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HLA-G coding region polymorphism is skewed in Autistic Spectrum Disorders

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Running title: *HLA-G isoforms in ASD*

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ABSTRACT

Different isoforms of HLA-G protein are endowed with a differential ability to induce allogenic tolerance during pregnancy. As prenatal immune activation is suggested to play a role in the onset of autistic spectrum disorders (ASD), we evaluated HLA *G*01:01-*01:06* allelic polymorphism in a cohort of Italian children affected by ASD (N=111) their mothers (N=81), and their healthy siblings (N=39). DNA sequencing analysis of *HLA-G* exon 2, 3 and 4 was used to obtain *HLA-G* allelic frequencies; alleles distribution was compared with that of two control groups of Caucasoid couples of multiparous women and their partners from Brazil and Denmark. *HLA-G* distribution was significantly different in ASD children compared to both control groups (Brazilian $p_c=1x10^{-4}$; Danish $p_c=1x10^{-3}$). Since *HLA-G* distribution was similar in the two control groups, their data were pooled. Results indicated that *HLA-G*01:01* was significantly less frequent ($p_c=1x10^{-4}$; OR:0.5, 95%CI: 0.3-0.7) whereas *HLA-G*01:05N* was significantly more frequent ($p_c=2x10^{-3}$; OR:7.3, 95%CI: 2.4-26.6) in ASD children compared to combined controls. Finally, no clear pattern emerged when *HLA-G* allelic distribution was analyzed in healthy sibs. Notably, *HLA-G* allelic distribution found in ASD mothers was similar to that observed in the control subgroup of women with recurrent miscarriages, whilst it was significantly different compared to women without miscarriages ($p_c=6x10^{-4}$ df=12). Since *HLA-G*01:01* is associated with the elicitation of KIR-mediated tolerogenic responses and *HLA-G*01:05N* correlates with NK cells activation, results herein indicate that an immune activating milieu during pregnancy is more likely observed in association with the development of ASD, similarly to what occurs in women with recurrent miscarriages.

Highlight: *A skewing of HLA-G alleles, resulting in an immune activating milieu during pregnancy, is observed in the context of ASD*

1. INTRODUCTION

An uncontrolled immune response is suspected to impair the central nervous system (CNS) development as observed in children with a diagnosis of Autism Spectrum Disorders (ASD) (Saresella et al., 2009; Westover et al., 2011; Onore et al., 2013). In line with this, a maternal immune attack on foetal tissues is suggested to be involved in the pathogenesis of ASD (Warren et al., 1996). This hypothesis is reinforced by the observation that autoantibodies (indicating an adaptive immune process) targeting neuronal components of the foetal brain, are observed in a subset of mothers of ASD patients (Braunschweig et al., 2013, Croen et al., 2008; Patterson, 2011; Singer et al., 2008).

Natural killer (NK) cells are effectors of innate immunity that are present in the peripheral blood and, during pregnancy, can also be identified within the uterine mucosa, at the foetal/maternal interface. NK cells are characterized by the expression of a receptor family (KIR) that interact with classical HLA-C and non-classical HLA-G molecules on foetal trophoblast. The net effect of these interactions determines whether NK will or will not be activated. A skewing of the KIR-HLA complexes, in which activating molecules prevail, was recently shown in ASD children (Torres et al., 2012a) and their mothers (Guerini et al., 2014). HLA-G, in particular, is a non-classical HLA Class Ib molecule expressed by the placental and decidual trophoblast cells (Yelavarthi et al., 1991) as well as by foetal cells (Van Wijk et al., 2001), which is responsible for the induction of allogenic tolerance during pregnancy. The immunomodulatory functions of HLA-G are mediated by the interaction with KIR receptors on NK cells, including KIR2DL and ILT-2 (Favier et al., 2010); these interactions lead to suppression of T cell lymphocyte proliferation and stimulation of interferon (IFN)-c production by uterine NK cells (UNK), with the net effect of favouring foetal tolerance (Akhter et al., 2012, Matter and Sharif, 2013, Dadelszen, 2008). Besides being expressed on placental cell surface, HLA-G molecules can also be secreted. Soluble HLA-G (sHLA-G) induces the apoptosis of both activated NK and CD8 T-cells and a decreased amount of sHLA-G is seen in serum of women undergoing recurrent spontaneous abortions (Hviid et al., 2002). These observations suggest that the HLA-G/NK interaction at the maternal–foetal interface is critical in determining the pregnancy outcome .

