Asthma and allergic rhinitis associate with the *rs2229542* variant that induces a p.Lys90Glu mutation and compromises AKR1B1 protein levels

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Abstract

Asthma and rhinitis are two of the main clinical manifestations of allergy, in which increased reactive oxygen or electrophilic species can play a pathogenic role. Aldose reductase (AKR1B1) is involved in aldehyde detoxification and redox balance. Recent evidence from animal models points to a role of AKR1B1 in asthma and rhinitis, but its involvement in human allergy has not been addressed. Here, the putative association of allergic rhinitis and asthma with *AKR1B1* variants has been explored by analysis of single-strand variants on the *AKR1B1* gene sequence in 526 healthy subjects and 515 patients with allergic rhinitis, 366 of whom also had asthma. We found that the *rs2229542* variant, introducing the p.Lys90Glu mutation, was significantly more frequent in allergic patients than in healthy subjects. Additionally, in cells transfected with expression vectors carrying the wild type or the p.Lys90Glu variant of AKR1B1, the mutant consistently attained lower protein levels than the wild type and showed a compromised thermal stability. Taken together, our results show that the *rs2229542* variant associates with asthma and rhinitis, and hampers AKR1B1 protein levels and stability. This unveils a connection between the genetic variability of aldose reductase and allergic processes.

Key words: Asthma, allergy, rhinitis, variant, Aldose reductase, protein levels and stability.

Introduction

Aldose reductase, also known as aldo-keto reductase 1B1 (AKR1B1, MIM# 103880, EC. 1.1.1.21), catalyzes the conversion of glucose to sorbitol utilizing NADPH as cofactor, which constitutes the first reaction of the polyol pathway. In addition, AKR1B1 metabolizes reactive aldehydes and ketones which could be deleterious to cells, and is therefore considered to play a protective role under physiological conditions (Petrash, 2004). AKR1B1 is itself a target for modification by several reactive species, which may impact positively or negatively on its activity, depending on the modifying moiety (Díez-Dacal et al., 2011; Sánchez-Gómez et al., 2016; Srivastava, Ramana, Chandra, Srivastava, & Bhatnagar, 2003), thus modulating its ability to cope with oxidative or electrophilic stress. Nevertheless, in pathophysiological situations, the activity of aldose reductase may display a negative side and participate in disease mechanisms (Ramana & Srivastava, 2010).

The pathological implications of AKR1B1 have been mainly studied in diabetes. In this condition, hyperglycemia forces glucose metabolism through the polyol pathway (Petrash, 2004), which elicits multiple consequences. First, excess sorbitol can induce osmotic stress. Moreover, consumption of the NADPH cofactor (Lee & Chung, 1999), which is needed for the regeneration of cellular antioxidant defenses, including glutathione (GSH), peroxiredoxins and thioredoxin (Pollak, Dolle, & Ziegler, 2007), can provoke redox imbalance reducing the cellular capacity to respond to oxidative stress. Aldose reductase can also contribute to oxidative stress-induced inflammation (Srivastava et al., 2011). Reactive oxygen species (ROS) generated in inflammatory situations lead to reactive oxidized lipid species, like 4-hydroxynonenal, which in turn can form GSH adducts. Both, the reactive aldehydes and their GSH adducts, serve as

substrates for aldose reductase. However, the resulting products, like 1,4-dihydroxynonene and glutathionyl-1,4-dihydroxynonane, can stimulate proinflammatory signaling cascades, originating a positive feedback of inflammation (Ramana et al., 2006). In addition, the finding that AKR1B1 metabolizes prostaglandins suggests novel implications of this enzyme in the balance of inflammatory mediators, not completely understood (Bresson, Lacroix-Pepin, Boucher-Kovalik, Chapdelaine, & Fortier, 2012). Lastly, the ability of several AKR enzymes to detoxify reactive species and drugs can contribute to tumor cell chemoresistance (Díez-Dacal et al., 2011; Díez-Dacal & Pérez-Sala, 2012).

Interestingly, recent work in cellular and animal models suggests the involvement of aldose reductase in allergy. In murine models, both genetic and pharmacological blockade of this enzyme play a protective role against airway inflammation, reducing eosinophil infiltration and airway hyper-responsiveness (Yadav, Aguilera-Aguirre, Boldogh, Ramana, & Srivastava, 2011; Yadav et al., 2009). In addition, aldose reductase inhibition has been reported to prevent allergic rhinitis, inhibit mast cell degranulation and tryptase release (Yadav et al., 2013). Yet, it remains unknown whether these protective outcomes arise from a general anti-inflammatory effect or from specific anti-allergic actions. The available evidence suggests that the protective effects of AKR1B1 inhibition or knockdown may arise from blocking its role as mediator of oxidative stress-induced inflammation (Sánchez-Gómez et al., 2016). Indeed, ROS and ROS-induced lipid peroxidation have been involved in the pathophysiology of asthma and allergic rhinitis (Bowler & Crapo, 2002; Sahiner, Birben, Erzurum, Sackesen, & Kalayci, 2011; Sánchez-Gómez et al., 2016). Nevertheless, whether AKR1B1 plays a pathogenic role in human allergy has not been assessed.

