Research Article
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Pharmacogenomics

Genetic variants in arachidonic acid pathway genes associated with NSAIDs-exacerbated respiratory disease

Aim: NSAIDs are the most frequent cause of hypersensitivity drug reactions. We have examined the association between NSAIDs-exacerbated respiratory disease (NERD) and genetic variants in arachidonic acid metabolism genes. **Patients & methods:** We included 250 NERD patients, 260 NSAID-tolerant asthmatic (NTA) subjects and 315 healthy controls. **Results:** Significant associations with NERD were identified for: *ALOX15* rs3892408 C/C homozygous genotype (NERD vs NTA; p = 0.0001, pc = 0.0011; NERD vs controls; p = 0.0001, pc = 0.0011), *PTGS-1* rs5789 A/A homozygous genotype (NERD vs NTA; p = 0.0001, pc = 0.0011; NERD vs controls; p = 0.0001, pc = 0.0011), *PTGS-1* rs10306135 A/A homozygous genotype (NERD vs NTA; p = 0.0009, pc = 0.0091; NERD vs controls; p = 0.0064, pc = 0.045). Differences in *ALOX5* copy number variations were also found (NERD vs NTA; p = 0.010; NERD vs controls; p = 0.0001). **Conclusion:** These results improve our understanding of the underlying mechanisms of NERD and may help develop a predictive test for this pathology.

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Keywords: copy number variations • hypersensitivity drug reactions • NSAIDs • NSAIDs-exacerbated respiratory disease • polymorphisms

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Hypersensitivity drug reactions (HDRs) are an important health problem affecting patients of all ages [1]. They may not be predicted before postmarketing surveillance and constitute one of the most frequent reasons for consultations in allergy services, particularly for adults, requiring many resources, including trained personnel and an appropriate setting for their correct evaluation and diagnosis [2]. A recent study carried out by our group has shown that NSAIDs are currently the drugs most frequently involved in HDRs, surpassing betalactam antibiotics [3].

Different types of HDRs to NSAIDs can be identified depending on the symptoms, number of NSAIDs involved and the presence of an underlying pathology. Two main groups can be distinguished: in the first, inflammatory mediators are produced in the absence of a specific immunological mechanism (cross intolerant [CI]). This includes several entities that are expressed with varied clinical manifestations: NSAIDs-exacerbated respiratory disease (NERD), previously known as ASA-induced asthma or ASA-exacerbated respiratory disease; NSAIDs-exacerbated cutaneous disease (NECD), manifesting as urticaria and angioedema in patients with chronic spontaneous urticaria; and NSAIDs-induced urticaria/angiodema (NIUA), occurring in patients without preexisting chronic spontaneous urticaria. In addition, combined reactions can occur involving respiratory airways and the skin, also known as blended reactions [4]. The second group of entities encompasses reactions involving immunological mechanisms (selective responders). These can be further classified into: Single-NSAID-induced urticaria/angioedema or anaphylaxis (SNIUAA) attributed to specific IgE responses; and Single-NSAID-induced delayed reactions (SNIDR) induced by sensi-

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tized T cells [5]. Our group recently published the analysis of a large cohort of almost 700 patients, showing that 76% of NSAIDs reactions belonged to the CI type compared with 24% for selective reactions [6]. Further classification has been proposed in order to better define the different phenotypes of NSAID-hypersensitive patients [7].

In this work, we will focus on NERD. Its prevalence is estimated to range from 0.3 to 0.9% in the general population, 4.3 to 11% in adult asthmatics and is about 25% in patients with asthma and nasal polyposis [8,9]. In NERD patients, NSAID intake induces nasal congestion and rhinorrhea, followed by difficulty breathing and progressive nasal obstruction within a short time interval [10]. This entity is characterized by an adult onset and patients have a previous history of bronchial asthma that is very frequently associated with chronic rhinosinusitis and/or nasal polyposis [11]. The gold standard for diagnosis is oral challenge; however, this is a time-consuming procedure and is not risk-free. The mechanism of NERD has been attributed to the inhibition of prostaglandin-endoperoxide synthases (PTGS) by NSAIDs in susceptible individuals, causing a reduction in prostaglandin E_2 production and shunting arachidonic acid metabolism toward the lipooxygenase pathway, resulting in the release of cysteinyl leukotrienes (CysLTs) [12]. This hypothesis is consistent with previous studies that have reported CysLT overproduction in NERD patients [13,14]. The first genetic studies showed functional variation in *LTC4S* and *ALOX5* genes to be associated with NERD [15,16]. Associated genetic variants have also been found in the leukotriene receptors *CysLTR1* and *CysLTR2*, *PTGS2* and prostaglandin receptor genes [17]. However, many of these associations have been inconsistently replicated, possibly due to the limited number of gene studied, and most studies have focused on Asian populations [17]. With regards to European populations, previous work by our group investigating genetic variation in NIUA patients found statistically significant associations for several SNPs in enzymes and receptors from the arachidonic acid pathway [18]. Copy number variations (CNVs), variations in the number of copies of a section of DNA in the genome, can affect gene expression and be associated with disease susceptibility [19]. However, they have not yet been studied in relation to NSAID hypersensitivity and their role as possible genetic markers for NERD has not been investigated.

In this work, we have investigated the association of various SNPs and CNVs in arachidonic acid-related genes with NERD, in a Spanish population. We found a number of significant variants that will be of use for establishing the genetic basis of NERD and enable us to compare and contrast NERD with other NSAIDs-hypersensitivity pathologies such as NIUA and NECD.

Patients & methods

Subjects

We included a total of 250 NERD patients, 260 NSAID-tolerant asthmatic (NTA) patients and 315 unrelated healthy subjects. These individuals were recruited between 2007 and 2013 from the Allergy services of six Spanish public hospitals. They are integrated in the national research network RIRAAF (Table 1).

