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Human leukocyte antigen-G 3' untranslated region polymorphisms are associated with asthma severity

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ABSTRACT

Asthma is a genetically complex chronic inflammatory airway disorder, and according to disease pathogenesis, clinical manifestations may vary according to asthma severity. A gene region close to the human leukocyte antigen-G (*HLA-G*) gene was identified as an independent susceptibility marker for asthma. Considering that the HLA-G immune checkpoint molecule may modulate inflammation, we evaluated the diversity of the *HLA-G* 3′ untranslated region (3′UTR) in asthmatic patients stratified according to disease severity. We evaluate the entire *HLA-G* 3′UTR segment in 115 Brazilian patients stratified into mild ($n=29$), moderate ($n=21$) and severe asthmatics ($n=65$), and in 116 healthy individuals. *HLA-G* 3′UTR typing was performed using Sanger sequencing. The multiple comparisons among patients stratified according to disease severity revealed several associations; however, after Bonferroni's correction, the following results remained significant: i) the +3010C and +3142G alleles were overrepresented in mild asthma patients when compared to controls; ii) the +3010G and +3142C alleles were overrepresented in severe asthma patients in comparison to patients with mild asthma. In conclusion, the +3010C/G and +3142C/G *HLA-G* 3′UTR variation sites were differentially associated according to asthma severity.

1. Introduction

Asthma is a heterogeneous chronic inflammatory disease, exhibiting various phenotypes (Gauthier et al., 2015). Asthma prevalence is increasing worldwide, and it has been predicted that by the year 2025, 400 million people will develop asthma worldwide (Global Initiative for Asthma, 2017). According to the 2017 Update of the Global Initiative for Asthma (GINA), asthma severity can be classified into mild, moderate and severe (Global Initiative for Asthma, 2017).

Asthma severity is retrospectively evaluated based on the treatment necessary to control symptoms and according to exacerbations after several months of follow-up, which may change along time. According to GINA steps 1 and 2 guidelines, mild asthma is well controlled requiring the need of short acting bronchodilators alone (step 1) or low intensity daily treatment, including low dose inhaled corticosteroid (ICS) (step 2). Moderate asthma is asthma well controlled with step 3 treatment, requiring low dose of ICS plus long-acting beta2-agonist (ICS + LABA) as first line of therapy. Most patients with severe asthma needs high-dose ICS-LABA (Step 4) to avoid that disease becomes uncontrolled, and some patients require add-on therapies including anti-leukotrienes, anti-cholinergic agents, and therapy with anti-IgE (omalizumab) or anti-IL-5 (mepolizumab, reslizumab) monoclonal antibodies (Step 5) (Global Initiative for Asthma, 2017).

Mild asthma patients may present allergic response to allergens and an eosinophilic inflammation that is usually associated with a Th2 polarization profile (Holgate, 2012). Severe asthma patients may exhibit increased number of exacerbations, decreased response to corticosteroid therapy, neutrophilia with predominance of a Th17 cytokine profile (Bell et al., 2011) and a Th1 polarization, involving IFN-γ production accompanied with a reduced Th2 and Th17 response (Gauthier

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Table 1
Clinical and laboratory features of the asthmatic patients, stratified according to disease severity.

	Whole Group	Mild	Moderate	Severe	*P-value*
Age, mean (± SD)	45.8 (17.01)	40.2 (18.17)	42.9 (19.09)	49.2 (15.09)	NS[a]
Gender (%)					NS[b]
Female	82 (71.30%)	22 (75.86%))	17 (80.95%)	43 (66.15%)	
Male	33 (28.70%)%)	07 (24.14%)	04 (19.05%)	22 (33.85%)	
Skin Color					NS[b]
White	91 (79.13%)	24 (82.76%)	17 (80.95%)	50 (79.13%)	
Mulatto	13 (11.30%)	04 (13.79%)	02 (9.52%)	07 (11.30%)	
Black	11 (9.57%)	01 (3.45%)	02 (9.52%)	08 (9.57%)	
Atopy					NS[b]
Yes	83 (72.17%)	19 (65.52%)	16 (76.19%)	48 (73.85%)	
No	16 (13.91%)	01 (3.45%)	03 (14.29%)	12 (18.46%)	
N/A	16 (13.91%)	09 (31.03%)	02 (9.52%)	05 (7.69%)	
Skin Prick Test					NS[b]
Positive	74 (64.35%)	18 (62.07%)	16 (76.19%)	40 (61.54%)	
Negative	19 (16.52%)	01 (3.45%)	03 (14.29%)	15 (23.08%)	
N/A	22 (19.13%)	10 (34.48%)	02 (9.52%)	10 (15.38%)	
Total IgE, geometric mean (range)*	182.4 (7.06-6340.00)	324.3 (27.9-4396.0)	139.3 (7.06-3010.00)	179.1 (9.66-6340.00)	NS[a]
Spirometry values					
FVC (% predicted), mean (± SD)**	85.3 (19.70)	101.2 (13.40)	92.0 (13.70)	76.9 (18.90)	< 0.0001[a]
FEV1 (% predicted), mean (± SD)**	65.4 (22.70)	88.1 (12.90)	74.0 (16.30)	53.6(19.30)	< 0.0001[a]
FEF 25-75 (% predicted), mean (± SD)**	37.8 (25.90)	60.0 (20.90)	44.5 (20.80)	27.0 (22.90)	< 0.0001[a]
FVC/FEV1 (% observed), mean (± SD)**	61.2 (17.80)	74.2 (11.00)	67.2 (15.80)	53.9 (17.00)	< 0.0001[a]
Smoking habits					NS[b]
Yes	10 (8.70%)	02 (6.90%)	02 (9.52%)	06 (9.23%)	
No	75 (65.22%)	20 (68.97%)	17 (80.95%)	38 (58.46%)	
Ex-smoker	25 (27.69%)	05 (17.24%)	02 (9.52%)	18 (27.69%)	
N/A	05 (4.35%)	02 (6.90%)	0 (0.00%)	93 (4.62%)	