AKR1B1 belongs to the AKR family of enzymes, which comprises more than 40 members that are highly polymorphic. The *AKR1B1* gene is located on chromosome 7q33 (dbSNP release 142) and contains 10 exons. Several *AKR1B1* variants have been found to associate with pathological conditions (Penning & Drury, 2007). For example, the *rs759853* variant (c.-144C>T), located at the gene promoter, is associated with the susceptibility to suffer diabetic complications, including diabetic retinopathy and nephropathy (Kao, Donaghue, Chan, Knight, & Silink, 1999; Moczulski et al., 1999), or other pathologies such as essential hypertension (Wang et al., 2016). However, the existence of *AKR1B1* variants related to allergic reactions has not been reported.

With this background we set out to explore the potential existence of aldose reductase variants associated to allergy. Allergic rhinitis provides an interesting field of study due to the importance of genetic factors in this condition and its frequent association with asthma (Bousquet et al., 2010; Van Cauwenberge et al., 2007). Therefore, we assessed the presence of three known *AKR1B1* variants in patients with allergic rhinitis and healthy subjects, namely, the *rs5053* variant (c.-49C>G) (Prasad et al., 2010) (Fig 1A), consisting in a G>C mutation at the 5'UTR region; the *rs150113325* variant (c.429+6A>G), that generates a splicing variant; and the variant *rs2229542* (c.268A>G), consisting in an A to G single mutation in the coding region, resulting in the p.Lys90Glu mutation. Among these three variants, only the *rs2229542* affects the *AKR1B1* mRNA (Fig 1B), whereas the other two are located in non-translated regions of the gene.

Here we report the association of the *rs2229542* (c.398A>G) variant with allergic rhinitis, as well as the potential impact of this change on protein levels. This is the first evidence of a variant in a member of the *AKR* family related to the development of an allergic response.

Materials and Methods

Clinical evaluation

A cohort of 1041 Caucasian individuals, including 526 healthy subjects and 515 allergic rhinitis patients, whose clinical features have been summarized previously (Amo et al., 2016), was studied. Patients were recruited from the Allergy Department, Infanta Cristina Hospital (Badajoz, Spain) and from the UGC Allergy, Regional Hospital (Málaga, Spain) (265 and 250 patients, respectively) and most of them have been included in previous studies (Gervasini et al., 2010). Diagnosis of allergic rhinitis within the cohort was undertaken by an allergy specialist following the current ARIA guidelines (Brozek et al., 2010). All patients had a positive skin prick test for at least one relevant aeroallergen. Among the 515 patients, 366 (71.1%) were diagnosed of allergic asthma, according to Global Initiative for Asthma guidelines (Reddel et al., 2015). Healthy subjects from the same ethnicity and geographic area were selected from the personnel staff and medical students belonging to the Hospitals and the Universities participating in the study. Their medical histories were obtained and examinations were carried out to discard pre-existing disorders. Subjects with familiar, up to second-degree relatives, or personal antecedents of allergic, atopic or autoimmune diseases were excluded.

All participants read and signed the written consent for their participation in the study. All the protocols and practices undertaken in this study were in accordance with the Declaration of Helsinki and the subsequent revisions, and were approved by the Ethics Committees of the participating institutions, namely, the “Comisión de Ética y de Investigación” of Hospital Regional Universitario Carlos Haya, Málaga, and the “Comisión de Bioética y Bioseguridad”, University of Extremadura, with approval number: 42/2012.

Genotype analysis

Genomic DNA from leukocytes was isolated and purified following standard procedures. Search for SNPs in the *AKR1B1* gene was performed by means of TaqMan assays (Life Technologies, Alcobendas, Spain). Variants studied, according to the Single Nucleotide Polymorphism Database, included: *rs5053* (chromosomal location 7:134143863, assay ID C_2363983_20, NM_001628.3:c.-49C>G), *rs150113325* (chromosomal location 7:134134466, assayed with custom designed primers, NM_001628.3:c.429+6A>G), and *rs2229542* (chromosomal location 7:134135621, assay ID C_16172477_20, NM_001628.3:c.268A>G) (K90E). Samples were analyzed in triplicate by real-time PCR (qPCR) in a 7500 Real Time qPCR system (Life Technologies, Alcobendas, Spain), following the manufacturer instructions. The variants *rs139346970* (splice acceptor), *rs73724958* (missense) and *rs115638419* (missense) were not studied due to their absence in Caucasians.

Database searching and theoretical predictions

AKR sequences were aligned using CLUSTALW. Predictions of p.Lys90 posttranslational modifications were obtained from GeneCards Human Gene Database (Weizmann Institute of Science) and UbPred: predictor of protein ubiquitination sites (Radivojac et al., 2010). Nuclear localization/export signals were predicted with NetNES and PSORT II. The impact of the mutation was evaluated with PredictProtein. Structural analysis was carried out with PyMOL (v1.7.435, Schrödinger LLC) using the human aldose reductase crystal structure (1PWL). The SNPeffect 4.0 server database was searched to obtain the theoretical impact of the p.Lys90Glu mutation on AKR1B1 stability. Detailed information regarding the software or servers used is provided in the Supp. Table S1.