All patients were diagnosed with asthma according to the Global Initiative for Asthma (GINA) guidelines [20]. NERD patients were required to have a history of at least two episodes of respiratory symptoms after NSAID intake and a positive nasal challenge with lysine-acetylsalicylate (L-ASA), as previously described [21]. The NTA group consisted of asthmatic patients who tolerated NSAIDs. Healthy subjects had no past history of asthma or NSAID hypersensitivity, a FEV_1 greater than 80% of the predicted value and a PC_{20} with methacholine greater than 25 mg/ml [22]. The presence of rhinosinusitis and nasal polyps was evaluated using a paranasal sinus X-ray and rhinoscopy. L-ASA was administered to NTA and healthy subjects to confirm tolerance before inclusion in the study.

Skin prick testing (SPT) was performed with a panel of 30 prevalent inhalant allergens and total serum IgE was measured using the CAP system (Phadia, Uppsala, Sweden). Atopy was defined as one or more positive SPT results or a total IgE value ≥ 130 kU/l. Eosinophil cationic protein and serum tryptase were measured by UniCAP (Phadia), and eosinophil count by flow cytometry.

Genotyping

A total of 33 SNPs in *PTGS1* (rs5789, rs5794, rs1236913, rs10306108, rs3842787, rs10306135), *PTGS2* (rs689465, rs689466), *ALOX5* (rs2115819, rs3780894, rs4986832), *ALOX5AP* (rs1132340), *ALOX12* (rs434473, rs1126667), *ALOX15* (rs3892408, rs11568131), *LTC4S* (rs730012), *CYSLTR1* (rs320995, rs2806489), *CYSLTR2* (rs912278), *PTGER1* (rs3810253, rs3810255), *PTGER2* (rs17197, rs1254598, rs1353411, rs2075797), *PTGER3* (rs959), *PTGER4* (rs4495224, rs7720838, rs45613037), *PTGDR* (rs8004654, rs34236606) and *PTGFR* (rs3753380) genes were studied. They were selected on the basis of their minor allele frequencies (MAF > 0.05) in European populations from the HapMap [23], 1000 Genomes project [24] or dbSNP databases [25] and their

Table 1. Clinical and demographic characteristics of study subjects.

	NERD (n = 250)	NTA (n = 260)	Controls (n = 315)	p-value (NERD vs controls)	p-value (NERD vs NTA)
Age (mean, SD)	(39.87,17.55)	(38.15,12.80)	(39.16,17.74)	0.8010	0.2054
Sex (% females)	65.70	59.68	62.45	0.4903	0.2104
Personal history					
Rhinitis (%)	63.12	94.31	na	na	<0.0001
Asthma (%)	67.43	100	na	na	<0.0001
Polyposis (%)	29.31	9.87	na	na	<0.0001
FEV1%	87.71	89.89	na	na	0.6954
Culprit NSAIDs (%)					
Propionic acid derivates	31.64	na	na	na	na
ASA	25.99	na	na	na	na
Metamizol	18.64	na	na	na	na
Paracetamol	4.80	na	na	na	na
Diclofenac	6.50	na	na	na	na
Type of reaction (%)					
Rhinitis	12.73	na	na	na	na
Asthma	57.58	na	na	na	na
Upper airways	7.27	na	na	na	na
Rhinitis + asthma	22.42	na	na	na	na
Total IgE (median, range) (kU/l)	82.0 (44.0–208.0)	228.0 (108.0–543.0)	27.5 (11.0–63.25)	<0.0001	<0.0001
SPT (% positive skin prick test)	60.8%	94.82%	34.98%	<0.0001	<0.0001
Phleum	5.6	3.21	6.36	0.7841	0.2832
Lolium	24.0	3.66	6.36	<0.0001	<0.0001
Olea europaea	24.0	71.55	8.63	<0.0001	<0.0001
Dermatophagoides pteronyssinus	20.0	67.43	7.27	0.0004	<0.0001
Lepidoglyphus destructor	6.4	67.43	2.72	0.0952	<0.0001
Dog	16.8	44.03	2.72	<0.0001	<0.0001
Cat	17.6	44.49	5	0.0001	<0.00010

ASA: Acetylsalicylate; IgE: Immunoglobulin E; na: Not applicable; NERD: NSAIDs-exacerbated respiratory disease; NTA: NSAID-tolerant asthmatic patients; SPT: Skin prick testing.

possible functional role according to previous reports and information obtained from SNPinfo [26].

Genotyping was carried out by PCR in a 7500 Fast Real-Time PCR System using TaqMan© allelic discrimination assays (Applied Biosystems, CA, USA). All samples were determined in triplicate and genotypes were assigned using 7500 software 2.0.1. CNVs were analyzed using TaqMan copy number assays (Applied Biosciences), which were designed to hybridize within the open reading frame in each gene. Amplification was carried out as described by the manufacturer, using RNAse P as a copy number reference assay. Results were analyzed using the CopyCaller Software (Applied Biosciences).

Statistical analysis

Demographic and clinical data were analyzed using the Mann-Whitney U-test implemented in SPSS 15.0 (SPSS Inc., IL, USA). The Hardy–Weinberg equilibrium was evaluated with the DeFinetti program [27]. Allelic and genotypic frequency analyses were performed by logistic regression using allelic, heterozygous and homozygous dominant and recessive models and adjusting for the atopic status (Table 2). Correction for multiple testing was performed by calculating the false-discovery rate (FDR) using QVALUE [28]. A corrected p-value (q-value) of ≤0.05 was considered statistically significant. Haplotype reconstruction was performed using Haploview 4.2 [29]. The statistical power

10.2217/PGS.15.43

Table 2. Genotype and minor allele frequencies of the SNPs in arachidonic acid pathway genes.