Different HLA-G protein isoforms were recently identified; in fact, four membrane-bound HLA-G isoforms (G1, G2, G3, and G4) and three different soluble forms (G5, G6 and G7) -resulting by alternative splicing and very few amino acid polymorphisms (Castelli et al., 2014)- can be distinguished. To date, 44 *HLA-G* alleles encoding 14 distinct functional proteins have been described. Nevertheless, only four variation sites in the coding region of the *HLA-G* locus are related to the amino acid exchange frequently found in worldwide populations: (i) the +292 A/T SNP at exon 2 (codon 31), exchanging a threonine for serine in the *G*01:03* allele; (ii) the +755 C/A SNP at exon 3 (codon 110), exchanging leucine/isoleucine in the *G*01:04* allele group; (iii) the DeltaC deletion at exon 3 in the G*01:05N allele; and (iv) the +1799 C/T SNP (codon 258), exchanging threonine/methionine in the *G*01:06* allele (Donadi et al., 2011). These isoforms are functionally different, as they are endowed with a differential ability to induce tolerance. Finally, a nucleotide insertion of 14 bp in the regulatory 3'UTR region of *HLA-G* gene (*HLA-G*14bp+*) is shown to reduce tolerogenic activity (Christiansen et al., 2012; Hylenius et al., 2004), and suggested to be involved in ASD development (Guerini et al., 2015).

We evaluated *HLA G*01:01-*01:06* allelic polymorphisms in a well-characterized cohort of Italian children affected by ASD and their relatives to investigate whether specific *HLA-G* alleles, which might play a role in ASD-associated immune activation, can be identified.

2. MATERIALS and METHODS

2.1 Patients and controls

A total of 111 ASD children (92 males/19 females; mean age: 8.7± 4.4 years) born in peninsular Italy and of Italian descent, 81 of their mothers, and 39 of their healthy siblings (13 males/26 females; mean age: 11± 5.9 years) were enrolled at the Don Gnocchi Foundation, at the C. Mondino National Neurological Institute and at the ANFFAS (National Association of the Families of People with Intellectual and/or Relational Disability).

In the 3 months preceding study enrollment, all ASD children underwent diagnostic evaluation to establish whether they could be included in the protocol. Psychiatric, neurological, and neuropsychological exams (covering, in particular, the domains of social interaction, imaginative play and communication) were administered by expert child neuropsychiatrists (see Guerini et al., 2015 for details). The Italian version of the Autism Diagnostic Interview Revised and the Childhood Autism Rating Scale were used to assess in detail specific symptoms of the autistic spectrum. In addition the Leiter-R scale, the Weschler Intelligence Scales, and Raven's Progressive Matrices, were used to assess global cognitive functioning.

The only inclusion criterion was to fit the DSM-5 (American Psychiatric Association, 2013) diagnostic criteria for Autism Spectrum Disorder. Exclusion criteria were (i) the presence of an etiologically defined disorder typically presenting with ASD features (including Rett Syndrome, PKU or Tuberous Sclerosis) (Chiappedi et al., 2010) and (ii) the presence of a neurodevelopmental psychiatric disorder without ASD (such as specific language or learning disorders, obsessive compulsive disorder, selective mutism, schizophrenia and intellectual disability).

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and has been approved by the local ethics committee. Written informed consent was obtained from all parents / legal guardians prior to inclusion in the

study and when possible, with respect to their age and functioning, a written informed consent was obtained also from children. All participants were volunteers and the possibility to obtain diagnostic and therapeutic interventions was not contingent upon participation in the study.

In order to avoid biases due to HLA-G molecules that may be involved in pregnancy-related disorders, control data were obtained by published datasets of two different cohorts of multiparous Caucasoid subjects. Thus, HLA-G allelic frequencies from 167 Brazilian individuals (Nardi et al., 2012)(Pop 1) and from 93 Danish individuals (Hviid et al, 2002)(Pop 2) were used as comparisons. Finally, the HLA-G allelic frequencies observed in our ASD mothers were compared to those obtained in two subgroups of these cohorts: (i) 98 women with recurrent miscarriages from Pop 1and Pop 2 ; (ii) 130 women without miscarriage from Pop 1 and Pop 2.

2.2 HLA-G Genotyping

Saliva samples or peripheral whole blood were collected from all enrolled subjects and stored at -20°C until processing. Genomic DNA was isolated from peripheral blood using standard phenol/chloroform procedure and from saliva using the ORAgene-DNA kits (DNA Genotek, Ottawa, Canada) following manufacturer's instructions. DNA concentration and quality were assessed with Qubit™ 3 fluorometer (Thermo Fisher Scientific, USA).