Cell culture, treatments and sample processing

Breast carcinoma MCF7 cells (ATCC) were cultured as previously described (Sánchez-Gómez, Díez-Dacal, Pajares, Llorca, & Pérez-Sala, 2010). For cytokine stimulation, cells were treated with 2 ng/ml IL-1 β plus 25 ng/ml TNF α for 6 h. Cell lysates were obtained and aliquots containing 30 μ g of protein (Bicinchoninic acid method, Thermo Fisher) were analyzed by SDS-PAGE and Western blot as detailed previously (Sánchez-Gómez, Gayarre, Avellano, & Pérez-Sala, 2007). Antibodies used were anti- β -actin (A2066) and anti-HSP70 (H5147) from Sigma, anti-AKR1B1 (ab62795) from Abcam, and anti-ICAM1 (sc-7891), anti-AKR1A1 (sc-365078) and anti-pan-AKR (sc-33219) from Santa Cruz Biotechnology.

Plasmids and transfections

The mammalian expression vector of human AKR1B1 was from Origene (Rockville, MD). The AKR1B1 p.Lys90Glu mutant was generated by site-directed mutagenesis using the QuikChange II mutagenesis kit (Agilent Technologies) and oligonucleotides containing the mutation (underlined): sense, 5'-GGGCCTGGTGGGAAGGAGCCTGCCAG-3'; antisense, 5'-CTGGCAGGCTCCTTTCCACCAGGCCC-3'. Both wild type (WT) and mutant expression vectors were fully sequenced to ensure identity of the remaining plasmid sequence. For transfections, cells at 60 % confluence were incubated with mixtures containing 1 μ g of DNA and 3 μ l of Lipofectamine 2000 reagent (Thermo Fisher), FuGene (Promega) or TransIT (Mirus Bio), in OPTIMEM, following manufacturer's instructions. After 6-h incubation, the transfection medium was replaced by DMEM plus 10 % (v/v) fetal bovine serum (Sigma) for another 48 h.

RNA isolation and qPCR

Transfected MCF7 cells were used for RNA purification. The quality and quantity of the RNA was analyzed by means of an automated electrophoretogram using a Bioanalyzer 2100 and reverse transcription and cDNA amplification were carried out as previously described (Delgado et al., 2008; Reytor, Pérez-Miguelsanz, Alvarez, Pérez-Sala, & Pajares, 2009). Expression changes were verified by qPCR using the ABI 7900HT Real-Time PCR system (Applied Biosystems) at the Genomic Service of Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM). TaqMan probes (ThermoFisher Scientific) for the following human genes were used: *AKR1B1* (Hs00739326_m1), *RNA18S1* (4352930E), *HPRT1* (Hs99999909_m1) and *GAPDH* (Hs99999905_m1). Relative expression ratios were normalized to the geometric mean of the reference genes (*RNA18S1*, *HPRT1* and *GAPDH*) used as controls following the method of Pfaffl et al. (Pfaffl, 2001).

Cellular Thermal Stability Assay (CETSA)

The effect of the mutation on protein stability was studied by means of CETSA. MCF7 cells transfected with WT or p.Lys90Glu AKR1B1 plasmids were harvested in PBS, containing protease and phosphatase inhibitors, as previously described (Sánchez-Gómez et al., 2007), but in the absence of detergents. Cells were homogenized by three freeze-thaw cycles using dry ice-ethanol. Lysates were centrifuged at 20000xg for 20 minutes at 4°C and protein concentration estimated as above. To ensure similar WT and p.Lys90Glu AKR1B1 protein levels in the CETSA experiment, WT AKR1B1 lysates were diluted (1:1) with lysates from mock-transfected cells. Forty µl aliquots from WT and p.Lys90Glu samples (15 µg of total protein) were exposed for three minutes to a range of temperatures between 0°C and 75°C, as depicted in Fig 6. Afterwards, the samples were centrifuged at 20000xg for 20 minutes at 4 °C to separate the soluble

(supernatants) and precipitated proteins (pellet). Both fractions were analyzed by Western blot with antibodies against AKR1B1 and two different control proteins, namely, AKR1A1 and HSP70. Densitometric analysis of the Western blot signals was performed using Scion Image software. Signals obtained were represented as percentage of the total protein signal (supernatant + pellet = 100 %) at each temperature and plotted against temperature using GraphPad Prism (GraphPad Software). Each data set was fitted to a variable slope sigmoidal curve by least squares using the following equation (1):

$$A = B + \frac{C - B}{1 + 10(Tm50 - D)^E} \quad (1)$$

The parameters in the equation were: A, the relative signal intensity at each temperature; B, value of the plateau at the bottom of the curve; C, value of the plateau at the top of the curve; Tm50, melting temperature; D, temperature; and E, the slope of the curve.