Abbreviations: SD: Standard deviation; FVC: Forced Vital Capacity; FEV1: Forced Expiratory Volume in the First Second; FEF 25–75%: Forced Expiratory Flow between 25% and 75% of the FVC; N/A: missing information in the medical records; NS: Non-significan.
Differences between groups were analyzed using the [a]Kruskal–Wallis test, followed by Dunn *post hoc* test, and the [b]chi-square test.
*This information was available for 64 patients in the medical records.
**This information was not available for 6 patients in the medical records.

et al., 2015; Raundhal et al., 2015).

As a heterogeneous genetically complex disorder, many immune and non-immune genes have been involved on asthma susceptibility, and the interaction of genetic predisposition with environment factors may account for the asthma phenotype (Kuhlen et al., 2014; Wenzel, 2012). Genome-wide association studies have disclosed several susceptibility genes associated with asthma, including *ADAM33* (Disintegrin and metalloproteinase domain-containing protein-33), *DPP10* (Inactive dipeptidyl peptidase-10), *PTGDR* (prostaglandin D receptor), *CTNNA* (Catenin alpha-3), *IL1RL1* (Interleukin 1 receptor-like 1), *PDE4D* (cAMP-specific 3′,5′-cyclic phosphodiesterase 4D), *TLE4* (Transducin-like enhancer protein 4), *DENND1B* (DENN domain-containing protein 1B), and the genes of the Major Histocompatibility Complex (MHC), including *HLA-DQ* and *HLA-DP* alleles (Lee et al., 2015). Besides the association with MHC class II genes, a study conducted on a large number of asthmatic patient together with their families identified the *HLA-G* gene region (6p21) as an independent susceptibility factor (Nicolae et al., 2005).

HLA-G is a non-classical histocompatibility class I molecule that plays a pivotal role on immune system regulation, acting as a checkpoint molecule. It is mainly expressed in the first and second trimester placental tissues, facilitating the maternal-fetal tolerance, inhibiting the cytotoxicity effects of T CD8+ and natural killer (NK) cells. However, HLA-G can expressed in several pathological conditions, such as cancer and viral infection, leading to the impairment of the immune response against tumor cells or pathogens (Morandi et al., 2016). Alternative splicing of the primary transcript may produce at least four membrane-bound (HLA-G1 to HLA-G4) and three soluble (HLA-G5 to HLA-G7) isoforms that modulate the immune system by interaction with inhibitory receptors (ILT-2 and ILT-4) present on the surface of several immune cells, including macrophages, monocytes, dendritic cells, T cells and NK cells (Donadi et al., 2011).

The *HLA-G* gene may be regulated by several transcriptional and post-transcriptional elements. Particularly, the 3′ untranslated region (3′UTR) exhibits several polymorphic sites that may influence the *HLA-G* expression profile by modifying mRNA stability and/or influencing the microRNAs (miRNA) binding to the mature mRNA (Rousseau et al., 2003; Tan et al., 2008; Yie et al., 2008). Individual patterns of HLA-G expression have been associated with *HLA-G* 3′UTR variation sites (Martelli-Palomino et al., 2013) and these variation sites have associated with many pathological situations, including autoimmune, chronic inflammatory and infectious disorders (Ciliao Alves et al., 2012; Dias et al., 2015; Donadi et al., 2011; Silva et al., 2013).

Considering that: i) HLA-G may modulate the severity of chronic inflammatory disorders (Morandi et al., 2016), ii) the inflammatory profile of asthmatic patients may be different according to asthma severity (Gauthier et al., 2015), and iii) a region close the *HLA-G* gene has been associated with susceptibility to asthma (Nicolae et al., 2005), we studied the role of *HLA-G* 3′UTR polymorphic sites and haplotypes, and their relationship with asthma severity.

2. Material and methods

2.1. Subjects

We studied 115 asthmatic patients (82 women), aged 9 to 82 years old (mean: 45.8 years, SD ± 17.0), selected from the Allergy Clinics of the University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil. Patients were stratified according to disease severity into mild ($n=29$), moderate ($n=21$) and severe asthmatics ($n=65$), according to the 2017 GINA guidelines (GINA, 2017).

Patients with severe asthma presented values of Forced Expiratory

Table 2
Allele frequency of variations sites observed at 3′ untranslated region (3′UTR) of *HLA-G* gene in asthmatic patients stratified according to disease.