Gene	SNP	Genotype	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
						p-value	OR (95% CI)	p-value	OR (95% CI)
ALOX5	**rs2115819**	A/A	29.6	33.6	30.5	0.160	0.67 (0.37–1.19)	0.900	0.97 (0.59–1.58)
		A/G	48.7	46.6	48.5				
		G/G	21.7	19.7	21.0				
		Allele frequency	46.0	43.0	45.25	0.641	0.89 (0.56–1.41)	0.911	0.97 (0.62–1.50)
	rs3780894	A/A	65.2	66.8	61.1	0.530	0.84 (0.49–1.44)	0.400	1.22 (0.77–1.94)
		A/G	32.2	30.4	34.5				
		G/G	2.6	2.8	4.4				
		Allele frequency	18.7	18.0	21.65	0.882	0.94 (0.53–1.68)	0.588	1.18 (0.68–2.04)
	rs4986832	A/A	64.7	60.6	59.8	0.790	1.07 (0.63–1.81)	0.300	1.28 (0.80–2.03)
		A/G	32.8	36.0	35.7				
		G/G	2.6	3.5	4.5				
		Allele frequency	19.0	21.5	22.35	0.667	1.18 (0.67–2.09)	0.499	1.24(0.72–2.15)
ALOX5AP	**rs1132340**	A/A	90.5	87.0	86.5	0.390	1.42 (0.63–3.20)	0.340	1.41 (0.68–2.92)
		A/G	9.5	12.6	13.6				
		G/G	0.0	0.4	0.0				
		Allele frequency	4.8	6.70	6.80	0.644	1.37 (0.52–3.60)	0.653	1.37 (0.53–3.53)
ALOX12	**rs434473**	A/A	37.2	32.1	32.0	0.591	1.16 (0.68–1.96)	0.250	1.26 (0.85–1.85)
		A/G	43.5	47.3	50.5				
		G/G	19.4	20.7	17.4				
		Allele frequency	41.1	44.3	42.6	0.557	1.13 (0.77–1.66)	0.849	1.05 (0.72–1.52)
	rs1126667	A/A	37.2	32.7	32.5	0.812	1.07 (0.63–1.83)	0.300	1.23 (0.84–1.80)
		A/G	43.5	46.9	50.2				
		G/G	19.4	20.4	17.3				
		Allele frequency	41.1	43.8	42.4	0.621	1.10 (0.75–1.63)	0.850	1.04 (0.72–1.51)
ALOX15	**rs3892408**	C/C	54.4	27.8	25.9	0.0001 pc = 0.0011	3.29 (1.93–5.60)	0.0001 pc = 0.0011	3.48 (2.08–5.80)
		C/T	31.6	52.3	52.6				
		T/T	14.0	19.9	21.5				
		Allele frequency	29.8	46.0	47.8	0.0001 pc = 0.0011	1.83 (1.38–2.43)	0.0001 pc = 0.0011	2.00 (1.52–2.62)
	rs11568131	A/A	71.7	73.1	71.7	0.840	1.06 (0.60–1.87)	0.880	1.04 (0.63–1.71)

Allele frequency represents the minor allele frequency for each SNP.
NERD: NSAIDs-exacerbated respiratory disease; NS: Not significant; NTA: NSAID-tolerant asthmatic patients; OR: Odds ratio.

Table 2. Genotype and minor allele frequencies of the SNPs in arachidonic acid pathway genes (cont.).

Gene	SNP	Genotype	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
						p-value	OR (95% CI)	p-value	OR (95% CI)
ALOX15 (cont.)	rs11568131 (cont.)	A/G	25.7	25.2	24.3				
		G/G	2.6	1.7	4.0				
		Allele frequency	15.4	14.3	16.1	0.750	0.90 (0.48–1.67)	0.980	1.02 (0.56–1.85)
PTGS1	rs5789	C/C	53.5	81.7	99.3	0.0001 pc = 0.0011	0.28 (0.18–0.44)	0.0001 pc = 0.0011	0.01 (0.00–0.03)
		C/A	43.0	18.3	0.7				
		A/A	3.5	0.0	0.0				
		Allele frequency	25.0	9.1	0.35	0.0001 pc = 0.0011	0.33 (0.22–0.48)	0.0001 pc = 0.0011	0.01 (0.00–0.05)
	rs5794	G/G	95.7	96.1	96.7	0.740	1.25 (0.33–4.66)	0.610	0.74 (0.24–2.33)
		G/A	4.3	3.9	3.3				
		A/A	0.0	0.0	0.0				
		Allele frequency	2.15	1.95	1.65	0.722	0.81 (0.19–3.48)	0.699	0.69 (0.16–2.96)
	rs1236913	G/G	95.7	96.1	96.7	0.460	0.78 (0.40–1.50)	0.720	0.90 (0.50–1.61)
		G/A	4.3	3.9	3.3				
		A/A	0.0	0.0	0.0				
		Allele frequency	2.1	1.9	1.6	0.841	0.87 (0.40–1.89)	0.846	0.89 (0.42–1.89)
	rs10306108	T/T	93.0	87.8	91.4	0.066	1.90 (0.94–3.84)	0.662	1.21 (0.52–2.83)
		T/C	7.0	11.7	8.3				
		C/C	0.0	0.5	0.4				
		Allele frequency	3.5	6.35	4.55	0.257	1.78 (0.69–4.56)	0.787	1.36 (0.43–4.23)
	rs3842787	C/C	92.0	85.9	91.4	0.041 pc = 0.144	1.91 (1.01–3.60)	0.220	1.67 (0.71–3.89)
		C/T	7.1	11.3	8.6				
		T/T	0.9	2.8	0.0				
		Allele frequency	4.4	8.4	4.3	0.013 pc = 0.069	2.12 (1.17–3.83)	0.192	1.99 (0.73–5.45)
	rs10306135	A/A	83.5	67.7	69.4	0.0009 pc = 0.0091	2.19 (1.37–3.52)	0.0064 pc = 0.045	2.12 (1.21–3.74)
		A/T	12.2	28.9	27.3				
		T/T	4.3	3.4	3.2				
		Allele frequency	10.4	17.8	16.85	0.0080 pc = 0.046	1.73 (1.15–2.59)	0.0081 pc = 0.046	1.65 (1.12–2.43)
PTGS2	rs689465	T/T	65.7	69.0	66.4	0.370	0.77 (0.44–1.35)	0.802	0.94 (0.58–1.52)
		T/C	30.6	27.2	29.1				
		C/C	3.7	3.8	4.4				

Allele frequency represents the minor allele frequency for each SNP.
NERD: NSAIDs-exacerbated respiratory disease; NS: Not significant; NTA: NSAID-tolerant asthmatic patients; OR: Odds ratio.