HLA-G alleles are normally defined based upon polymorphisms located in exon 2 and exon 3 of the *HLA-G* gene. PCR amplifications of exon 2 and 3 were performed using the following two sets of primers: exon 2: HLAGEX2A (5'-GGGTCGGGCGGGTCTCAA-3') and BHLAGEX2 (5'-GGCCTCGCTCTGGTTGTAG-3'); exon 3: 5HLGIN2 (5'-CCCAGACCCTCTACCTGGGAGA-3'); GI3/3 (5'GGCCAGGCTGAGAGGTCTACAGGAGATCA-3') (Hylenius et al, 2004). PCR products were a fragment of 303bp for exon 2 and a fragment of 343bp of exon 3. For the amplification of both exon 2 and exon 3 the PCR conditions were: a final volume of 24 ul containing 2.5 U of @Taq polymerase and 2.5 ul of 10X Buffer (EuroClone s.p.a, Italy), 0.75 ul of 10 mM dNTPs,

0.75 ul of 1.5 mM $MgCl_2$, 10 picomoles of each primer and 13.5 ul of ddH_2O. 250ng of DNA isolated from blood or from saliva were used in each PCR experiment. Thermocycling conditions were: initial denaturation at 96°C for 5 min, 30 cycles of 96°C for 15s, 60°C for 30 s, 72°C for 45s, and a final extension step at 72°C for 7 min.

PCR products of exons 2 and 3 of the HLA-G gene were directly sequenced (Hylenius et al, 2004), using an ABI Prism Big Dye Terminator cycle sequencing kit (Thermo Fisher Scientific, USA) and an ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific, USA)The primers used in the direct DNA sequencing reaction were: exon 2: (5'-AGATCACGACCCCCACCTCCAT-3'); and exon 3: (5'-GGTGGGTCCGGGCGAGGGCGAGGCT-3') and in some instances for control sequencing, primers HLAGEX2A and GI3/3 (Hylenius et al, 2004). *HLA-G* 01:06*, identified by the codon 258 in exon 4, was detected by RFLP with the restriction endonuclease Eco72I (Hviid et al, 2002). In this study six isoforms of HLA-G protein (HLA-G**0101, *0102, *0103, *0104, *0105N, *0106*) differing for one non-synonymous nucleotide substitution in the coding region were genotyped at four-digit resolution. On the contrary, alleles exhibiting synonymous nucleotide substitutions in the coding sequence, producing no modification of the amino acid sequence of the encoded protein, were genotyped at six digit resolution for *HLA-G:01*01* (*:01, :02, :03, :05, :08, :14*) and for *HLA-G*01:04* isoforms *(:01, :03, :04*).

2.3 Statistical Analysis

Chi-square analysis was applied to confirm Hardy Weinberg equilibrium (HWE) of *HLA-G* genotypes distribution in ASD children and their sibs.

Chi-square and p-value calculation (with Bonferroni's correction, p_c, for degrees of freedom, df) was used to evaluate the association of *HLA-G* polymorphisms with ASD. Fisher's exact test (p_f) for small sample sizes (lower than 5 units per cell) was applied to the 2x2 table as appropriate. The risk of association was reported as an Odds Ratio (OR) with its 95% Confidence Interval (CI). All

statistical analyses were performed using the IBM SPSS 23 statistical software. The significance threshold was set at $p < 0,05$.

3. RESULTS

3.1 HLA-G distribution in ASD children and control group

HLA-G allelic distribution was analyzed both in the group of 111 ASD children and in their healthy siblings and was compared to that reported in two Caucasoid population of multiparous women and their partners from Brazil (N=167) (Nardi et al., 2012)(Pop 1) and Denmark (N= 93) (Hviid et al 2002)(Pop 2) (Figure 1). A statistically different *HLA-G* distribution was observed when ASD children were compared to either Pop1 (p_c=1x10^{-4} df=12) or Pop 2 (p_c=1x10^{-3} df=12). In particular, the *HLA-G*0105N* allele was statistically more frequent in ASD children (5.4%) than in either Pop 1 (0.6%)(p_f=1x10^{-3} p_c=1x10^{-2}; OR:9.5, 95%CI:2.4-63.2) or Pop 2 (1.1%). However, in the latter comparison difference was not statistically significant after p value correction for 12 degree of freedom (df) (p_f=3x10^{-2}, p_c=0.36; OR:5.2, 95%CI:1.3-34.9). Conversely, the *HLA-G*01:01:02* allele, one of the most common *HLA-G* alleles, was statistically less frequent in ASD children (17,6%) compared to either Pop 1 (26.0%)(p=2x10^{-2}, p_c=0.24; OR:0.6, 95%CI:0.4-0.9) or Pop 2 (26.9%) (p=2x10^{-2}, p_c=0.23; OR:0.6, 95%CI:0.4-0.9), though these difference were not statistically significant after p value correction.

