining a regression $p = 0.005$. The statistical power was 81% for an error $\alpha = 0.05$ with an FPRP = 8.5% for a prior probability of 10%.

To confirm whether these differences were due to BL hypersensitivity or to atopy, we compared atopic and non-atopic patients with BL allergy. We observed again the same statistically significant differences above mentioned. In addition, we found an association with the SNP 1902 *IL4RA* (Supplementary table 3)

The GG genotype of SNP 1902 of *IL4RA* was more frequent in atopic patients (15.8%) than in non-atopic patients (1.8%), $p = 0,007$. When adjusting for age and sex, the significance was maintained, obtaining a regression $p = 0.012$. This fact suggests that these associations are due to atopy.

In the group of non-atopic patient differences were not found between BL allergic and BL tolerant patients.

ACKNOWLEDGES:

This work was partially supported by Institute de Salud Carlos III (ISCIII) by a grant of the “Red temática de investigación en salud Asma, Reacciones Adversas y Alérgicas (ARADYAL)” RD16/0006. JA Cornejo-García is a researcher from the Miguel Servet Program (Ref CP14/00034), the Carlos III National Health Institute, Spanish Ministry of Economy and Competitiveness.

AUTHOR CONTRIBUTIONS

The authors MLR-R, EM, CS, AG-S, JAC-G, MI-G and ID have contributed to the conception and design of this work, as well as to the acquisition, analysis and interpretation of data, to the drafting and critical revision of the manuscript and have approved the final version to be published.