Genotypes	C		W		ML		MD		SV		MD/SV		ML/MD	
and alleles	*n* (*n* = 116)	Freq.	*N* (*n* = 115)	Freq.	*n* (*n* = 29)	Freq.	*n* (*n* = 21)	Freq.	*n* (*n* = 65)	Freq.	*n* (*n* = 89)	Freq.	*n* (*n* = 50)	Freq.
14-bp-Del-Del	41	0.3534	35	0.3043	6	0.2069	7	0.3333	22	0.3385	29	0.3372	13	0.2600
14-bp-Del-Ins	52	0.4483	66	0.5739	20	0.6897	9	0.4286	37	0.5692	46	0.5349	29	0.5800
14-bp-Ins-Ins	23	0.1983	14	0.1217	3	0.1034	5	0.2381	6	0.0923	11	0.1279	8	0.1600
14-bp-Ins	98	0.4224	94	0.4087	26	0.4483	19	0.4524	49	0.3769	68	0.3820	45	0.4500
+3001C-C	114	0.4914	113	0.9826	28	0.9655	21	1.0000	64	0.9846	85	0.9884	49	0.9800
+3001C-T	2	0.0086	2	0.0174	1	0.0345	0	0.0000	1	0.0154	1	0.0116	1	0.0200
+3001T-T	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
+3001C	230	0.9914	228	0.9913	57	0.9828	42	1.0000	129	0.9923	177	0.9944	99	0.9900
+3003C-C	1	0.0043	3	0.0261	0	0.0000	1	0.0476	2	0.0308	3	0.0349	1	0.0200
+3003C-T	29	0.1250	17	0.1478	2	0.0690	3	0.1429	12	0.1846	15	0.1744	5	0.1000
+3003T-T	86	0.3707	94	0.8174	26	0.8966	17	0.8095	51	0.7846	68	0.7907	43	0.8600
+3003T	201	0.8664	207	0.9000	56	0.9655	37	0.8810	114	0.8769	157	0.8820	93	0.9300
+3010C-C	36	0.1552	42	0.3652	17	0.5862	9	0.4286	16	0.2462	25	0.2907	26	0.5200
+3010G-C	56	0.2414	42	0.3652	10	0.3448	5	0.2381	27	0.4154	32	0.3721	15	0.3000
+3010G-G	24	0.1034	20	0.1739	1	0.0345	7	0.3333	12	0.1846	19	0.2209	8	0.1600
+3010C	128	0.5517	137	0.5957	45	0.7759	23	0.5476	69	0.5308	98	0.5506	68	0.6800
+3027A-A	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
+3027C-A	13	0.0560	6	0.0522	0	0.0000	3	0.1429	3	0.0462	6	0.0698	3	0.0600
+3027C-C	103	0.4440	109	0.9478	29	1.0000	18	0.8571	62	0.9538	80	0.9302	47	0.9400
+3027C	219	0.9440	224	0.9739	58	1.0000	39	0.9286	127	0.9769	172	0.9663	97	0.9700
+3032G-G	116	0.5000	114	0.9913	29	1.0000	21	1.0000	64	0.9846	85	0.9884	50	1.0000
+3032G-C	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
+3032C-C	0	0.0000	1	0.0087	0	0.0000	0	0.0000	1	0.0154	1	0.0116	0	0.0000
+3032G	232	1.0000	228	0.9913	58	1.0000	42	1.0000	128	0.9846	176	0.9888	100	1.0000
+3035C-C	87	0.3750	82	0.7130	19	0.6552	16	0.7619	47	0.7231	63	0.7326	35	0.7000
+3035C-T	23	0.0991	31	0.2696	10	0.3448	5	0.2381	16	0.2462	21	0.2442	15	0.3000
+3035T-T	6	0.0259	1	0.0087	0	0.0000	0	0.0000	1	0.0154	1	0.0116	0	0.0000
+3035C	197	0.8491	197	0.8565	48	0.8276	37	0.8810	112	0.8615	149	0.8371	85	0.8500
+3121C-C	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
+3121T-C	1	0.0043	1	0.0087	0	0.0000	0	0.0000	1	0.0154	1	0.0116	0	0.0000
+3121T-T	115	0.4957	114	0.9913	29	1.0000	21	1.0000	64	0.9846	85	0.9884	50	1.0000
+3121T	231	0.9957	229	0.9957	58	1.0000	42	1.0000	129	0.9923	177	0.9944	100	1.0000
+3142C-C	24	0.1034	19	0.1652	1	0.0345	7	0.3333	11	0.1692	18	0.2093	8	0.1600
+3142C-G	56	0.2414	53	0.4609	11	0.3793	5	0.2381	37	0.5692	42	0.4884	16	0.3200
+3142G-G	36	0.1552	40	0.3478	15	0.5172	9	0.4286	16	0.2462	25	0.2907	24	0.4800
+3142G	128	0.5517	138	0.6000	45	0.7759	23	0.5476	70	0.5385	99	0.5562	68	0.6800
+3187A-A	66	0.2845	66	0.5739	21	0.7241	11	0.5238	34	0.5231	45	0.5233	32	0.6400
+3187A-G	36	0.1552	44	0.3826	8	0.2759	6	0.2857	30	0.4615	36	0.4186	14	0.2800
+3187G-G	14	0.0603	5	0.0435	0	0.0000	4	0.1905	1	0.0154	5	0.0581	4	0.0800
+3187A	168	0.7241	176	0.7652	50	0.8621	28	0.6667	98	0.7538	132	0.7416	78	0.7800
+3196C-C	62	0.2672	57	0.4957	13	0.4483	9	0.4286	35	0.5385	44	0.5116	22	0.4400
+3196C-G	45	0.1940	50	0.4348	14	0.4828	10	0.4762	26	0.4000	36	0.4186	24	0.4800
+3196G-G	9	0.0388	8	0.0696	2	0.0690	2	0.0952	4	0.0615	6	0.0698	4	0.0800
+3196C	169	0.7284	164	0.7130	40	0.6897	28	0.6667	96	0.7385	130	0.7303	68	0.6800
+3227A-A	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
+3227G-A	5	0.0216	7	0.0609	3	0.1034	0	0.0000	4	0.0615	4	0.0465	3	0.0600
+3227G-G	111	0.4784	106	0.9217	25	0.8621	21	1.0000	60	0.9231	81	0.9419	46	0.9200
+3227G	227	0.9784	223	0.9696	55	0.9483	42	1.0000	126	0.9692	174	0.9775	97	0.9700