Table 2. Genotype and minor allele frequencies of the SNPs in arachidonic acid pathway genes (cont.).

Gene	SNP	Genotype	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
						p-value	OR (95% CI)	p-value	OR (95% CI)
***PTGS2* (cont.)**	**rs689465 (cont.)**	**Allele frequency**	19.0	17.4	18.9	0.648	0.87 (0.48–1.58)	0.960	0.98 (0.56–1.73)
	rs689466	T/T	69.0	66.1	66.9	0.861	1.05 (0.62–1.78)	0.930	1.02 (0.63–1.65)
		T/C	29.3	30.5	30.5				
		C/C	1.7	3.4	2.6				
		Allele frequency	16.3	18.6	17.8	0.876	0.96 (0.52–1.77)	0.771	1.12 (0.63–2.01)
CYSLTR1	**rs320995**	A/A	59.1	68.8	74.4	0.110	0.66 (0.39–1.10)	0.0039 pc = 0.030	0.50 (0.31–0.80)
		A/G	31.3	21.6	16.9				
		G/G	9.6	9.5	8.8				
		Allele frequency	25.2	20.3	17.2	0.335	0.76 (0.45–1.29)	0.0135 pc = 0.069	0.66 (0.48–0.91)
	rs2806489	T/T	60.2	57.5	64.9	0.552	1.39 (0.47–4.08)	0.491	0.85 (0.53–1.35)
		T/C	30.1	25.0	22.9				
		C/C	9.7	17.5	12.2				
		Allele frequency	24.7	30.0	23.6	0.795	0.94 (0.56–1.56)	0.800	0.93 (0.57–1.53)
CYSLTR2	**rs912278**	A/A	26.1	29.9	28.8	0.571	0.85 (0.48–1.49)	0.610	0.88 (0.53–1.45)
		A/G	56.5	50.4	50.5				
		G/G	17.4	19.7	20.7				
		Allele frequency	45.6	44.9	45.9	0.909	0.95 (0.61–1.49)	0.980	1.00 (0.65–1.55)
LTC4S	**rs730012**	A/A	46.1	51.9	59.0	0.173	0.71 (0.43–1.17)	0.025 pc = 0.107	0.60 (0.38–0.94)
		A/C	43.5	41.5	36.3				
		C/C	10.4	6.6	4.8				
		Allele frequency	32.1	27.3	22.9	0.382	0.79 (0.49–1.29)	0.0156 pc = 0.080	0.69 (0.52–0.93)
PTDGR	**rs8004654**	C/C	35.6	28.3	23.8	0.681	1.15 (0.59–2.25)	0.021	1.77 (1.09–2.88)
		C/T	44.4	55.7	54.2				
		T/T	20.0	16.0	22.0				
		Allele frequency	42.2	43.8	49.1	0.909	1.05 (0.67–1.65)	0.266	1.30 (0.84–2.01)
	rs34236606	C/C	87.9	84.8	85.9	0.510	1.29 (0.60–2.75)	0.680	1.15 (0.59–2.25)
		C/T	12.1	15.2	13.7				
		T/T	0.0	0.0	0.4				
		Allele frequency	6.05	7.6	7.25	0.659	1.32 (0.53–3.29)	0.828	1.25 (0.51–3.03)

Allele frequency represents the minor allele frequency for each SNP.
NERD: NSAIDs-exacerbated respiratory disease; NS: Not significant; NTA: NSAID-tolerant asthmatic patients; OR: Odds ratio.

Table 2. Genotype and minor allele frequencies of the SNPs in arachidonic acid pathway genes (cont.).