Since the *HLA-G* allelic distribution was similar in Pop 1 and Pop 2 (p_c=0.10 df=13), data of these two control groups were pooled together and compared with the results obtained in ASD and their healthy sibs. The *HLA-G* distribution was different in ASD children as compared to the pooled control group (p_c=3x10^{-6} df=13). Two alleles, *HLA-G*01:05N* and *HLA-G*01:01:02,* were mostly responsible for this difference. In fact, the *HLA-G*01:05N* allele was more frequent in ASD children (5.4%) than in controls (0.8%) (p_f=4x10^{-4}, p_c=5x10^{-3}; OR: 7.3, 95%CI: 2.4-26.5), whereas the *HLA-G*01:01:02* allele was less frequently carried by ASD children (17.6%) compared to controls (26.3%) although the difference was not significant after p value correction (p=1x10^{-2}, p_c=0.13; OR:0.6, 95%CI:0.4-0.9)(Table 1).

The frequency of these two alleles was intermediate in healthy sibs (*HLA-G*0105N*=2.5% , *HLA-G*01:01:02*= 15.4%) between that of ASD and of controls (Figure 1)

3.2 HLA-G allelic grouped distribution in ASD children and controls

Overall frequency of the *HLA-G:01:01* grouped alleles (eight allelic subtypes from *01:01:01 to *01:01:14) are shown in Table 2. Results indicate that the *HLA-G:01:01* skewing (ASD: 69.8%; controls: 83.5%) results in a significantly protective effect (p_c=1x10^{-4} OR: 0.5, 95%CI:0.3-0.7). Notably, the overall *HLA-G:01:01* allelic subtypes frequency is also lower (73.1%) in healthy siblings than other controls(83.5%), though this difference is not significant after p value correction for 4 degree of freedom (p=2x10^{-2}, p_c=0.1 OR: 0.5, 95%CI: 0.3-0.9)(Table 1s). In contrast, the overall frequency of the *HLA-G*01:04* alleles (three allelic subtypes: **01:04:01, *01:04:03, *01:04:04*) was similar in all analysed individuals. A unique allelic form of *HLA-G*01:03, HLA-G*01:05N* and *HLA-G*01:06* is known (Donadi et al., 2011); the frequencies of such alleles are the same shown in Table 1.

In healthy sibs, a statistical discrepancy was observed only versus combined controls (p_c=2x10^{-2}) (Table 1s), but no specific allelic association reached the statistical significance, perhaps due to the low number of healthy sibs.

Finally, we evaluated the distribution of the *HLA-G*0101-G*0106* genotype together with the *HLA-G14bp* ins/del genotypes in ASD children and their healthy siblings. Genotypes distributions were in Hardy Weinberg equilibrium in both groups. No specific association was observed between *HLA-G* and *HLA-G14bp* loci either in ASD children or in their healthy sibs (Table 2s).

3.3 HLA-G and HLA class I haplotype analysis.

Since both *HLA-G* and the classical *HLA class I* alleles are localized on the same chromosome, we evaluated the presence of possible haplotypes that could distinguish ASD children; particular attention was given to those haplotypes including *HLA-G*0101* and **0105N,* together with **G14bp+* (Table 3s). Results show no allelic skewing between ASD children and their healthy siblings; this, however, could be the consequence of the small sample size of children, particularly that of ASD sibs, analyzed with a haplotype counting approach.

3.4 HLA-G distribution in ASD mothers, women with recurrent miscarriages, and multiparous women without miscarriages

HLA-G distribution was analyzed in 81 ASD mothers, 98 women with recurrent miscarriages (WRM), and 130 multiparous women without miscarriages (WWM) from Pop 1 and Pop 2. Results showed that while the *HLA-G* distribution pattern was similar between ASD mothers and WRM (p_c=0.46 df=12), it was significantly different when compared to WWM mothers (ASD *vs*. WWM, p_c=6x10^{-4} df=12). In particular, the *HLA-G*01:01:01* allele was less frequent in ASD mothers (35.8%) than in WWM (48.4%)(p=1x10^{-2}, p_c=0.12; OR:0.6, 95%CI: 0.4-0.9), whereas the *HLA-G*01:05N* allele was more frequently observed in ASD mothers (4.9%) than in WWM (0.8%) (p_f=2x10^{-2}, p_c=0.07; OR: 6.6 95%CI: 1.5-46.5). Results are shown on Figure 2.