Statistical analysis

Statistical analyses were performed with the SPSS software version 19.0 for Windows (SPSS, Chicago, Ill, USA). Genotype, allelic and Hardy-Weinberg equilibrium analyses were performed with PLINK40 software. Comparison of alleles and genotypes frequencies between controls and patients was undertaken with the χ^2 statistics or Fisher exact test, as appropriate. The putative association between the SNPs and the risk of developing allergic rhinitis was estimated by the odds ratio (OR) and the 95 % confidence interval (CI). False discovery rate (FDR) correction was used for the multiple comparison adjustments (Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001).

Results from MCF7 cell transfection experiments are expressed as mean $\pm$ SEM of three independent experiments. Differences between experimental conditions were analyzed by Student's *t*-test “paired test” when normality tests were not supported by the data and considered significant when $p \leq 0.05$.

Results

Study of the association between AKR1B1 variants and rhinitis

The cohort of patients considered has been included in a previous study (Amo et al., 2016) where the general parameters regarding clinical characterization have been described. Briefly, there were no statistically significant differences between patients and control subjects regarding gender or age, whereas the percentage of smokers was lower in rhinitis plus asthma patients than in control subjects (Chi-Square test $p=0.002$) (Amo et al., 2016).

The frequencies for *AKR1B1* genotypes in control subjects and overall rhinitis patients are summarized in Table 1. Statistical analyses showed a significant increase in the frequency for the non-synonymous variant *rs2229542* in heterozygosity in overall patients (Table 1). The *rs5053* variant showed a slightly higher frequency, although non-statistically significant.

Overall patients were classified into two subgroups considering their clinical manifestations: patients with allergic rhinitis alone and patients presenting rhinitis plus asthma. Analysis of the distribution frequency of each variant in the sub-cohort of patients with rhinitis alone showed a statistically significant higher association of the *rs2229542* variant in heterozygosity compared to controls, similar to that observed in the overall population (Table 1). For the other two variants there is a trend towards a higher frequency in heterozygous patients compared to control subjects.

In the second subset of patients, presenting rhinitis plus asthma, there was also a statistically significant higher frequency for the variant *rs2229542* in heterozygosity compared to the control population (Table 1). Again, a trend for a higher frequency of the *rs5053* variant was observed in the patients' group.

Altogether, the analyses of variants in the overall population and in the two population subsets support the association between the p.Lys90Glu mutation and the presence of allergic rhinitis.

Study of the p.Lys90Glu aldose reductase mutant

The association between rhinitis and the *rs2229542* variant suggests that the p.Lys90Glu (K90E) mutation could have structural or functional implications. To gain insight into the potential structural changes induced by this mutation we first examined the available crystal structure of AKR1B1 (1PWL) (Fig 2). The p.Lys90Glu substitution exchanges the native positive charge of the lysine residue for the negative charge of the glutamic acid carboxylic group (Fig 2A,B). Moreover, this mutation implies a change in the orientation of the amino acid lateral chain, which can also be observed in the ribbon plots (Fig 2C,D), and alters the balance between charges in this area (Fig 2 E,F).

Putative functional effects of the p.Lys90Glu mutation were explored by transfecting expression vectors for the WT and mutant AKR1B1 in MCF7 cells (Fig 3), in which endogenous AKR1B1 levels are virtually undetectable (Fig 3B, lanes 1 and 4). The response of these cells to proinflammatory stimuli was then evaluated upon cytokine treatment (IL-1 β plus TNF α) (Sánchez-Gómez, Cernuda-Morollón, Stamatakis, & Pérez-Sala, 2004). This treatment elicited a clear increase in the levels of the cell surface inflammatory marker ICAM-1 (Fig 3A). Interestingly, transfection of AKR1B1 moderately reduced ICAM-1 induction compared to mock-transfected cells, whereas AKR1B1 p.Lys90Glu transfection did not mimic this protective effect. To investigate the reasons for this differential effect, we first monitored the levels of WT and mutant AKR1B1 proteins. Unexpectedly, we noted that AKR1B1 p.Lys90Glu transfection consistently resulted in lower protein levels than those attained by the WT, as assessed by immunoblot with either specific anti-AKR1B1 (Fig 3B) or pan-AKR antibodies (Fig

4A). This effect was independent from the transfection reagent used (Lipofectamine 2000, Fugene or TransIT) (Fig 4A), and also occurred in bovine aortic endothelial cells, indicating that the p.Lys90Glu mutation yielded lower protein levels in several cell types (Fig 4B). Thus, the low protein levels achieved could contribute to the failure of AKR1B1 p.Lys90Glu transfection to attenuate ICAM-1 induction.

To explore whether the lower protein levels of the p.Lys90Glu mutant resulted from reduced expression, qPCR was performed to measure the steady-state mRNA levels. No differences were found between *AKR1B1* transcript levels in cells transfected with the WT or the p.Lys90Glu forms, whereas they were virtually undetectable in mock-transfected cells. These results were consistent with any of the three reference genes used in the assay, namely, *RNA18S1* ribosomal subunit, *HPRT1* and *GAPDH* (Fig 5). Hence, these data indicate that the differences in protein levels reached by the WT and p.Lys90Glu AKR1B1 are due to posttranscriptional mechanisms, potentially affecting protein translation or stability.