Gene	SNP	Genotype	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
						p-value	OR (95% CI)	p-value	OR (95% CI)
PTGER1	**rs3810253**	A/A	60.9	67.2	74.5	0.642	0.88 (0.52–1.49)	0.0038 pc = 0.030	0.49 (0.28–0.74)
		A/C	37.4	29.3	22.9				
		C/C	1.7	3.5	2.6				
		Allele frequency	20.4	18.1	16.0	0.565	0.85 (0.49–1.49)	0.0012 pc = 0.011	0.57 (0.41–0.80)
	rs3810255	C/C	77.4	77.2	86.0	0.831	0.94 (0.52–1.70)	0.05	0.56 (0.32–0.99)
		C/T	22.6	22.4	13.6				
		T/T	0.0	0.4	0.4				
		Allele frequency	11.3	11.6	7.2	0.960	1.05 (0.52–2.11)	0.0009 pc = 0.010	0.48 (0.31–0.73)
PTGER2	**rs17197**	A/A	79.3	76.7	73.9	0.840	1.06 (0.58–1.94)	0.215	1.41 (0.82–2.41)
		A/G	19.8	22.5	25.0				
		G/G	0.9	0.8	1.1				
		Allele frequency	14.4	12.0	13.6	0.863	1.09 (0.54–2.18)	0.620	1.25 (0.64–2.45)
	rs1254598	A/A	70.7	67.1	65.1	0.027 pc = 0.103	0.10 (0.01–0.94)	0.271	1.31 (0.81–2.12)
		A/G	26.7	32.5	31.6				
		G/G	2.6	0.4	3.3				
		Allele frequency	15.9	16.6	19.1	0.980	1.04 (0.57–1.89)	0.569	1.21 (0.68–2.15)
	rs1353411	G/G	71.3	66.5	66.0	0.760	1.09 (0.63–1.87)	0.300	1.29 (0.79–2.10)
		G/A	22.6	30.1	30.6				
		A/A	6.1	3.4	3.3				
		Allele frequency	17.4	18.4	18.6	0.883	1.09 (0.61–1.95)	0.776	1.10 (0.62–1.95)
	rs2075797	C/C	81.7	79.1	75.3	0.640	1.16 (0.62–2.20)	0.151	1.49 (0.85–2.61)
		C/G	16.5	20.1	23.6				
		G/G	1.7	0.8	1.1				
		Allele frequency	9.9	10.8	12.9	0.978	1.05 (0.51–2.16)	0.610	1.27 (0.63–2.55)
PTGER3	**rs959**	T/T	68.7	70.8	62.7	0.940	0.98 (0.55–1.73)	0.210	1.35 (0.84–2.18)
		C/T	28.7	24.1	35.1				
		C/C	2.6	5.0	2.2				
		Allele frequency	16.9	17.0	19.7	0.980	0.98 (0.53–1.80)	0.671	1.18 (0.67–2.08)
PTGER4	**rs4495224**	A/A	36.8	41.0	46.5	0.400	0.80 (0.48–1.35)	0.067	0.65 (0.41–1.03)

Allele frequency represents the minor allele frequency for each SNP.
NERD: NSAIDs-exacerbated respiratory disease; NS: Not significant; NTA: NSAID-tolerant asthmatic patients; OR: Odds ratio.

Table 2. Genotype and minor allele frequencies of the SNPs in arachidonic acid pathway genes (cont.).

Gene	SNP	Genotype	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
						p-value	OR (95% CI)	p-value	OR (95% CI)
***PTGER4* (cont.)**	**rs4495224 (cont.)**	A/C	50.0	47.3	45.4				
		C/C	13.2	11.7	8.1				
		Allele frequency	38.2	35.3	30.8	0.637	0.88 (0.55–1.39)	0.046 pc = 0.170	0.75 (0.57–0.99)
	rs7720838	T/T	27.2	27.9	32.7	0.610	0.86 (0.48–1.52)	0.230	0.74 (0.45–1.21)
		T/G	47.4	51.4	47.4				
		G/G	25.4	20.7	19.9				
		Allele frequency	49.0	46.0	43.6	0.646	0.90 (0.57–1.41)	0.370	0.81 (0.52–1.25)
	rs45613037	G/G	26.1	22.6	22.4	0.008 pc = 0.046	2.41 (1.22–4.76)	0.410	1.24 (0.74–2.09)
		G/A	56.5	50.2	50.7				
		A/A	17.4	27.1	26.8				
		Allele frequency	45.6	52.2	52.0	0.029 pc = 0.011	1.35 (1.03–1.77)	0.317	1.28 (0.82–1.98)
PTGFR	**rs3753380**	C/C	44.4	57.6	52.9	0.015 pc = 0.071	0.63 (0.43–0.92)	0.020 pc = 0.09	0.58 (0.37–0.92)
		C/T	47.0	34.9	36.4				
		T/T	8.7	7.5	10.7				
		Allele frequency	32.2	25.0	28.9	0.024 pc = 0.099	0.71 (0.53–0.95)	0.546	0.86 (0.54–1.38)

Allele frequency represents the minor allele frequency for each SNP.
NERD: NSAIDs-exacerbated respiratory disease; NS: Not significant; NTA: NSAID-tolerant asthmatic patients; OR: Odds ratio.

for two-tailed associations for the presence of the SNPs (MAF = 0.1) identified in this study was 87.0%.

The study was conducted according to the principles of the Declaration of Helsinki. All participating patients gave their informed consent and protocols were approved by the ethics committees of the institutions involved in the study.

Results

The study included a total of 250 Caucasian NERD patients, 260 NTA subjects and 315 unrelated, ethnically matched healthy subjects. No statistically significant differences were found between the three groups for age or sex (Table 1). The most frequent clinical entity in the NERD group was asthma only (57.6%) followed by rhinitis and asthma (22.4%). Propionic acid derivatives were the most frequent culprit drugs (31.6%), followed by ASA (26.0%) and pyrazolones (18.70%) (Table 1). Regarding the atopic status, we found statistically significant differences in SPT results between NERD patients and controls (Table 1).

Out of the 33 SNPs studied, 17 were in genes encoding enzymes from the arachidonic acid pathway and 16 were in genes encoding CysLT and prostaglandin receptors. No significant deviation from Hardy–Weinberg equilibrium was found for any of the SNPs in controls.

Genotypic frequencies are shown in Table 2. We found three statistically significant associations according to a dominant genetic model: *ALOX15* rs3892408 C/C homozygous genotype (NERD vs NTA; OR: 3.29; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 3.48; p = 0.0001, pc = 0.0011), *PTGS1* rs5789 A/A homozygous genotype (NERD vs NTA; OR: 0.28; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 0.01; p = 0.0001, pc = 0.0011), *PTGS1* rs10306135 A/A homozygous genotype (NERD vs NTA; OR: 2.19; p = 0.0009, pc = 0.0091; NERD vs controls; OR: 2.12; p = 0.0064, pc = 0.045).