4. DISCUSSION

HLA-G coding region polymorphisms were analyzed in ASD children, in their healthy siblings and in 81 of their mothers; results were also compared to those obtained in two different datasets of multiparous women with different ethnic backgrounds. Results showed the presence of a significant skewing of *HLA-G* polymorphisms in ASD children, being the *HLA-G*0105N* allele significantly more and the *HLA-G*0101* allele significantly less frequent, respectively. Notably, the same skewing was detected in ASD mothers compared to those with or without a history of recurrent miscarriages. Specifically, *HLA-G* polymorphisms distribution was similar between ASD mothers and those with recurrent miscarriages, whereas *HLA-G*0105N* allele was significantly more frequent and *HLA-G*0101* significantly less frequent in ASD mothers compared to those without a recurrent miscarriages.

The full-length molecule coded by *HLA-G*01:01* largely predominates over the other isoforms in the general population (Castelli et al., 2014), indicating that a strong selective pressure for the conservation of this protein sequence was exerted during evolution. *HLA-G*0105N* is much less frequent and is defined by a C deletion at exon 3: this deletion disrupts the reading frame and results in incomplete formation of the HLA-G1, HLA-G4, and HLA-G5 isoforms. Notably, particular *HLA-G*0105N* alleles have been associated with significantly lower serum concentrations of sHLA-G proteins (Arjmand et al., 2016); this condition seems to characterize women undergoing recurrent spontaneous abortions (Hviid et al., 2002). A different clinical context is associated with HLA-*G*01:01*, whose presence results in higher sHLA-G levels (Rebmann et al., 2001) and a lower rate of recurrent miscarriages. As *HLA-G*01:01* is associated with the elicitation of KIR-mediated tolerogenic responses (Hunt et al., 2003) whereas *HLA-G*01:05N* correlates with a reduced inhibition of NK cells activation (Aldrich et al., 2001), results herein indicate that an immune activating milieu during pregnancy is significantly more frequently observed in association with the development of ASD. To this regard, we have recently shown that a 14bp polymorphism, located in the 3'UTR regulatory region of *HLA-G* gene, can be observed in ASD mothers (Guerini et al., 2015) as well as in women with recurrent spontaneous abortions (Christiansen et al., 2012; Hylenius et al., 2004). Possibly, this might contribute to the generation of a poorly tolerogenic foetal environment, which is associated with a lower expression of the soluble form of HLA-G protein.

Our study results, together with recently published data (Guerini et al., 2015) suggest that *HLA-G*0101, HLA-G*0105N*, and *HLA-G14bp+* may play a role in ASD-associated immune activation. In a hypothetical scenario, these three factors might interact and *HLA-G*0105N* could code for a non-functional HLA-G molecule, whilst *HLA-G14bp+* would results in a lower expression of HLA-G protein. The activating effect of this allele combination would be partially counteracted by *HLA-G*0101*, whose product is a protein that interacts with KIR molecules, inhibiting NK cell activation (Hunt et al., 2003). Alternatively, these genetic variants could act independently. To better understand these mechanisms we evaluated the association of *HLA-G14bp* polymorphism and *HLA-G* alleles in ASD children and their healthy siblings. However, no associations emerged between these polymorphisms in either groups; haplotype analysis evaluating both *HLA-G* and *HLA class I* alleles, which are located on the same chromosome, did not show any skewing between ASD children and their healthy siblings either (see supplementary Table 3). These results might be the consequence of the low number of enrolled healthy sibs; larger cohort analysis will be required to definitely clarify this issue.

Besides being suspected to play a role in the pathogenesis of ASD, immune activation during pregnancy is indeed assumed to be involved in recurrent spontaneous abortions. Immune activation and inflammation also play a fundamental role in autoimmune disorders. Notably, both spontaneous multiple abortions and autoimmune diseases are more frequently observed in mothers of ASD children (Comi et al., 1999, Croern et al., 2005, Atladóttir et al., 2009, Altevogt et al., 2008, Sweeten et al., 2003). The *G*0105N* allele, in particular, though not frequently observed in the general population, was previously shown to be significantly more frequent in women who had multiple abortion, as compared to normal fertile controls (Arjmand F et al., 2016); this allele was observed to reduce the HLA-G expression (Rebman et al., 2001) and increase the risk for miscarriage, possibly as a consequence of immune activation (Le Discorde et al., 2005). It is also worth observing that *HLA-G*01:01* allele was less frequently carried by women with recurrent abortions; in this group we

have also observed a frequency similar to that registered in ASD mothers. Therefore, we speculate that both women with recurrent abortions and ASD mothers may share a *HLA-G* polymorphism-driven uncontrolled immune activation against foetal tissues.

Autism is a multifactorial and multigenic disorder (Torres et al., 2016) in which pathogenic roles for immune activation and neuroinflammation during pregnancy are suspected. Relatedly, cytokines and chemokines produced by activated NK cells might strongly influence CNS development and plasticity as well as foetal development (Gesundheit et al., 2013). These immune proteins are also known to modulate brain function and to affect cognitive and emotional processing.

