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HLA-G 14bp deletion/insertion polymorphism and mother-to-child transmission of HIV

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Abstract

The human leukocyte antigen HLA-G, highly expressed at the maternal-fetal interface, has a pivotal role in mediating immune tolerance. In this study we investigated the influence of *HLA-G* 14 bp insertion polymorphism in HIV-1 mother-to-child HIV-1 transmission.

The 14 bp insertion polymorphism was analyzed among 99 HIV-1 positive mothers and 329 infants born to HIV-positive mothers in Zambia, among whom vertical transmission status and timing had been determined. *HLA-G* 14bp insertion polymorphism was detected using a custom TaqMan SNPs genotyping assay. Logistic regression was conducted to examine the associations between *HLA-G* alleles and the risk of HIV transmission.

The 14bp insertion allele was more frequent in HIV exposed-uninfected infants than in infected infants, and was associated with reduced risk of both in utero and intrapartum HIV transmission, after adjusting for maternal CD4 cell count and plasma viral load. Maternal *HLA-G* 14bp insertion genotype and *HLA-G* concordance between mother and child were not associated with the risk of perinatal HIV transmission.

The presence of the 14 bp insertion associates with protection towards in utero and intrapartum HIV infection in children from Zambia, suggesting that *HLA-G* could be involved in the vertical transmission of HIV.

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The authors declare that they have no conflict of interest.

Authors contribution:

LS conceived the study and drafted the manuscript; LZ performed DNA extraction and *HLA-G* genotyping; HYK conducted the statistical analyses; EC participated in *HLA-G* genotyping; DT Designed original study; CK Conducted original study; GA Undertook all laboratory analyses on original study; LK Designed original study and conducted statistical analysis; SC critically revised the manuscript.

Keywords

HIV-1; HLA-G; 14bp insertion/deletion; genetic polymorphism; vertical transmission

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Introduction

Globally, 1.4 million HIV-infected pregnant women each year give birth and can transmit HIV during pregnancy, delivery and post-natally via breastfeeding [1]. Without antiretroviral interventions, ~20% of infants will be infected during pregnancy and delivery with an additional ~15% through breastfeeding [1]. In richer countries, a well-developed infrastructure makes prophylactic and/or therapeutic antiretroviral interventions almost universally available reducing the risk of vertical transmission to <2% [2]. However, there are still many barriers to implement these programs to reduce vertical HIV transmission in resource-limited settings [3].

Over the past few years, many studies have investigated possible underlying mechanisms for intrauterine transmission, but these remain as yet poorly understood. Various HIV-1 sequences were observed within the placentae of HIV-infected pregnant women, independent of the virological status of the infants [4, 5], but only selected maternal HIV-1 variants were detected to cross the placental barrier [6]. In addition, while the trophoblast cells in the first trimester of pregnancy seem to be susceptible to HIV infection [7], some studies failed to infect cytotrophoblast cells through the use of HIV-1 isolates *in vitro* [8,9] indicating that the placental trophoblast layer may have regulatory mechanisms and functions as an effective barrier to HIV transmission [10,11].

Although vertical transmission of HIV-1 has been associated with several viral, obstetric, socioeconomic, and behavioral factors (reviewed in [12]), numerous studies suggested that host genetic factors such as human leukocyte antigen (HLA) class I and II alleles contribute to determining vertical transmission [13]. In particular, HLA-G molecules are mainly expressed on cytotrophoblasts cells at the maternal-fetal interface where classical HLA class I and II antigens are absent and play an important role in the modulation of the maternal immune system during pregnancy [14]. During pregnancy, the HLA-G molecules inhibit the cytotoxic activity of maternal T lymphocytes and Natural Killer cells towards fetal cells and their proliferation by binding to specific receptors such as immunoglobulin-like transcript 2 (ILT2) and killer cell immunoglobulin-like receptor (KIR2DL4) thus conferring immunological tolerance towards the semi-allogeneic fetus [15-17]. Thus, the preferential expression of HLA-G at the level of the placenta and its immunomodulatory function suggest that it could play an important role in mother-to-child HIV-1 transmission.

HLA-G gene encodes for seven isoforms (four membrane-bound, G1-G4, and three soluble, G5-G7; resulting from alternative splicing; the main isoform expressed on trophoblast cells is the full-length membrane-bound HLA-G1 isoform [18].)