When the allelic model was considered statistical significant differences were also observed for the SNPs *ALOX15* rs3892408 (NERD vs NTA; OR: 1.83; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 2.00; p = 0.0001, pc = 0.0011), *PTGS1* rs5789 (NERD vs NTA; OR: 0.33; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 0.01; p = 0.0001, pc = 0.0011) and *PTGS1* rs10306135 (NERD vs NTA; OR: 1.73; p = 0.0080, pc = 0.046; NERD vs controls; OR: 1.65; p = 0.0081, pc = 0.046). In addition, these three SNPs were not in linkage disequilibrium in this population [rs3892408-rs5789 (r^2 = 0.003) and rs3892408-rs10306135 (r^2 = 0.001)].

Other SNPs in *CYSLTR1* rs320995, *PTGER1* rs3810253 and *PTGER1* rs3810255 showed significantly different distributions of genotypes and allele frequencies in NERD and control subjects, but not when NERD and NTA patients were compared.

We found three statistically significant associations following FDR multiple testing correction. Moreover, when using a more conservative correction such as Bonferroni, the associations of *ALOX15* rs3892408, *PTGS1* rs5789 and *PTGS1* rs10306135 remained statistically significant according to a dominant genetic model.

In order to further evaluate the possible functional effects of these three significant SNPs (rs3892408, rs5789 and rs10306135), we analyzed their association with the following clinical parameters: eosinophil count, eosinophil cationic protein and tryptase serum levels. However, we could not find any association between these three SNPs and changes in these clinical parameters between NERD and controls (data not shown).

We also analyzed the association of CNVs in *PTGS1, PTGS2, LTC4S, ALOX5* and *PTGER1–4* with NERD (Table 3). All control subjects had two copies of each gene analyzed. Regarding NERD patients, we identified significant differences between the different groups for *ALOX5*: NERD vs NTA (OR: 0.17; p = 0.010), NERD vs controls (OR: 0.03; p = 0.0001). We also found three NERD individuals with one copy of the *LTC4S* gene, although these differences were not significant compared with controls.

Discussion

NERD has long been the most highly studied model of NSAID hypersensitivity with a large number of association studies of genetic variants published in the last few years, which have mainly focused on genes related to the arachidonic acid pathway [17]. However, in many of these studies, the associations were inconsistently replicated and the number of gene variants was limited [17]. Genome wide association studies (GWAS) have also been carried out. The first GWAS was carried out in a small cohort of Korean NERD patients, where the authors found an association for SNPs in the *CEP68* gene and confirmed the association of several others [30]. More recently, another GWAS in Korean NERD patients showed the HLA-DPB1 rs1042151 polymorphism to be a putative genetic factor [31]. Nevertheless, there is a lack of genetic association studies for this clinical entity in Spanish populations. Recently, we have reported a genetic study involving variants of *CEP68* and HDR to NSAIDs for NIUA, NERD and blended phenotype patients [32]. We have also reported a study that found genetic variants involved in arachidonic acid pathways that were associated with NIUA [18]. These

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findings support that using a small number of SNPs selected based on evidence from previous studies of hypersensitivity reactions to NSAIDs and related phenotypes can be used to search for genetic variants associated with the related pathology NERD.

In this study, we found significant association between NERD and a number of SNPs in genes related to arachidonic acid pathways, including *ALOX15* and *PTGS1*.

These genes are important in the first steps of arachidonic acid metabolism (see Figure 1). This lipid can be metabolized by different pathways including lipoxygenases (5-LOX, 15-LOX and 12-LOX), PTGS (PTGS-1 and PTGS-2), cytochrome oxidases and nonenzymatic pathways [33].

Concerning the lipoxygenases pathway, *ALOX15* encodes 15-lipoxygenase (15-LOX), an iron metalloenzyme that catalyzes the generation of the proinflammatory metabolites eoxins [34] and lipoxin 15-hydroxyeicosatetraenoic acid (15-HETE), a precursor of active lipoxins and functional antagonists of CysLTs [14,35]. In presence of L-ASA, eosinophils activated from NERD patients generate higher levels of lipoxin 15-HETE and eoxin EXC4 [36]. Also, exhaled 15-HETE levels have been shown to be higher at baseline in NERD than in NTA subjects [37]. Thus, the activation of 15-LOX appears to be an important step in 15-HETE generation by ASA in NERD patients [38]. Moreover, 15-LOX catalyzes the main formation route of resolvins, lipid mediators characterized by their anti-inflammatory and proresolving properties [39,40]. Moreover, PTGS-2 has been shown to enhance resolvin formation under the ASA therapy [41]. In this context, SNPs in *ALOX15* could act by modulating eicosanoid generation in NERD patients. We found an association between the NERD and the *ALOX15* rs3892408 polymorphism, which gives rise to the missense amino acid substitution Val239Met located in a metal-binding protein domain [42]. Whether this association implies an impaired 15-LOX function in NERD patients requires further research. Recently, a haplotype comprising promoter polymorphisms of *ALOX15* was found to be associated with NERD and related to increased eosinophil infiltration in Korean patients [43]. Nevertheless, to our knowledge, this is the first study that shows an association between the nonsynonymous *ALOX15* rs3892408 polymorphism and NERD.

Population frequency data for the SNP rs3892408 are scarce. The data available on NCBI show a minor allele frequency of 0.40, similar to our reported a minor allele frequency in the control group of 0.48. Our findings show a significant association between this SNP and NERD patients (NERD vs NTA; OR:

Table 3. Copy number variations in *PTGS1*, *PTGS2*, *LTC4S*, *ALOX5* and *PTGER1-4* genes in controls, NERD and NTA subjects.