Effect of the p.Lys90Glu mutation on AKR1B1 protein stability

To explore putative effects of the p.Lys90Glu (K90E) mutation in protein stability, we carried out *in silico* calculations using the amino acid sequences of the WT and mutant AKR1B1 proteins, and the SNPeffect web tool. Theoretical calculations indicated a marginal variation (0.65 kcal/mol) in stability between the WT and p.Lys90Glu proteins, suggesting a slight reduction in stability for the mutant form. In order to confirm this effect, we assessed protein stability experimentally by performing Cellular Thermal Shift Assays (CETSA), using cell lysates from MCF7 cells transfected with the WT or p.Lys90Glu forms of AKR1B1 (Fig. 6). Thus, cell lysates containing WT or p.Lys90Glu AKR1B1 were exposed to the indicated temperatures for 3 min, and the amount of the respective proteins remaining in the soluble fraction was taken as an

index of their stability. The melting temperatures (T_m) for both protein forms were determined and the values obtained were 57.84 ± 1.12 °C for WT AKR1B1 and 54.22 ± 0.45 °C for the p.Lys90Glu mutant (Fig 6C). Remarkably, these data indicate that the p.Lys90Glu mutant displayed a tendency towards denaturation with temperatures ~3-4°C lower than the WT protein, suggesting a lower stability. This difference is especially evident near the T_m value ($p=0.025$ at 59°C). The specificity of this effect was assessed by estimating the stability of two additional proteins, namely, AKR1A1, a ubiquitous member of the AKR family, and the chaperone HSP70, in the same lysates used for determination of AKR1B1 stability. We observed no significant differences in the melting curves of AKR1A1 or HSP70 at or around their respective T_m values, thus indicating the selective effect of the p.Lys90Glu mutation on AKR1B1 stability (Fig 6B,C).

Discussion

Increased aldose reductase activity is a well-established pathogenic factor for several diseases, for which it is considered a crucial drug target (Diez-Dacal et al., 2016). Accumulating evidence indicates that it plays a complex role in inflammation (Sánchez-Gómez et al., 2016; Srivastava et al., 2011). Also, reports from animal models put forward a role for aldose reductase in allergy (Yadav et al., 2011; Yadav et al., 2013). Given that AKR enzymes are considered highly polymorphic, we have addressed for the first time the potential association of several known *AKR1B1* variants with allergic rhinitis in patients and identified a relevant clinical association of the c.268A>G variant (*rs2229542*). This finding constitutes the first connection of aldose reductase with human allergy and paves the way for studies exploring the involvement of *AKR1B1* variants in allergic diseases.

Given their role in detoxification, AKR variants could afford inter-individual variability in the metabolism of endogenous carbonyl-containing compounds, including ketosteroids, ketoprostaglandins, and retinoids, as well as of exogenous chemicals (Penning & Drury, 2007; Ruiz, Porte, Pares, & Farres, 2012). Interestingly, variants of other detoxifying enzymes, like glutathione transferases (GST) or glutathione reductases, are associated with allergic diseases (Sánchez-Gómez et al., 2016; Wu et al., 2007). For instance, the I105V GSTP1-1 variant, resulting in defective conjugation of electrophilic drugs and GSH metabolites (Dragovic, Venkataraman, Begheijn, Vermeulen, & Commandeur, 2014), has been associated with atopic (Hoskins, Wu, Reiss, & Dworski, 2013), air pollution and acetaminophen-induced asthma (Kang et al., 2013; Su et al., 2013).

According to the NCBI database (32) the *rs2229542* variant presents an allele frequency of 4.7%. It produces the substitution of lysine 90 for glutamic acid (p.Lys90Glu), a

drastic change affecting the charge and reactivity of the residue. Interestingly, the p.Lys90 residue is conserved in vertebrates from human to *Xenopus laevis*, whereas the zebrafish homolog displays a conservative change (arginine) (Fig 7A). In addition, p.Lys90 is conserved or substituted by arginine in several AKR family members, including AKR1B3, AKR1B7, AKR1B8, AKR1B10 (Petrash, 2004) and AKR1B15 (Fig 7B), supporting the importance of a positively-charged residue at this position. However, the available information on the functional importance of this change is limited. In a previous study, no significant differences were found between the capacities of purified WT and p.Lys90Glu AKR1B1 to metabolize the antitumoral drug doxorubicin (Bains, Grigliatti, Reid, & Riggs, 2010). Nevertheless, this type of assay cannot detect differences related to cellular expression levels or alterations in protein-protein interactions involving this enzyme.

According to the available crystal structure (1PWL), p.Lys90 is surface-exposed, and hence, putatively involved in interactions or subjected to posttranslational modifications. The organization of charged, polar, and polarizable residues in the tertiary structure of a protein creates large internal electrostatic fields, expected to affect many aspects of protein function, including molecular interactions and subcellular localization (Goryaynov & Yang, 2014; Reytor et al., 2009; Valero et al., 2010). The available AKR1B1 structure (Fig 2E,F) shows the amino group at p.Lys90 lateral chain surrounded by negative charges. Therefore, the p.Lys90Glu mutation will drastically affect this area increasing the negative character, putatively eliciting repulsive effects between lateral chains of adjacent residues. Moreover, PredictProtein assigns a high score to the functional influence of the p.Lys90Glu mutation. Nevertheless, to the best of our knowledge, the *rs2229542* variant has not been reported in association with other pathological situations.