Abbreviations: *HLA-G*: Human Leukocyte Antigen G; 14-bp-I: 14 pair bases Insertion; 14-bp-D: 14 pair bases Deletion; Freq.: frequency; C: healthy individuals; W: whole group of asthmatic patients; ML: mild asthmatic patients; MD: moderate asthmatic patients; SV: severe asthmatic patients; MD/SV: moderate and severe asthmatic patients group; ML/MD: mild and moderate asthmatic patients group.

Volume in One Second (FEV1) < 60%; Forced Expiratory Flow (FEF) between 25% and 75% of the Forced Vital Capacity (FVC) FEF 25–75% < 30%; VEF1/CVF < 70%; and severe exacerbations. Skin prick tests were carried out on patient forearm using a prick lancet, with a panel of inhalant allergenic extracts (FDA Allergenic, Rio de Janeiro, Brazil). Positive (histamine hydrochloride 10 mg/mL) and negative (saline solution) controls were included in all tests. Skin prick tests were considered to be positive when a wheal of ≥ 4 mm accompanied by erythema developed 15 min following application of the extract. Total IgE levels > 100 UI/mL or the positive result of the skin prick test were considered to classify allergic status. Demographic and clinical features, spirometry values, skin prick test results and total IgE levels are shown in Table 1.

A group of 116 healthy individuals from the same geographic region, presenting age and ethnic characteristics compatible to the studied patients, was also evaluated. This group is a sample of a larger dataset, in which the entire *HLA-G* variability was characterized in Brazil (Castelli et al., 2017) by using massive parallel sequencing procedures.

The study protocol was approved by the local Research Ethics Committee (Protocols #12398/2004 and 6323/2011), and all subjects or their legal guardians gave written informed consent to participate in the study.

2.2. Assignments of 3′UTR HLA-G variation sites

For patients, the identification of polymorphic sites at the *HLA-G* 3′UTR segment was performed using PCR-amplified DNA and Sanger sequencing, as previously reported (Castelli et al., 2010).

Table 3
Comparison of allele frequencies of the *HLA-G* 3′ untranslated region (3′UTR) polymorphisms. Significant results are highlightened in bold.

Allele	Comparisons	*P-value*	OR	95% CI
14-bp-Del-Ins	ML *versus* C	0.0232	2.7350	1.1485 to 6.5130
+3003C-T	ML *versus* C	0.0414	0.2222	0.0498 to 0.9925
	MD/ML *versus* C	0.0352	0.3333	0.1208 to 0.9198
+3010G-G	MD *versus* ML	0.0067	14.0000	1.5647 to 125.2615
	SV/MD *versus* ML	0.0029	7.9403	1.0133 to 62.2196
+3010G-C	MD/ML *versus* C	0.0397	0.4592	0.2267 to 0.9303
+3010C-C	ML *versus* C	0.0090	3.1481	1.3630 to 7.2716
	MD/ML *versus* C	0.0141	2.4074	1.2195 to 4.7525
	SV *versus* ML	0.0022	0.2305	0.0910 to 0.5841
	SV/MD *versus* ML	0.0069	0.2893	0.1208 to 0.6928
	SV *versus* MD/ML	0.0034	0.3014	0.1366 to 0.6651
+3010G	ML *versus* C	0.0025	0.3636	0.1860 to 0.7111
	MD/ML *versus C*	0.0384	0.5878	0.3586 to 0.9637
	SV *versus* ML	**0.0006***	3.4993	1.7122 to7.1517
	MD *versus* ML	0.0184	2.7960	1.1747 to 6.6551
	SV/MD *versus* ML	**0.0012***	3.3021	1.6543 to 6.5909
	SV *versus* MD/ML	0.0063	2.1647	1.2456 to 3.7622
+3010C	ML *versus* C	0.0025	2.7500	1.4063 to 5.3776
	MD/ML *versus C*	0.0384	1.7012	1.0377 to 2.7888
	SV *versus* ML	**0.0006***	0.2858	0.1398 to0.5840
	MD *versus* ML	0.0184	0.3577	0.1503 to 0.8513
	SV/MD *versus ML*	**0.0012***	0.3021	0.1517 to 0.6045
	SV *versus* MD/ML	0.0063	0,4620	0.2658 to 0.8028
+3142C-C	MD *versus* ML	0.0067	14.0000	1.5647 to 125.2615
+3142C-G	SV *versus* MD	0.0116	4.2286	1.3828 to 12.9313
	SV *versus* MD/ML	0.0089	2.8080	1.2991 to 6.0695
+3142G-G	ML *versus* C	0.0499	2.3810	1.0405 to 5.4482
	SV *versus* ML	0.0166	0.3048	0.1213 to 0.7658
	SV/MD *versus* ML	0.0413	0.3825	0.1612 to 0.9079
	SV *versus* MD/ML	0.0108	0.3537	0.1603 to 0.7805
+3142C	ML *versus* C	**0.0006***	0.1270	0.0296 to 0.5451
	SV *versus* ML	0.0038	2.6939	1.3468 to 5.3884
	MD *versus* ML	0.0306	2.5373	1.0778 to 5.9728
	SV/MD *versus ML*	**0.0006***	8.4460	1.9439 to 36.6940
	SV *versus* MD/ML	0.0310	1.8214	1.0577 to 3.1367
+3142G	ML *versus* C	**0.0006***	7.8750	1.8347 to 33.8016
	SV *versus* ML	0.0038	0.4309	0.2203 to 0.7658
	MD *versus* ML	0.0306	0.4035	0.1712 to0.9511
	SV/MD *versus ML*	**0.0006***	0.1184	0.0273 to 0.5144
	SV *versus* MD/ML	0.0310	0.5490	0.3188 to 0.9455
+3187G-G	SV *versus* MD	0.0118	0.0664	0.0070 to 0.6336
+3187G	ML versus C	0.0403	0.4200	0.1887 to 0.9347
	MD versus ML	0.0276	3.1250	1.1679 to 8.3614
	SV/MD versus ML	0.0493	2.2820	1.0060 to 5.1770
+3187A	ML versus C	0.0403	2.3810	1.0699 to 5.2987
	MD versus ML	0.0276	0.3200	0.1196 to 0.8562
	SV/MD versus ML	0.0493	0.4383	0.1931 to 0.9944