A few studies have examined the role of HLA-G in mother-to-child HIV transmission. HLA-G1 expression was up-regulated almost four times in placenta of HIV-1 transmitting mothers, compared to non-transmitting mothers [19]. Presence of *HLA-G*01:01:01:03* allele or *HLA-G*01:05N* allele was also associated with a reduced risk of vertical transmission of HIV-1 [20]. Another study reported that the deletion of the 14bp at the 3'-untranslated region (UTR) in exon 8 of the *HLA-G* gene was associated with a protective effect against vertical transmission of HIV-1 in Brazilian population [21]. The 14 bp deletion/insertion polymorphism has been reported to influence the stability of *HLA-G* mRNA, and HLA-G expression [14, 22-24]

In this study, we analyzed the 14bp deletion/insertion polymorphism and evaluated the association between *HLA-G* gene and vertical mother-to-child HIV-1 transmission among 99 HIV-1 positive mothers and 329 infants born to HIV-positive mothers in Zambia.

Materials and methods

Study Design and Population

A subset of the study population was selected from the Zambia Exclusive Breastfeeding Study, a randomized clinical trial to examine whether exclusive breastfeeding to 4 months could reduce HIV transmission and child mortality relative to longer breastfeeding through a median of 16 months. In brief, 958 HIV-infected women, recruited during pregnancy at two antenatal care clinics in Lusaka, Zambia between May 2001 and September 2004, were followed to delivery and through 24 months postpartum with their infants. Infants were tested regularly for HIV. All women were counseled to breastfeed to at least 4 months. Thereafter half of the women were randomized to stop all breastfeeding and the other half to continue breastfeeding for as long as they usually would. Women were given only single-dose nevirapine as prophylaxis to prevent transmission to the child as antiretroviral therapy was not available in the public sector at the time this study was done.

For this analysis, we selected a total of 329 infants who were born before the end of October 2002 with confirmed vertical transmission status. Of these 329 infants, 23 (6.9%) had intrauterine transmission (defined as a positive PCR result within 2 days of birth), 24 (7.5%) had intrapartum transmission (defined as a positive PCR result within 42 days of birth with an earlier negative result), and 38 (14.4%) had postnatal (breastfeeding) transmission (defined as a positive PCR results older than 42 days with a negative earlier result in a breastfed child). The remainder were HIV-exposed uninfected children. Only 99 mothers of the 329 infants included in this study were randomly selected for genotyping. All women provided written informed consent for their participation and all the investigators' Institutional Review Boards approved the study.

DNA extraction and HLA-G genotyping

DNA of both children and mothers were extracted from dried blood spot using the "DNA extract all reagents" kit (Applied Biosystem, USA) following manufacturer instructions. Briefly, a small portion of Guthrie card (approximately 4mm × 4mm) was incubated for 6 minutes at 96°C in 50 µl of lysis solution; after that 50 µl of stabilizing solution were added. Samples were then diluted 1:2 with sterile distilled water and stored at -20°C until used.

HLA-G 14bp deletion/insertion polymorphism (rs1704) was detected using a custom TaqMan SNPs genotyping assay, constituted by forward primer TGAAACAGCTGCCCTGTGT, reverse primer AGTCAGGGTTCTTGAAGTCACAAAG, insertion-specific probe AGTGGCAAGATTTGT (VIC labeled), deletion-specific probe ACTGAGTGGCAAGTC (FAM labeled) (Life Technologies, Carlsbad, California, U.S.) on the ABI7900HT Real Time PCR platform (Applied Biosystems) with an initial step for Taq polymerase activation at 95°C for 10 minutes, followed by 40 cycles with 15 seconds of 95°C for denaturation and 1 minute at 60° C for extension.

Allelic discrimination was done both manually and automatically with the SDS detection software (Applied Biosystems). The 14 bp inserted allele was named I while the deleted allele named D.

Classification of mother-child HLA-G matching

Matching between mother and child *HLA-G* alleles was assessed following the method reported by Luo et al. [20]. Briefly, if the two *HLA-G* alleles of the child matched two alleles of the mother, they were considered concordant. If the mother was homozygous at *HLA-G*, she was also considered to be concordant with her child.

Statistical analyses

Student's *t* and nonparametric Wilcoxon test were used to compare continuous variables. Pearson's χ^2 statistics and Fisher's exact test were used for categorical variables. Logistic regression was conducted to examine the association between *HLA-G* alleles and the risk of HIV transmission. Unadjusted and adjusted odds ratios (OR) were reported with 95% confidence intervals. Maternal health indicators including CD4 cell count, hemoglobin level, plasma viral load, and clinical stage at baseline were investigated as potential confounders. Women were defined as symptomatic if they experienced weight loss, more than 30 days of diarrhea, fever or cough in the prior 6 months or had a history of thrush or tuberculosis [25].