We propose that at least three set of genes: *HLA-C* (Guerini et al., 2014) and *HLA-G* (Guerini et al., 2015) expressed by foetal cells as well as *KIR* (Guerini et al., 2014, Torres et al., 2013) present in placental and decidual maternal cells are involved in immune activation during pregnancy. We might also hypothesize that different combinations of these genetic patterns will result in different degrees of immune activation, possibly playing a role in determining the high heterogeneity of neuropsychiatric impairment in ASD children (Guerini et al., 2017). The worst such genetic combination may be incompatible with life, bringing the foetus to spontaneous abortion, whereas mild to lower degrees of detrimental immunogenetic combination may lead to variegated alterations of neuronal development. This deserves to be further investigated by analyzing *HLA-G, HLA C* and *KIR* patterns in relation to different degrees of clinical, behavioral and neuropsychiatric impairment. It would be of great interest to evaluate a possible correlation of *HLA-G* polymorphisms with the clinical severity of autism. However, due to the high rate of polymorphism of such alleles and the complementarity of many other variables to be considered (as age, gender, other genes, education, et cetera), a tailored study in a bigger patients cohort should be designed.

Conflict of interest statement

No author declares any conflict of interest

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- *HLA-G alleles are skewed in ASD children*
- *HLA-G alleles seen in ASD result in immune activation*
- *The skewing of HLA-G alleles seen in ASD might play a pathogenic role*