Abbreviations: *HLA-G:* Human Antigen Leukocyte-G; C: healthy individuals; W: whole group of asthmatic patients; ML: mild asthmatic patients; MD: moderate asthmatic patients; SV: severe asthmatic patients; MD/SV: moderate and severe asthmatic patients group; ML/MD: mild and moderate asthmatic patients group; OR: odds ratio; 95% CI: 95% Confidence Interval; for alleles.
*Significant results after Bonferroni's correction for multiple tests involving genotypes and alleles highlighting in bold: αc = 0.0014 for genotypes and αc = 0.0020.

2.3. Statistical analysis

Differences among patient characteristics according to asthma severity were performed using the Kruskal–Wallis test followed by the Dunn *post hoc* test. Total IgE levels and spirometry values were compared by the *Chi-square* test using the GraphPad Prism 5.0 software (Table 1).

Allele and genotype frequencies at each variable site were estimated by direct counting. Adherences of genotypic proportions to Hardy-Weinberg equilibrium (HWE) expectations were estimated by the exact test of Guo and Thompson, using the ARLEQUIN 3.5.1.2 software (Excoffier and Lischer, 2010). Linkage disequilibrium (LD) between each pair of *loci* was evaluated for each group by means of a likelihood ratio test of linkage disequilibrium, implemented by HAPLOVIEW program (Barrett et al., 2005).

Data for control subjects have been previously phased into extended haplotypes as described elsewhere (Castelli et al., 2017). For patients, given the positive LD between alleles, but unknown gametic phase, haplotypes were reconstructed for each individual using a coalescence-based method implemented by the PHASE v2 software (Stephens et al., 2001). Ten independent runs were performed and the following configurations were used: random seed values for each run, number of interactions set to 1000, thinning interval set to 1, and burn-in value set to 100.

We constructed 2 × 2 contingency tables to compare the frequency of each allele, genotype or haplotype between patients and controls. *P*-values were calculated by the two-sided Fisher exact test, using the GraphPad Prism 5.0 software, which was also used to estimate the Odds Ratio (OR) and its 95% Confidence Interval (95%CI). For all analyses, *P*-values were considered to be significant at a 0.05 significance level ($\alpha = 0.05$). The correction for multiple tests was performed using the Bonferroni procedure, which resulted in adjusted significance levels of $\alpha_c = 0.0014$ for genotypes, $\alpha_c = 0.0020$ for alleles and $\alpha_c = 0.0036$ for haplotypes, according to the equation $\alpha_c = 0.05/n$, in which n corresponds to the total number of genotypes ($n = 36$), alleles ($n = 24$) or haplotypes ($n = 14$) analyzed. The OR was used as association measure between multiple comparisons, for which an OR ≥ 1.0 was associated with susceptibility to disease development and an OR ≤ 1.0 conferred protection against disease development.

3. Results

Considering patients and controls, we observed twelve variation sites along the *HLA-G* 3′UTR segment, including the 14-bp-Del/Ins (rs66554220) and the following single nucleotide polymorphisms (SNPs): +3001C/T (rs567747015), +3003T/C (rs115689421), +3010C/G (rs116152775), +3027C/A (rs115810666), +3032G/C (rs146339774), +3035C/T (rs115100128), +3121T/C (rs138249160), +3142G/C (rs115928989), +3187A/G (rs114317070), +3196C/G (rs115045214) and +3227G/A (rs1233331).