Our initial experiments on the potential effect of *AKR1B1* in the inflammatory response showed that the transfection of the WT enzyme attenuated cytokine-elicited ICAM-1 induction, whereas the p.Lys90Glu mutant did not. This lack of effect could be due, at least in part, to the lower protein levels consistently attained with the mutant construct. However, *AKR1B1* transcript levels in cells transfected with WT or mutant constructs were indistinguishable, suggesting that the transcription efficiency of the two plasmids is similar. Our data suggest that, at least in cellular models, reduced levels of the p.Lys90Glu mutant protein could be related to translational or posttranslational mechanisms. Thus, the presence of the mutated codon could affect the folding or translation efficiency of the transcript. Additionally, the study of protein stability accomplished by CETSA showed that the presence of the p.Lys90Glu mutation moderately increased the susceptibility of the protein to thermal denaturation. This compromised stability could contribute to reduced protein levels and an alteration in the overall *AKR1B1* function, including the detoxification of inflammatory mediators or, ultimately, the redox balance. Finally, it could be hypothesized that these alterations could influence the inflammatory processes underlying the development of the allergic responses. Missense mutations resulting in decreased protein stability have been identified in several human diseases. Of note, a p.Cys164Tyr variant of the AKR family member *AKR1C3*, which has been detected in amyotrophic lateral sclerosis, shows decreased thermal stability, although it is not known whether this property leads to decreased protein levels in patients or contributes to disease mechanisms (Endo et al., 2017).

Notably, in animal models the allergic response is ameliorated by strategies inhibiting *AKR1B1*, pointing to a deleterious role of *AKR1B1* activity in this process, whereas our results could suggest a protective role. Nevertheless, it should be taken into account

that, as other enzymes involved in the metabolism of electrophilic species and redox regulation, AKR1B1 could play a dual role in the modulation of inflammation. On one hand, this enzyme can detoxify reactive species, thus contributing to defense mechanisms (Kolb, Hunsaker, & Vander Jagt, 1994). However, processing of high levels of reactive species could lead to generation of proinflammatory metabolites and/or depletion of antioxidant defenses, for which a positive inflammatory feedback may occur (Ramana et al., 2006). Remarkably, AKR1B1 itself is a target for covalent modification by electrophilic species, which can stimulate or inhibit its activity depending on the modifying moiety (Díez-Dacal et al., 2016; Srivastava et al., 2003). Therefore, understanding the involvement of AKR1B1 in a particular pathology requires detailed consideration of the multiple factors involved.

In summary, to the best of our knowledge, the present work is the first to report an association of the c.268A>G variant with allergic rhinitis in a patient cohort. These findings pave the way for new efforts to elucidate the potential association of this variant with other types of allergic reactions and to establish the role of *AKR1B1* in the allergic response.

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Conflict of interest

Authors declare that no conflict of interest exists

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Figure legends

Figure 1. Gene location of the variants studied. (A) *AKR1B1* gene structure and location of *rs5053*, *rs2229542* and *rs150113325* SNPs. The exons (numbered and filled rectangles) together with the introns (lines with arrow heads) are depicted. A short description is shown over each SNP. (B) The resultant mRNA (canonical transcript) is represented, indicating the position of the exons, the “untranslated regions” at 5’ and 3’ extremes, the “ATG” and the “STOP” signals location, and the only polymorphism present in this structure of the three analyzed.

Figure 2. Structural impact of the p.Lys90Glu mutation. The human AKR1B1 crystal structure (1PWL) was used to prepare the figure using PyMOL. The lysine residue of interest was numbered according to the AKR1B1 sequence as p.Lys90 (red sticks) and the corresponding mutant as p.Lys90Glu (K90E; orange sticks) in all the panels. (A) Molecular surface of wild-type AKR1B1. (B) Molecular structure of the p.Lys90Glu mutant. (C) Ribbon structure of the wild-type protein, showing the p.Lys90 lateral chain. (D) Ribbon structure including the lateral chain of the p.Lys90Glu mutant. (E) Ribbon plot of the sequence comprising residues p.Lys77 to p.Leu101 of wild-type AKR1B1 (left), including the α -helix containing p.Lys90, and molecular surface of the area surrounding p.Lys90 (right), highlighting the negative (red) and positive charges (blue). (F) Ribbon plot of the same sequence stretch (left), and molecular surface area (right) but including the mutated lateral chain. In all cases, the mutated lateral chains depicted include the protonated form of glutamic acid.