Gene	N° copies	NERD	NTA	Controls	NERD vs NTA		NERD vs controls	
					p-value	OR (95% CI)	p-value	OR (95% CI)
PTGS1	2 copies	250	260	315	NS		NS	
	1 copy	0	0	0				
PTGS2	2 copies	250	260	315	NS		NS	
	1 copy	0	0	0				
LTC4S	2 copies	247	258	315	NS		NS	
	1 copy	3	2	0				
ALOX5	2 copies	239	258	315	0.010	0.17 (0.0–0.8)	0.0001	0.03 (0.0–0.5)
	1 copy	11	2	0				
PTGER1	2 copies	250	257	315	NS		NS	
	1 copy	0	3	0				
PTGER2	2 copies	250	260	315	NS		NS	
	1 copy	0	0	0				
PTGER3	2 copies	250	260	315	NS		NS	
	1 copy	0	0	0				
PTGER4	2 copies	250	260	315	NS		NS	
	1 copy	0	0	0				

NERD: NSAIDs-exacerbated respiratory disease; NTA: NSAID-tolerant asthmatic patients; NS: Not significant; OR: Odds ratio.

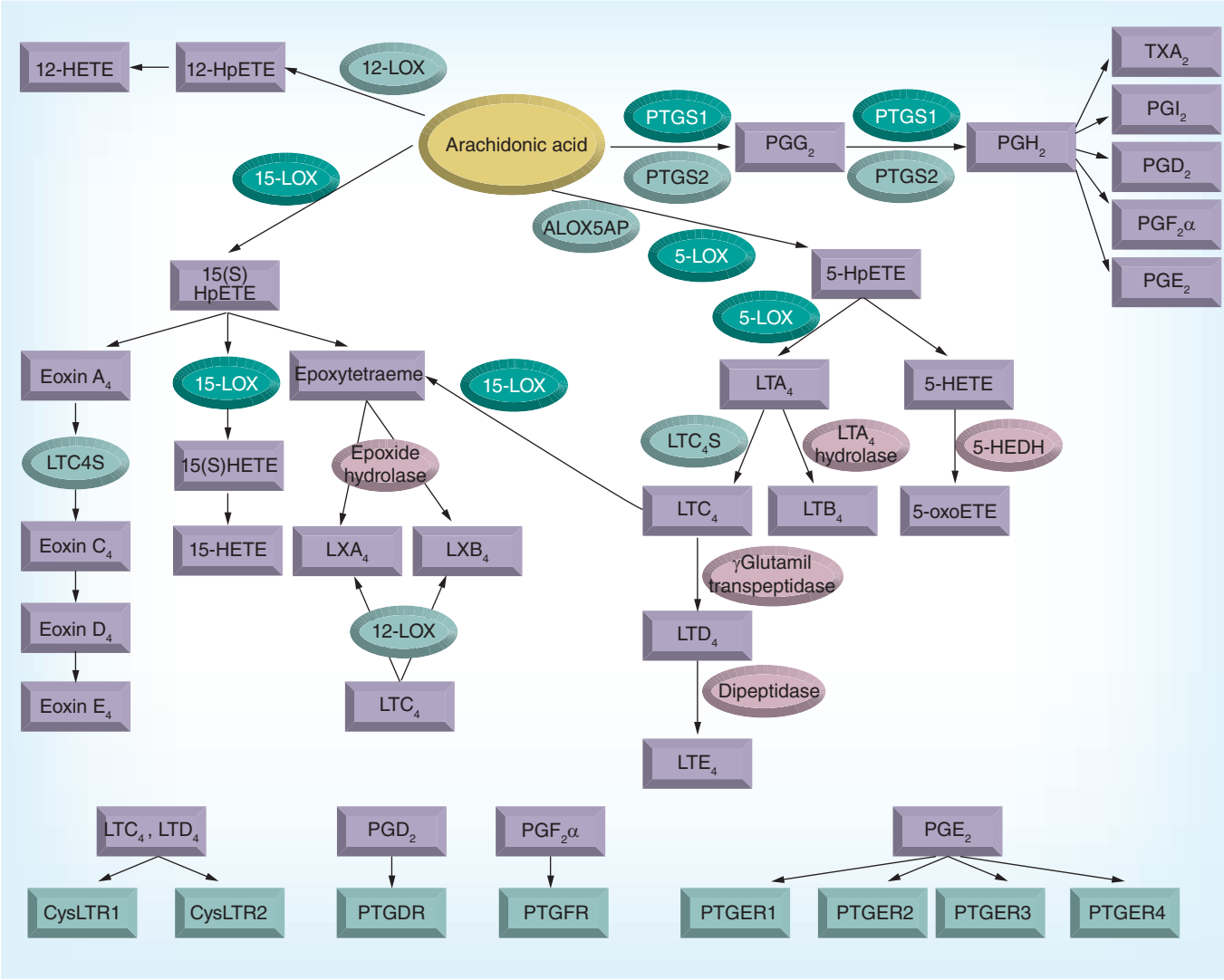

Figure 1. Arachidonic acid metabolic pathway: the metabolites involved in the NERD pathology are indicated in rectangles. The enzymes and receptors in which encoding genes have been analyzed in this study are indicated in light turquoise. The enzymes in which encoding genes have shown association with NERD in this study are indicated in dark turquoise.
NERD: NSAIDs-exacerbated respiratory disease.

2.49; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 2.83; p = 0.0001, pc = 0.0011); even adjusting for atopic status (NERD vs NTA; OR: 3.29; p = 0.0001, pc = 0.0011; NERD vs controls; OR: 3.48; p = 0.0001, pc = 0.0011). The H-W equilibrium is maintained in the NERD subjects (p = 0.430) and is slightly off for these patients when they are adjusted for atopic status (p = 0.032). These patients and controls have been genotyped by PCR using TaqMan allelic discrimination assays in triplicate, minimizing the likelihood of this being due to technical error. However, we are currently recruiting more NERD patients in order to validate these findings in a larger series of patients, as recommended by the NCI-NHGRI Working Group on Replication in Association Studies [44].

Although several SNPs in *ALOX5AP, ALOX5* and *ALOX12* have been shown to be related to chronic rhinosinusitis and/or asthma [45,46], we did not find statistical significant differences between NERD, NTA and healthy subjects.