Results

Study population

HLA-G 14bp deletion/insertion polymorphism frequency distributions were in Hardy Weinberg equilibrium in both the mother and child samples. The baseline characteristics of the 329 infants with *HLA-G* data and their mothers are shown in Table 1. HIV transmission status significantly correlated with maternal CD4 count and plasma viral load (Table 1). Maternal viral loads were significantly higher in transmitters than in non-transmitters ($p < 0.0001$).

Child's HLA-G genotype

HLA-G 14bp deletion/insertion polymorphism was genotyped in 329 infants born to HIV positive mothers. We found a significant association between infant *HLA-G* genotype and HIV transmission (table 2). The I allele was more frequent in HIV exposed-uninfected (EU) infants than in infected infants, (40.0% vs. 25.9%, p -value=0.001) and showed a protective association on mother-to-child HIV transmission (Odds Ratio [OR]=0.53; 95% CI 0.36-0.77). The associations observed with the I allele were compatible with the dominant genetic model (I/D + I/I vs. D/D, OR= 0.44 [95% CI 0.27-0.73], $p = 0.001$): the presence of the I allele was associated with reduced risk of vertical HIV transmission both in the homozygous I/I genotype (OR= 0.30 [95% CI 0.10-0.79], $p = 0.008$) and the heterozygous D/I genotype (OR= 0.49 [95% CI 0.28–0.86], $p = 0.008$), compared to the D/D genotype. This association was still significant after adjusting for maternal CD4 cell count and plasma viral load (OR= 0.29 [95% CI 0.11-0.81], $p = 0.01$ for I/I; OR= 0.51 [95% CI 0.28-0.91], $p = 0.02$ for I/D genotype).

HIV-infected children were further stratified according to timing of vertical transmission (table 3): 23 children were infected *in utero* (IU), 24 intrapartum (IP) and 38 postpartum (PP). The I allele was significantly more frequent in EU children (40.0%) than in IU (23.9%) and IP (20.8%) ($p = 0.039$ and 0.012 respectively). The I allele was not significantly more frequent in PP (30.3%) relative to EU ($p = 0.128$). The distribution of I/I, I/D, D/D genotypes in IP infants was significantly different compared to EU infants [$p = 0.027$] but did not differ in IU [$p = 0.095$] or PP infants [$p = 0.21$] relative to EU.

Mothers' HLA-G genotype

Of 99 HIV-infected mothers, 26 mothers (26.3%) transmitted HIV to their infants; 26.9% (7/26) were intrauterine transmission, 30.8% (8/26) were intrapartum transmitted, and 42.3% (11/26) were postpartum-transmitted via breastfeeding. The median CD4 cell counts among mothers with I/I, I/D, and D/D genotypes were 348, 355 and 309 cells/mm³, respectively, and the difference was not significant among groups ($p=0.22$ in I/I vs. D/D and $p=0.86$ in I/D vs. D/D). Maternal plasma viral load was also similar among groups ($p=0.70$ in I/I vs. D/D and $p=0.48$ in I/D vs. D/D).

HIV transmission status was not significantly different by maternal *HLA-G* 14bp deletion/insertion genotype ($p=0.31$) however the direction and magnitude of the association was consistent with what was observed in the larger number of children (Table 2).

Concordance of mother-child HLA-G

The possibility that mother-child *HLA-G* concordance could influence the risk of perinatal HIV transmission was also evaluated. *HLA-G* concordance between mother and child was not associated with the risk of perinatal HIV transmission in the 97 mother-child pairs. The concordance was 76.9% (20/26) in mother-child pairs where HIV was transmitted, and 78.9% (56/71) in pairs where the infection did not occur ($p=1.00$). When the timing of transmission was considered (IU, IP or PP), still no association was detected.

Discussion

HLA-G expression has been associated with several autoimmune and inflammatory diseases, as well as susceptibility to viral infections. Homozygosity for the 14-bp deletion (D/D genotype) in children was a risk factor for hepatitis C virus (HCV) vertical transmission [26] and high level of soluble HLA-G in amniotic fluid correlated with congenital transmission of *Toxoplasma gondii*, although sHLA-G is important in immunomodulation to avoid fetal loss [27].