Figure 3. Effect of AKR1B1 wild-type or p.Lys90Glu transfection on cytokine-induced ICAM levels and AKR1B1 abundance in MCF7 cells. MCF7 cells were transfected with wild-type (WT) or p.Lys90Glu (K90E) forms of AKR1B1, or mock-transfected, and stimulated with TNF α plus IL1 β as described in Materials and

Methods. **(A)** Western blot analysis of ICAM-1 protein levels and of actin as a house-keeping protein control. The graph shows the ICAM-1 signal corrected against the actin signal. Results shown are mean $\pm$ SEM of three independent experiments. $*p < 0.05$ vs. mock-transfected cells not stimulated with cytokines, and $\#p < 0.05$ vs. cells transfected with WT and stimulated with cytokines. **(B)** The levels of AKR1B1 WT and p.Lys90Glu attained by transfection were assessed by western blot. Actin was used as a house-keeping protein control. The histogram depicts the AKR1B1 signal corrected by that of actin. Results show the mean $\pm$ SEM of three independent experiments. $*p < 0.05$ vs. cells transfected with the WT form not stimulated with cytokines, and $\#p < 0.05$ vs. cells transfected with WT and stimulated with cytokines.

Figure 4. Levels of AKR1B1 attained upon transfection of wild-type and p.Lys90Glu constructs using different transfection agents and in several cell types.

(A) AKR1B1 wild-type (WT) or p.Lys90Glu (K90E) expression vectors were transfected in MCF7 cells utilizing Lipofectamine 2000, FuGene or TransIT transfection agents. Levels of AKR1B1 were assessed by western blot with a specific antibody (upper panel) or with a pan-AKR antibody (middle panel) to ensure that the differences observed were not due to poor recognition of the p.Lys90Glu mutant by the specific antibody. **(B)** Bovine aortic endothelial cells were transfected with AKR1B1 WT or p.Lys90Glu vectors, or mock-transfected, and AKR1B1 protein levels were analyzed by western blot. Actin was used as a house-keeping protein control. Results are representative of at least two experiments.

Figure 5. Analysis of AKR1B1 mRNA levels by real-time RT-PCR. *AKR1B1*

expression levels were analyzed by real-time RT-PCR in MCF7 cells mock-transfected (-) or transfected with plasmids containing the ORF sequences for the wild-type (WT) or the c.398A>G (K90E) AKR1B1 mutant. The fold changes were evaluated using the

HPRT1 (A), *GAPDH* (B) and *18S* (C) genes as reference. The figure shows the mean $\pm$ SEM of three independent experiments. * $p < 0.05$ vs. mock-transfected cells.

Figure 6. Evaluation of AKR1B1 wild-type and p.Lys90Glu protein stability by Cellular Thermal Shift Assay. Lysates from MCF7 cells transfected with AKR1B1 wild-type (WT) or p.Lys90Glu (K90E) plasmids were subjected to different temperatures and centrifuged to obtain the supernatant and pellet fractions as described in the “Materials and Methods” section. (A) Supernatant (left) and pellet (right) fractions were analyzed by Western blot using antibodies against AKR1B1; (B) As a control, levels of AKR1A1 and HSP70 present in supernatant fractions from lysates of cells transfected with AKR1B1 WT or p.Lys90Glu, as indicated, were assessed by Western blot. Signals obtained upon densitometric scanning of the blots were represented as percentage of the total protein signal (supernatant + pellet = 100 %) at each temperature. (C) The melting temperature (T_m) is depicted for the three proteins studied. The graphs show the mean $\pm$ SEM of the T_m values obtained in three independent experiments. * $p < 0.05$ vs. WT samples.

Figure 7. Amino acid alignment of several AKR proteins. (A) CLUSTALW alignment of the amino acid sequences of AKR1B1 from different species. (B) CLUSTALW alignment of the amino acid sequences of several human AKR proteins. The sequences shown correspond to the amino acids surrounding the AKR1B1 p.Lys90 residue (box) or the equivalent residue from the other proteins. Conservative variations in amino acid sequence are highlighted in light grey and non-conservative in dark grey and white fonts, respectively, according to the protein alignment performed with BLAST tool.

A **B**

A **B** **C** **D** **E** **F**

A **B** **C**

A

<i>H. sapiens</i>	AKR1B1 (P15121)	REELFIVSKLWCTYHEKGLVKGACQKTLSDLKLDYLDLYLI
<i>P. troglodites</i>	AKR1B1 (H2QVE6)	REELFIVSKLWCTYHEKGLVKGACQKTLSDLKLDYLDLYLI
<i>P. abelli</i>	AKR1B1 (Q5RAB3)	REELFIVSKLWCTYHEKGLVKGACQKTLSDLKLDYLDLYLI
<i>G. gorilla</i>	AKR1B1 (G3R7U6)	REELFIVSKLWCTYHEKGLVKGACQKTLSDLKLDYLDLYLI
<i>B. taurus</i>	AKR1B1 (P16116)	REDLFIVSKLWCTYHDKSLVKGACQKTLSDLKLDYLDLYLI
<i>M. musculus</i>	AKR1B1 (P45376)	RQDLFIVSKLWCTFHDQSMVKGAFQKTLSDLQLDYLDLYLI
<i>R. norvegicus</i>	AKR1B1 (P07943)	RQDLFIVSKLWCTFHDQSMVKGACQKTLSDLQLDYLDLYLI
<i>O. cuniculus</i>	AKR1B1 (P15122)	REELFIVSKLWCTSHDKSLVKGACQKTLNDLKLDYLDLYLI
<i>O. hannah</i>	AKR1B1 (V8NPC0)	REDLFIVSKLWCTFHEKSLVKGACQATLKALKLNLYLDLYLI
<i>X. laevis</i>	AKR1B1 (Q6PAB5)	REDLFIVSKLWNTFHDQSMVKGACQKTLSDLKLDYLDLYLV
<i>D. rerio</i>	AKR1B1 (Q6IQU1)	REDLFIVSKLWCTFHEKHLVKGACEKTLSDLKLDYVDLYLM
<i>B. latifrons</i>	AKR1B1 (A0K8W2Q6)	REELFIISKLWNTFHKPELVGSALETTLKLNRLDYLDLYLM
<i>Trichinella sp.</i>	AKR1B1 (A0A0V1P759)	REDLFIVTKLHLMCVFVNIHNPVLPQ