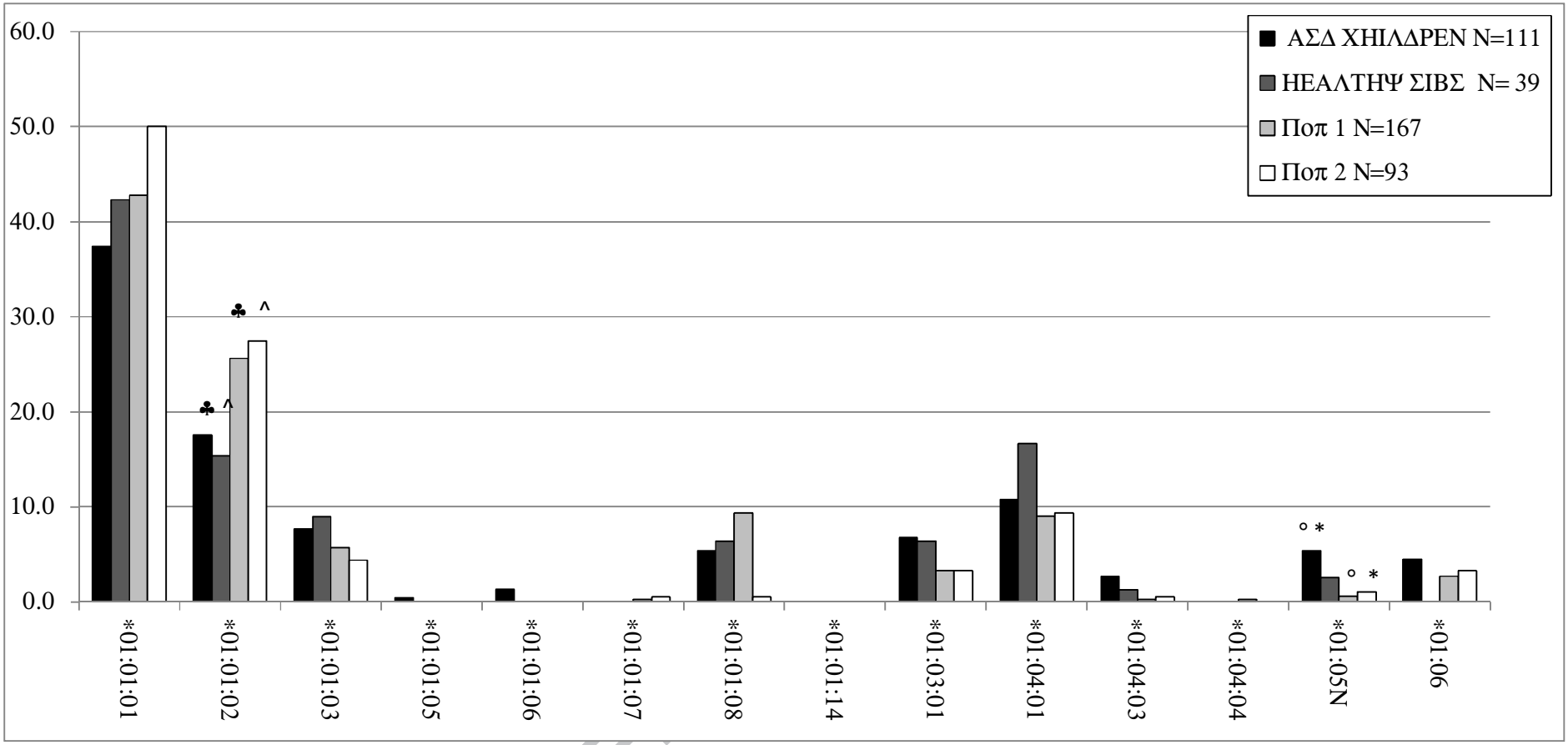

Figure 1: *HLA-G* distribution in 111 ASD children and 39 healthy sibs compared to control group from Brazil (Pop 1: Nardi et al, 2012) and from Denmark (Pop 2: Hviid et al, 2002). Frequency of distribution expressed as percentage

A statistical different distribution was observed between ASD children and both Pop1 (chi square= 39.3 p_c=1x10^{-4} df=12) and Pop 2 group (chi square=36.1, p_c=1x10^{-3} df=12).

§ ASD vs Pop1 : $p=2x10^{-2}$, $p_c=0.24$; OR:0.6, 95%CI:0.4-0.9; ^ASD vs Pop2: $p=2x10^{-2}$, $p_c=0.23$; OR:0.6, 95%CI:0.4-0.9)

°ASD vs Pop1 : $p_f=1x10^{-3}$ $p_c=1x10^{-2}$; OR:9.5, 95%CI:2.4-63.2; *ASD vs Pop2: $p_f=3x10^{-2}$, $p_c=0.36$; OR:5.2, 95%CI:1.3-34.9