To evaluate whether 3′UTR polymorphisms were associated with asthma and disease severity, we compared allele, genotype and haplotype frequencies of the whole group of asthmatic patients with healthy controls. Subgroups of asthmatic patients, stratified according to disease severity (mild, moderate, severe asthma, and combined mild/moderate and moderate/severe asthma) were compared to each other and with healthy controls.

The results revealed that most of the studied polymorphisms fit the Hardy-Weinberg equilibrium expectations (data not shown), except for the following polymorphisms: i) the +3032G/C in the whole asthma group ($P = 0.0045$), in patients with severe asthma ($P = 0.0077$), and in the moderate/severe asthma group ($P = 0.0058$); and ii) the +3010C/G in the patients with moderate asthma ($P = 0.0251$) and in the mild/moderate asthma group ($P = 0.0469$), exhibiting a deficit in heterozygosis for these variation sites.

Allele and genotype frequencies of all these polymorphic sites evaluated in this study are shown in Table 2. After allele, genotype and haplotype multiple comparisons, none of the *HLA-G* 3′UTR polymorphic sites was associated with asthma *per se*. In contrast, the comparisons of patients stratified according to disease severity revealed several associations as shown in Table 3; however, few associations remained significant after applying the Bonferroni's correction. Compared to healthy controls, the +3142G allele was overrepresented in mild asthma ($P = 0.0006$, OR = 7.8750, 95%IC = 1.8340–33.8016). Regarding the comparisons among asthma subsets, we observed that compared to mild asthma: i) the +3010G allele was overrepresented in patients with severe asthma ($P = 0.0006$, OR = 3.4993, 95% IC = 1.7122 to 1.517) and in patients with moderate/severe asthma

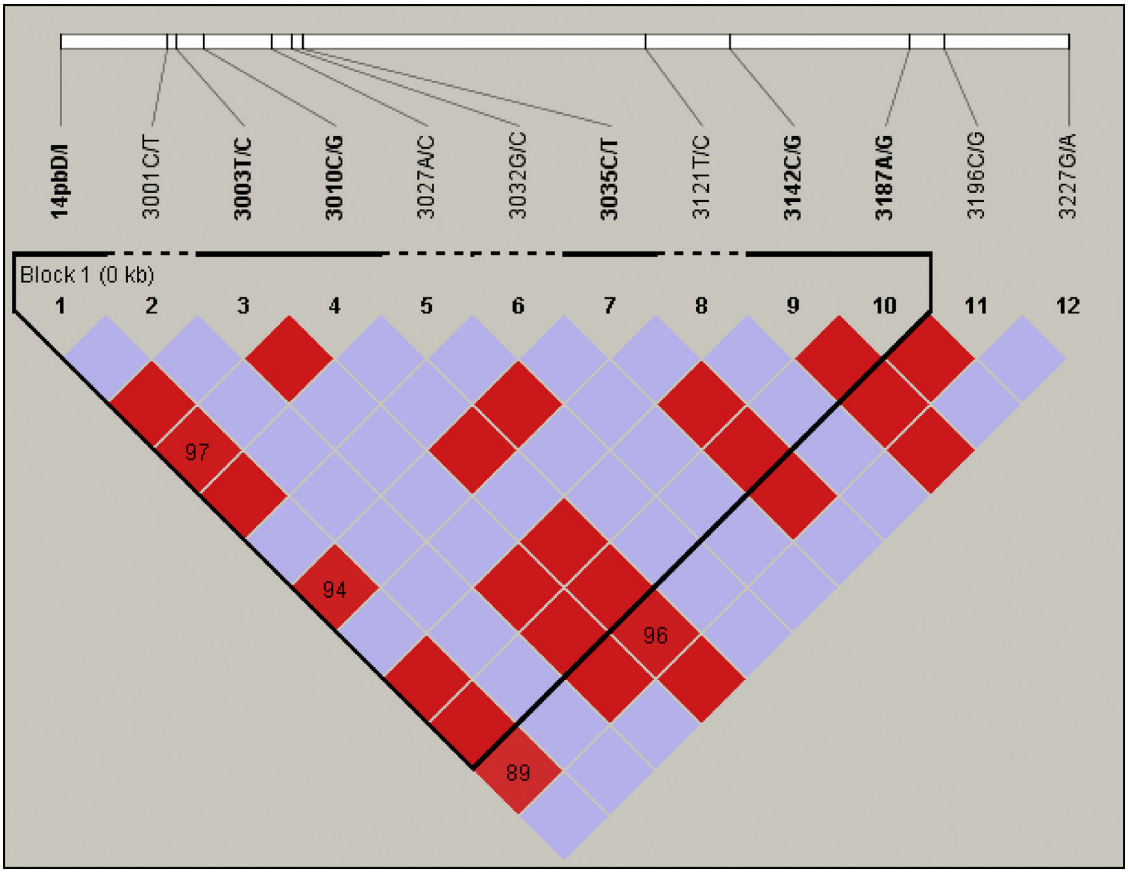

Fig. 1. Linkage disequilibrium (*D*′) plot of the *HLA-G* 3′UTR segment in the whole asthmatic patient group. The Haploview program generated the images. Areas in dark red indicate strong LD (LOD ≥ 2, $D' = 1$), shades of pink indicate moderate LD (LOD ≥ 2, $D' < 1$), blue indicates weak LD (LOD < 2, $D' = 1$), and white indicates no LD (LOD < 2, $D' < 1$). The haplotype blocks were defined by the confidence interval method implemented by Haploview (Barrett et al., 2005). *D*′ values different from 1.00 are represented inside the squares as percentages. LOD, log of the odds; *D*′, pairwise correlation between SNPs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
Haplotype frequencies in patients with asthma stratified according to severity of disease and in healthy individuals.