B

<i>H. sapiens</i>	AKR1B1 (P15121)	REELFIVSKLWCTYHEKGLVKGACQKTLSDLKLDYLDLYLI
	AKR1B3 (Q3UDY1)	RQDLFIVSKLWCTFHDQSMVKGAFQKTLSDLQLDYLDLYLI
	AKR1B7 (Q5RJP0)	REDLFIVSKLWSTFFEKSLNKGAFQKTLSDLKLDYLDLYLI
	AKR1B8 (P45377)	REDLFIVSKLWPTCFEKKLLKGAFAQKTLSDLKLDYLDLYLI
	AKR1B10 (O60218)	REDLFIVSKLWPTFFERPLVRKAFEKTLKDLKLSYLDVYLI
	AKR1B15 (C9JRZ8)	REDLFIVSKLWPTFFERPLVRKAFEKTLKDLKLSYLDVYLI

	Group	Non-mutated (frequency)	Heterozygous (frequency)	Homozygous (frequency)	MAF	Comparison Allele frequency OR (95% C.I.); <i>p</i>
<i>AKR1B1</i> genotypes in control individuals.						
<i>rs5053</i>	Controls	0.884	0.112	0.004	0.060	
<i>rs150113325</i>	Controls	0.996	0.004	0.000	0.002	
<i>rs2229542</i>	Controls	0.992	0.008	0.000	0.004	
<i>AKR1B1</i> genotypes in overall rhinitis patients.						
<i>rs5053</i>	Patients	0.854	0.138	0.008	0.077	1.30 (0.93-1.84); 0.128
<i>rs150113325</i>	Patients	0.996	0.004	0.000	0.002	1.02 (0.14-7.27); 1.00
<i>rs2229542</i>	Patients	0.965	0.035	0.000	0.017	4.99 (1.67-14.50); 0.0013
<i>AKR1B1</i> genotypes in patients with rhinitis alone (all rhinitis patients).						
<i>rs5053</i>	Patients	0.836	0.158	0.007	0.086	1.47 (0.91-2.38); 0.116
<i>rs150113325</i>	Patients	0.993	0.007	0.000	0.003	1.80 (0.16-19.97); 1.00
<i>rs2229542</i>	Patients	0.952	0.048	0.000	0.024	6.57 (1.87-22.14); 0.00068
<i>AKR1B1</i> genotypes in patients with rhinitis+asthma (all rhinitis+asthma patients)						
<i>rs5053</i>	Patients	0.862	0.130	0.008	0.073	1.24 (0.85-1.81); 0.263
<i>rs150113325</i>	Patients	0.997	0.003	0.000	0.001	0.71 (0.07-7.87); 1.00
<i>rs2229542</i>	Patients	0.970	0.030	0.000	0.015	4.01 (1.26-12.50); 0.011

Table 1. *AKR1B1* genotypes in control individuals, overall rhinitis patients, rhinitis alone patients and rhinitis+asthma patients. *p* values correspond to stratified Chi-Square Test. OR: odds ratio. CI: 95 % confidence interval.

Database	Reference ID	Software	URL
GenBank	NM_001628.3	UbPred	www.ubpred.org
GenBank	NM_001628.3	NetNES 1.1 server	www.cbs.dtu.dk/services/NetNES
GenBank	NM_001628.3	PSORT II Prediction	www.psort.hgc.jp/form2.html
GenBank	NM_001628.3	PredictProtein	www.predictprotein.org
NCBIdbSNP	rs2229542	SNPeffect 4.0 server	www.snpeffect.swichlab.org

Supplemental Table S1. Websites used for *in silico* studies. Different online bioinformatic tools were used to analyze the AKR1B1 sequence and the impact of the mutation. UbPred was utilized to predict potential ubiquitination targets. Studies in NetNES 1.1 and PSORT II were run to identify putative nuclear localization/export sequences in the protein. The PredictProtein server was used to estimate the potential effect of the mutation. SNPeffect database was searched to obtain information regarding the impact of the mutation on protein stability. The reference ID for the sequence or variants used in each case, the servers or databases and their websites are detailed.