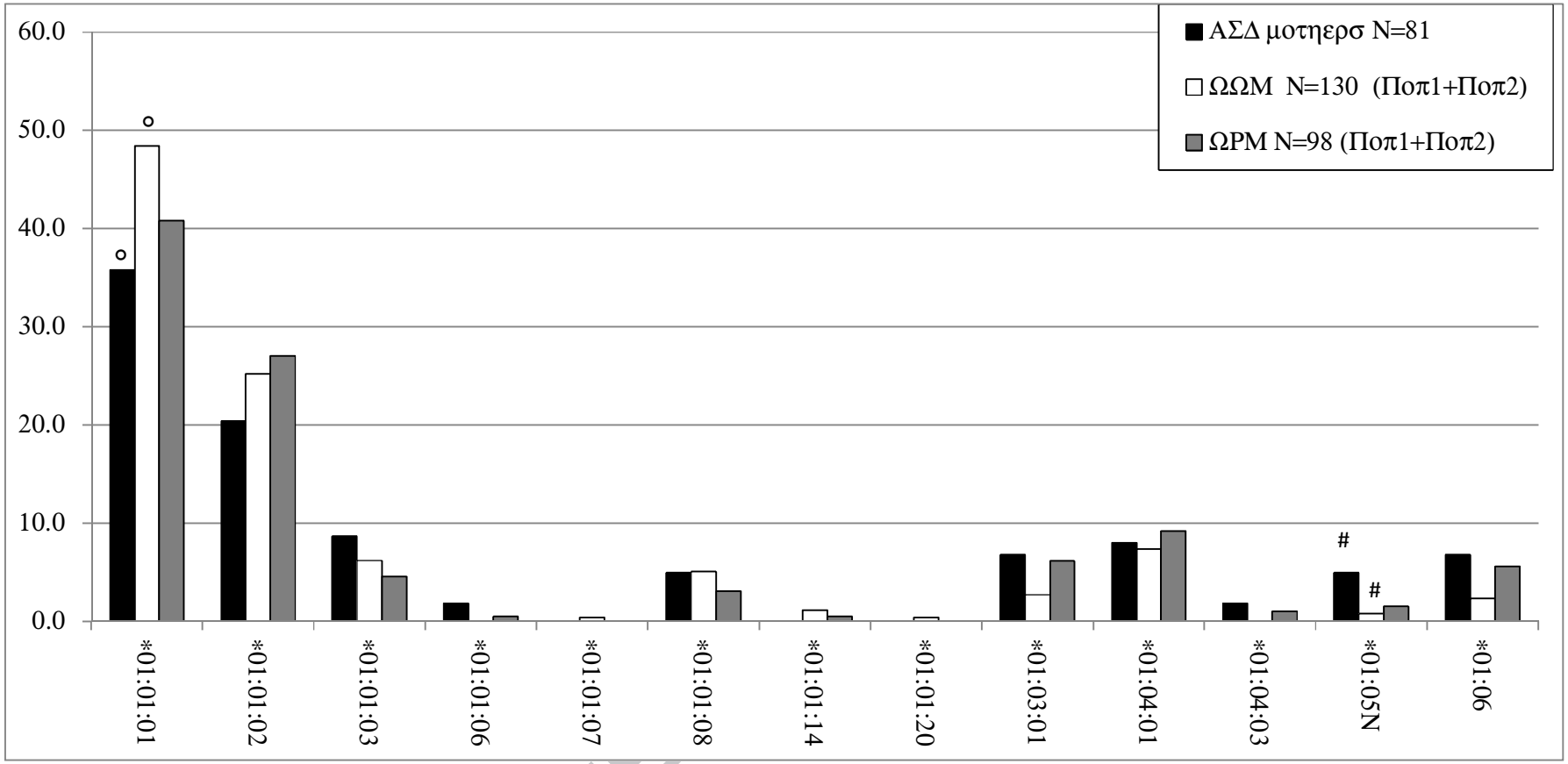

Figure 2: *HLA-G* allelic distribution in 81 ASD mothers compared to 130 multiparous women without miscarriage (WWM) (chi square=34,2, p_c=6x10^{-4} df=12) and to 98 women with recurrent miscarriage (WRM) (chi square=11.7, p_c=0,46 df=12) both pooled from Brazil (Pop 1)(Nardi et al, 2012) and from Denmark (Pop 2)(Hviid et al, 2002). Frequency of distribution expressed as percentage

°p=1x10^{-2} p_c=0.12; OR:0.6, 95%CI: 0.4-0.9 ; # p_f=2x10^{-2} p_c=0.07; OR: 6.6, 95%CI: 1.5-46.7

HLA-G	ASD children (N=111)		Controls (N=260) (Nardi et al., 2012, Hviid et al 2002)		Chi square	p value	p_c value	OR	95% CI
	N	%	N	%					
***01:01:01**	83	37,4	233	44,8					
***01:01:02**	39	17,6	137	26,3	6,26	$1X10^{-2}$	0,13	0,6	0,4-0,9
***01:01:03**	17	7,7	27	5,2					
***01:01:05**	1	0,5	0	0,0					
***01:01:06**	3	1,4	0	0,0		$p_f = 5X10^{-2}$	0,7	UND	
***01:01:07**	0	0,0	2	0,4					
***01:01:08**	12	5,4	32	6,2					
***01:01:14**	0	0,0	3	0,6					
***01:03:01**	15	6,8	17	3,3	4,58	$3x10^{-2}$	0,42	2,1	1,0-4,4
***01:04:01**	24	10,8	47	9,0					
***01:04:03**	6	2,7	2	0,4		$p_f = 2X10^{-2}$	0,27	7,2	1,5-51,6
***01:04:04**	0	0,0	1	0,2					
***01:05N**	12	5,4	4	0,8		$p_f = 4X10^{-4}$	**$5x10^{-3}$**	7,3	2,4-26,5
***01:06**	10	4,5	15	2,9					
Total	222		520		50.1		**$3x10^{-6}$**		

Table 1: *HLA-G* allelic subtype distribution in ASD children compared to pooled controls of couples of multiparous women without pregnancy complications and their partners. Only statistically significant comparisons were reported. N: absolute number, Chi square with 1 degree of freedom, p_c= p value corrected for 13 degree of freedom (df) . p_f= Fisher exact test. OR: Odds ratio and interval of confidence at 95% (95% CI) were reported only for comparison with p<0,05; UND: impossible to be defined

HLA-G	ASD children (N=111)		Controls (N=260) (Nardi et al2012, Hviid et al 2002)		Chi square	p value	p_c value	OR	95% CI
Alleles	N	%	N	%					
***01:01**	155	69,8	434	83,5	17,7	$3x10^{-5}$	**$1x10^{-4}$**	0,5	0,3-0,7
***01:03**	15	6,8	17	3,3	4,57	$3x10^{-2}$	0,12	2,1	1,03-4,4
***01:04**	30	13,5	50	9,6					
***01:05N**	12	5,4	4	0,8		$p_f = 4x10^{-4}$	**$2x10^{-3}$**	7,3	2,4-26,6
***01:06**	10	4,5	15	2,9					
	222		520		26.9		**$2x10^{-5}$**		

Table 2: *HLA-G* allelic distribution in ASD children compared to pooled controls of couples of multiparous women without pregnancy complications and their partners. Only statistically significant comparisons were reported. N: absolute number, Chi square with 1 degree of freedom, p_c= p value corrected for 4 degree of freedom (df) . p_f= Fisher exact test. Only p values <0,05 were reported OR: Odds ratio ; 95% Confidence Interval (95% CI).