Haplotype	3′UTR *HLA-G* variation sites												Groups											
													C		W		ML		MD		SV		MD/SV		ML/MD	
													n	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.
	14-bp	3001	3003	3010	3027	3032	3035	3121	3142	3187	3196	3227	*2n=232*		*2n=230*		*2n=58*		*2n=42*		*2n=130*		*2n=172*		*2n=100*	
UTR-1	Del	C	T	G	C	G	C	T	C	G	C	G	64	**0.2759***	54	0.2348	8	**0.1379***	14	**0.3333***	32	0.2462	46	**0.2674***	22	0.2200
UTR-2	Ins	C	T	C	C	G	C	T	G	A	G	G	63	0.2716	61	0.2652	17	0.2931	14	0.3333	30	0.2308	44	0.2558	31	0.3100
UTR-3	Del	C	T	C	C	G	C	T	G	A	C	G	30	**0.1293***	39	0.1696	17	**0.2931***	4	**0.0952***	18	0.1385	22	**0.1279***	21	0.2100
UTR-4	Del	C	C	G	C	G	C	T	C	A	C	G	31	0.1336	23	0.1000	2	0.0345	5	0.1190	16	0.1231	21	0.1221	7	0.0700
UTR-5	Ins	C	T	C	C	G	T	T	G	A	C	G	19	0.0819	23	0.1000	8	0.1379	2	0.0476	13	0.1000	15	0.0872	10	0.1000
UTR-6	Del	C	T	G	C	G	C	T	C	A	C	G	4	0.0172	6	0.0261	0	0.0000	0	0.0000	6	0.0462	6	0.0349	0	0.0000
UTR-7	Ins	C	T	C	A	G	T	T	G	A	C	G	13	0.0560	6	0.0261	0	0.0000	3	0.0714	3	0.0231	6	0.0349	3	0.0300
UTR-8	Ins	C	T	G	C	G	C	T	G	A	G	G	0	0.0000	1	0.0043	0	0.0000	0	0.0000	1	0.0077	1	0.0058	0	0.0000
UTR-10	Del	C	T	C	C	G	C	T	G	A	G	G	0	0.0000	4	0.0174	1	0.0172	0	0.0000	3	0.0231	3	0.0174	1	0.0100
UTR-13	Del	C	T	C	C	G	T	T	G	A	C	G	0	0.0000	1	0.0043	1	0.0172	0	0.0000	0	0.0000	0	0.0000	1	0.0100
UTR-17	Ins	T	T	C	C	G	T	T	G	A	C	G	2	0.0086	2	0.0087	1	0.0172	0	0.0000	1	0.0077	1	0.0058	1	0.0100
UTR-18	Del	C	T	G	C	G	C	T	C	A	C	A	5	0.0216	7	0.0304	3	0.0517	0	0.0000	4	0.0308	4	0.0233	3	0.0300
UTR-20	Del	C	T	G	C	C	C	T	C	A	C	G	0	0.0000	2	0.0087	0	0.0000	0	0.0000	2	0.0154	1	0.0058	0	0.0000
UTR-44	Ins	C	T	C	C	G	T	C	G	A	C	G	1	0.0043	1	0.0043	0	0.0000	0	0.0000	1	0.0077	2	0.0116	0	0.0000

($P = 0.0012$, OR = 3.3021, 95%IC = 1.6543–6.5909), and ii) the +3142G allele was underrepresented in patients with moderate/severe asthma group ($P = 0.0006$, OR = 0.1184, 95%IC = 0.0273 to 0.5144).

Overall, positive linkage disequilibrium (LD) was observed for most *loci* in patients (Fig. 1), allowing the application of computational haplotype inference procedures. Haplotypes were inferred by PHASE in all samples with an average probability of 1.0. Fourteen different *HLA-G* 3′UTR haplotypes were observed in the total sample, and about 50% of these haplotypes presented frequencies higher than or equal to 2% (Table 4). Haplotypes were named according the nomenclature previously proposed by our group (Castelli et al., 2017 2010). Although haplotype associations were observed as shown in Table 4, significance was lost after applying the Bonferroni correction for multiple comparisons ($\alpha_c = 0.0036$).

4. Discussion

The regulatory regions of the *HLA-G* gene present several polymorphic sites, potentially related to protein production, which may contribute to susceptibility to or protection against inflammatory and

non-inflammatory disorders. In fact, differential frequencies of polymorphic sites at *HLA-G* 3′UTR have been reported in autoimmune diseases, organ transplantation outcome, viral infections, parasitic disorders and cancer, among other diseases (Ciliao Alves et al., 2012; Dias et al., 2015; Donadi et al., 2011; Silva et al., 2013). Few studies have evaluated the association of asthma with the *HLA-G* regulatory region polymorphic sites (Nicolae et al., 2005; Tan et al., 2008; Zheng et al., 2010), however, none of these studies evaluated the entire *HLA-G* 3′UTR segment in asthmatic patients stratified according to disease severity.