Concerning the cyclooxygenases pathway, *PTGS1* encodes the PTGS-1 enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, prostacyclins and thromboxane. NSAIDs inhibit its activity, leading to CysLTs overproduction in NERD patients, which is considered the trigger of NSAID hypersensitivity [12]. The PTGS-1 isoform is constitutively expressed by almost all tissues involved in homeostasis [47]. Despite this fact, some studies have found a small increase in its expression under inflammatory conditions [48]. Human

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bronchial epithelial cells from NERD patients show a downregulation of PTGS-1-mRNA [49] and an altered regulation of *PTGS1* in nasal polyps from NERD patients has been suggested to contribute to the low levels of PGE_2 detected in these patients [50]. Moreover, the cyclooxygenase pathway results in the formation of other prostaglandins and higher concentration of PGD_2 in the sputum of NERD patients than in NTA and healthy subjects has been reported [51].

Therefore, *PTGS1* SNPs could contribute to the underlying mechanism of NERD. In this study, we found that the rs10306135 polymorphism was associated with NERD. This SNP is located close to the promoter region of *PTGS1*, and therefore may affect gene expression [52]. Nevertheless, there are no *in vitro* or *in vivo* functional studies evaluating the effect of this SNP.

For the rs5789 *PTGS1* polymorphism, we have found that the minor allele is more common in NERD patients than in NTA and control subjects. Interestingly, this allele has been shown to be related to a decrease of arachidonic acid metabolism in hemodynamic responses in the neurovascular system [53], although the effect of a decreased function in PTGS-1 caused by rs5789 has not been studied in NERD patients. Further functional studies are needed to elucidate the role of these SNPs in the underlying mechanism of NERD.

CysLTs contribute to inflammation and are effectors of the symptoms that characterize NERD patients [5]; therefore, we analyzed polymorphisms in the *LTC4S*, *CYSLTR1* and *CYSLTR2* genes. Although the SNP rs730012 in *LTC4S* has been shown to be associated with NERD in both Polish and Japanese populations [54,55], we found no association, in common with other studies in different ethnic groups [56,57]. The effects of CysLTs may be modulated by genetic variation in their receptors, supporting this hypothesis the polymorphism rs912278 in *CYSLTR2*, which affects the transcription and stability of *CYSLTR2* mRNA has been found to be associated with NERD in a Korean population [58]. Nevertheless, this association was not found in this study. With regard to prostaglandin receptors, associations have been found for variants in *PTGER2, PTGER3* and *PTGER4* in Japanese and Korean populations [59–61]. We analyzed these SNPs and other functional polymorphisms [62–64]; however, these associations were not found in our population. Considering the *PTGFR* rs3753380 SNP, our findings show that the minor allele is more frequent in NERD patients. This allele is related to a lower transcriptional activity of the *PTGFR* gene [64]. However, when we adjust for multiple comparisons, this association did not remain statistically significant.

Finally, we investigated CNVs in *PTGS1–2, LTC4S, ALOX5* and *PTGER1–4*. To the best of our knowledge, this is the first time that CNVs have been studied in NSAID hypersensitivity. We found an association between CNVs in *ALOX5* and NERD patients. 5-LOX catalyzes leukotriene and lipoxin production, playing a key role in pathogenesis of NERD [35]. Further studies are required to investigate the potential functional effect of the CNVs in the *ALOX5* gene found here.

These associations were not found in a previous study of Spanish NIUA patients, suggesting that NERD and NIUA may have different underlying mechanisms at the molecular level [18]. However, more studies including both larger sample sizes and more genetic variants are needed to further explore this hypothesis.

Conclusion

We found significant associations for common genetic variants in genes related to the arachidonic acid pathway in a well-phenotyped group of Spanish NERD patients. This is the first work evaluating both SNPs and CNVs in genes encoding enzymes and receptors from the arachidonic acid pathway for NSAIDs-hypersensitivity patients.

Future perspective

NSAIDs are one of the most highly used medicines and it is estimated that tens of millions of individuals worldwide consume them daily [65]. They are also the most frequent drugs involved in HDR [3]. Therefore, NSAID hypersensitivity constitutes an important health problem. Focusing on NERD, its prevalence reaches 14.89% among patients with severe asthma [66]. Currently, a drug provocation test is required for the accurate diagnosis of NERD, which has a number of drawbacks. Thus, knowledge of genetic markers that can provide information about susceptibility to this disorder may consequently help minimize patient risk by providing an alternative diagnosis tool. In this study, we report that certain genetic variants in genes involved in arachidonic acid metabolism are associated with NERD. Future studies will complement genetic studies by adding information on gene expression and methylation [67]. These advances will help us to unravel the full genetic basis of the disease, the relationship between genotype and phenotype, and to establish novel diagnostic techniques for HDRs to NSAIDs, an important step in the development of personalized medicine.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Results

- Significant associations were found between *ALOX15* SNP rs3892408 and *PTGS1* SNPs (rs10306135 and rs5789) and the risk of NSAIDs-exacerbated respiratory disease (NERD) in a Spanish population.
- Copy number variations (CNVs) were analyzed for *PTGS1*, *PTGS2*, *LTC4S*, *ALOX5*, *PTGER1*, *PTGER2*, *PTGER3* and *PTGER4* genes. This is the first time such work has been conducted for NERD. No CNVs were found in healthy control subjects.
- *ALOX5* CNVs occur in NERD subjects (4.6%) and NSAID-tolerant asthmatic patients (NTA; 0.8%), and there are statistically significant differences between NERD patients, NTA and controls. These CNVs are attributable to gene deletion.
- *LTC4S* CNVs are less frequent in NERD and NTA subjects and these differences were not significant compared with controls. These CNVs are attributable to gene deletion.

Conclusion

- This study shows several associations between common genetic variants, including SNPs and CNVs, in genes related to the arachidonic acid pathway and the risk of NSAIDs-exacerbated respiratory disease in a Spanish population.

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