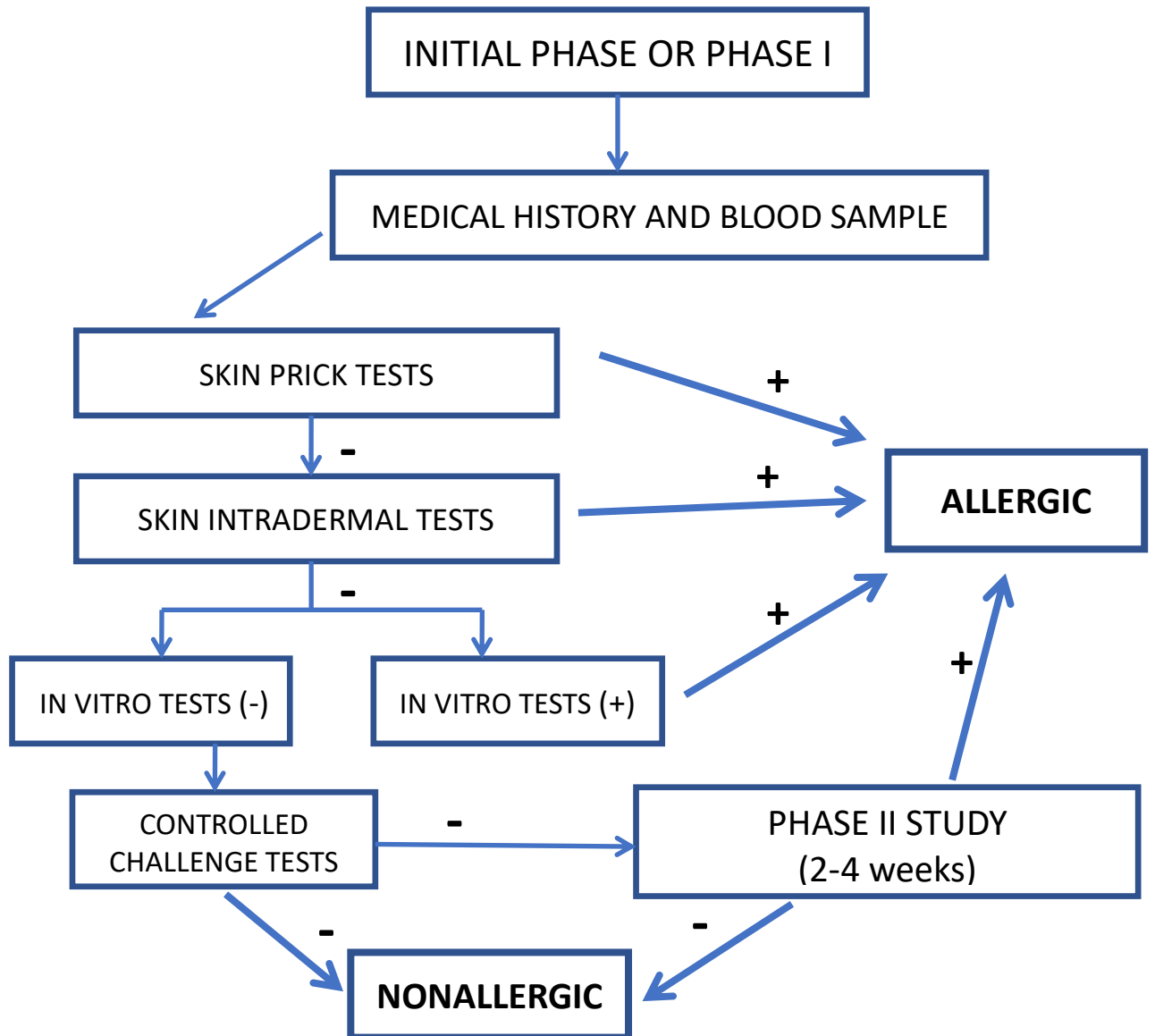
CONFLICTS OF INTEREST

None related to this work

REFERENCES

1. Moreno E, Laffond E, Muñoz-Bellido F, Gracia MT, Macías E, Moreno A, et al. Performance in real life of the European Network on Drug Allergy algorithm in immediate reactions to beta-lactam antibiotics. *Allergy*. 2016;71:1787-1790.
2. Skin tests used in type I allergy testing Position paper. Sub-Committee on Skin Tests of the European Academy of Allergology and Clinical Immunology. *Allergy*, 1989. 44 (Suppl 10): p. 1-59.
3. Brockow K, Garvey LH, Aberer W, Atanaskovic-Markovic M, Barbaud A, Bilo MB, Bircher A, et al. Skin test concentrations for systemically administered drugs -- an ENDA/EAACI Drug Allergy Interest Group position paper. *Allergy*. 2013;68:702-12.
4. Blanca M, Romano A, Torres MJ, Fernández J, Mayorga C, Rodriguez J, et al. Update on the evaluation of hypersensitivity reactions to betalactams. *Allergy*. 2009;64:183-93.
5. Torres MJ, Blanca M, Fernández J, Romano A, Weck A, Aberer W, et al. Diagnosis of immediate allergic reactions to beta-lactam antibiotics. *Allergy*. 2003;58:961-72.
6. Fleiss, J.L. *Statistical Methods for Rates and Proportions* (Second Edition, 1981).
7. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res*. 1989;17:2503-16.

8. Patton S. Draft Best Practice Guidelines for Laboratory Internal Quality Control. European Molecular Genetics Quality Network, 2002.
9. Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst.* 2004;96:434-42.
10. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res.* 2005;15:97-8.



Supplementary figure 1: Diagnostic algorithm for allergic reactions to beta-lactam. Phase II study consisted in the same study performed 3-4 weeks later

Supplementary table 1. Descriptive analysis of the population of patients with BL hypersensitivity and controls

Clinical Features (CF)	Controls N=104	Patients BL N=98	P-Value
Age	Median: 43; RI: 33	Median: 49; RI: 29	0.33
Sex	♂ ♀ 33%, 67%	♂ ♀ 42%, 58%	0.19
Total IgE (KU/L)	Mean: 98.94 SD: 139.22	Mean: 165.83 SD: 342.91	0.10
Atopy	27.3%	20.2%	0.31

Supplementary table 2. Types of reactions and involved drugs

Clinical Features	Patients BL (+) (N = 98)
<u>Type of reaction</u>	
✓ Urticaria/angioedema	54.1% (53)
✓ Anaphylaxis	32.5% (32)
✓ Rash	9.4% (9)
✓ Nonspecific reactions*	4% (4)
<u>Beta-lactam involved</u>	
• Bencylpenicillin	3.1% (3)
• Amoxicillin	79.5% (78)
• Cloxacillin	2.1% (2)
• Cephalosporins	9.2% (9)
• Amoxicillin-clavulanate	3.1% (3)
• Unknown	3% (3)

*Other reactions involved were

Supplementary table 3. Comparative study of allelic and genotypic frequencies in BL allergic patients with and without atopy

SNP	p-Value	Frequency (Allelic)	Frequency (Genotypic)
pst1 +1970 <i>IL1R</i>	Allelic: 0.08		
	Genotypic: 0.024*		TT: 21.1% A/ 8.9% NA
+874 <i>IFNG</i>	Alélica: 0.034*	A: 65% A/ 44.4% NA	
	Genotypic: 0.43		
-33 <i>IL4</i>	Allelic: 0.038*	T: 20.4% NA/ 5.6% A	
	Genotypic: 0.11		
+1902 <i>IL4RA</i>	Allelic: 0.83		
	Genotypic: 0.007*		GG: 15.8% A/ 1.8% NA

***p-Value < 0.05**

A= atopic patients; NA= nonatopic patients