A genome-wide study reported that the promoter *HLA-G* -964G-G genotype conferred susceptibility to asthma development in children born from asthmatic mothers, while the -964A-A genotype was associated with susceptibility to asthma development in children born from non-asthmatic mothers (Nicolae et al., 2005). Regarding the *HLA-G* 3′UTR segment, few studies evaluated only the 14bp-Del/Ins (Tan et al., 2008; Zheng et al., 2010) and the +3142G/C (Nicodemus-Johnson et al., 2013; Tan et al., 2008) variation sites. The evaluation of the entire *HLA-G* 3′UTR, as performed in this study, revealed several associations; however, only few remained significant after the correction for multiple comparisons, and these results were primarily associated with the +3010C/G (rs116152775) and +3142G/C (rs115928989) variation sites. Compared to healthy controls, the +3142G allele was associated to mild asthma development, and comparisons among asthma subgroups revealed that +3010G and the +3142C alleles were overrepresented in patients with moderate or severe asthma when compared to mild asthma. In addition, the evaluation of the polymorphic sites observed at the *HLA-G* 3′UTR did not reveal association with asthma *per se*.

The *HLA-G* +3142G has been described as protective allele for asthmatic children born from asthmatic mothers, while the *HLA-G* +3142C allele was associated with susceptibility to asthma in children born from non-asthmatic mothers (Tan et al., 2008). Noteworthy, these authors also identified by functional studies that *HLA-G* +3142G allele was associated with decreased HLA-G expression, due to the local action of specific miRNAs, including miR-148a, mIr-148b and miR-152 (Tan et al., 2008). These data corroborate another study which reported that adult asthmatic patients presenting the +3142 G allele exhibited lower soluble levels of HLA-G (sHLA-G) in bronchoalveolar fluid when compared to the +3142C allele (Nicodemus-Johnson et al., 2013). In contrast, the evaluation of the differentially expressed miRNAs in bronchoalveolar fluid or bronchial epithelial cells obtained from asthmatic patients stratified according to disease severity disclosed no differential expression of the miR152 family (Jardim et al., 2012; Levänen et al., 2013; Martinez-Nunez et al., 2014). Regarding the association between protein expression and the variation sites at the +3142G/C polymorphism, healthy individuals bearing the +3142G allele and +3142G-G genotype exhibited decreased plasma levels of soluble HLA-G (sHLA-G) (Martelli-Palomino et al., 2013). In asthmatic patients stratified according to disease severity, the sHLA-G levels were increased in mild asthma (White et al., 2010) and decreased in severe asthma (Rizzo et al., 2009). Then, considering that: i) the +3142G allele was associated with lower expression of sHLA-G (Nicodemus-Johnson et al., 2013; Tan et al., 2008) due miR152 local action; ii) the +3142G is a susceptible allele to mild asthma development, as observed in this study; iii) increased levels of sHLA-G have been observed in patients with mild asthma (White et al., 2010); and iv) miRNAs that can modulate sHLA-G production are not observed in mild asthma (Haj-Salem et al., 2015; Jardim et al., 2012; Levänen et al., 2013), we may infer that the high HLA-G expression in mild asthma may be due absence or low function of miRNAs that target the *HLA-G* 3′UTR +3142G/C variation site.

The *HLA-G* +3010C/G variation site has not been functionally evaluated in terms of mRNA stability or miRNA regulation; however, *in silico* studies have shown that the +3010G allele may be targeted by four miRNAs (miR-5703, miR-193a-5p, miR-5001-5p and miR6510-5p) and the +3010C by miR-5787 (Porto et al., 2015). The evaluation of the differentially expressed miRNAs in bronchoalveolar fluid or bronchial epithelial cells obtained from asthmatic patients stratified according to disease severity disclosed no differential expression of these miRNAs (Haj-Salem et al., 2015; Jardim et al., 2012; Levänen et al., 2013; Martinez-Nunez et al., 2014). In our study, the +3010G allele was a susceptibility factor to severe asthma development. Although, this allele, plus with the +3010GG genotype, have been associated with high sHLA-G levels in healthy individuals (Martelli-Palomino et al., 2013), the sHLA-G levels in severe asthmatic patients has been described as decreased (Rizzo et al., 2009), indicating that other transcriptional or posttranscriptional mechanisms may be involved on the pathogenesis of severe asthma besides miRNA level regulation.

Considering that the *HLA-G* 3′UTR variable sites are in linkage disequilibrium, as shown by our study (Fig. 1) as well as by others (Castelli et al., 2011; Sabbagh et al., 2014), we further evaluated the role of the ensemble of these variable sites (3′UTR haplotypes) on susceptibility to asthma and on asthma severity. Although haplotype associations were observed, significance was lost after applying the Bonferroni correction for multiple tests, probably due to the small size of the subgroups. Similarly, variation sites observed at the *HLA-G* promoter region are also in linkage disequilibrium with those at the 3′UTR (Castelli et al., 2011; Sabbagh et al., 2014). Noteworthy, the *HLA-G* 3′UTR +3142G/C and the promoter -964C/G variation sites were in linkage disequilibrium in asthmatic children (Nicolae et al., 2005), corroborating the influence of other variation sites along the *HLA-G* regulatory regions in asthma pathogenesis.

Concluding, the study of the 3′UTR segment of the *HLA-G* gene revealed that the 3010C/G and +3142G/C variation sites were differentially associated with asthma severity.

Conflict of interest

The authors declare absence conflict of interest regarding this paper publication.

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