Despite the high level of HLA-G expression at the maternal-fetal interface and its role in mediating immune tolerance, relatively few studies have investigated the influence of *HLA-G* on HIV-1 vertical transmission. An up-regulation of *HLA-G1* expression in placenta has been noticed among HIV-1 infected transmitting mothers compared to non-transmitting mothers, suggesting that down-regulation of *HLA-G* could have protective effect on vertical transmission of HIV-1 [19].

In the current study we found that the presence of the *HLA-G* 14 bp insertion polymorphism (in heterozygous or homozygous genotypes, according to the dominant model) among HIV-exposed children was associated with a reduced risk of perinatal HIV transmission. This protective association remained significant after adjusting for CD4 cell count or plasma viral load.

The 14 bp insertion polymorphism has been reported to increase *HLA-G* mRNA instability, resulting in down-regulation of HLA-G and reduced production of most membrane-bound and soluble mRNA isoforms in trophoblast samples [14,24]. Recently, Svendsen et al. [28] observed an incremented membrane bound HLA-G1 expression and an inhibition of NK cytotoxicity in 14 bp ins transfected K562 cells compared to 14 bp del transfectants. The authors also observed that the HLA-G1 14 bp deletion allele had a higher sHLA-G1/mHLA-G1 ratio than the 14 bp insertion allele. Based on these findings we can speculate that the 14bp ins polymorphism, associated in our study with protection toward HIV vertical transmission, could be responsible of a major expression of membrane bound isoforms of HLA-G, thus leading to a T-helper type 2 immune response with the production of anti

inflammatory cytokines at the maternal fetal interface that could prevent the risk of vertical transmission.

However the study by Svendsen et al. was conducted in cell culture and consecutively the effects of HLA-G 3' UTR polymorphisms on sHLA-G or mHLA-G production have been observed only in vitro. Moreover, the study of Svendsen et al. investigated the significance of the 14 bp ins/del polymorphism independently of other sequence variation. It has been proposed that the 14 bp del/ins polymorphism (together with other at the 3'UTR of the gene) may account for functional differences in soluble and membrane-bound HLA-G and that the balance between these forms, as well as between different splicing form of HLA-G, may affect HLA-G levels and functionality. Thus, it is possible that the 14 bp allele association with HLA-G expression might be different in different cell types/tissues, e.g. trophoblast cells versus immune cells and that this could in turn affect HIV infection in different ways; since however HLA-G expression and transcriptional regulation are not clear yet, every hypothesis, remains purely speculative.

Moreover, at present, it is not fully clarified whether the 14 bp ins/del polymorphism has itself a functional impact or if it is just a genetic marker for other HLA-G polymorphisms harboring functional significance. The 14 bp ins/del polymorphism is in strong linkage disequilibrium with other HLA-G polymorphisms in the 5'-upstream regulatory region (5'URR) and in the 3'UTR and this can possibly affect HLA-G expression in vivo [23].

Contrary to the results presented here, our group has previously reported that the *HLA-G* 14bp I allele and I/I genotype of a child was associated with an increased risk of perinatal HIV transmission in Brazilian population [21].

The 14bp insertion polymorphism has been reported to vary in frequency between populations, ranging from 12% in Japanese, to 32% in Europeans up to 43% in Africans (according to Ensembl database) and also within populations [29,30] and this could be one explanation for the different results obtained in the two studies involving Brazilian and Zambian subjects. However, although in some circumstances a dramatic racial effect can be seen for the same genotype, we do not think this is the case of the HLA-G 14 bp polymorphism, and believe it is more likely due to population stratification bias in our Brazilian study.

The Brazilian population, previously analyzed by our research group, was an admixture of African, Caucasian and Amerindian genomes estimated as 44, 36 and 20% respectively [31]; however, the different genome percentage can vary from individual to individual. Thus even with adequate ethnic-matching of cases and controls, the risk of population stratification bias persists. This inference is supported by the fact that HLA-G frequencies were not in Hardy Weinberg equilibrium in heterogenetic Brazilian children, while they were in the less heterogenetic Zambian population. Moreover, differently from the Zambian study, biological samples and clinical information were not available for the Brazilian mothers, and it was not possible to adjust analyses for possible confounding.

Our results support a possible role for HLA-G in protection against HIV infection via maternal and infant immune responses during pregnancy and delivery. The protective association we observed between the presence of *HLA-G* I allele in children from Zambia was strongest for intrauterine and intrapartum HIV transmission but weaker and non-significant for post-natal transmission. This differential association can be explained considering HLA-G expression patterns and HIV transmission route: in the post-natal period HIV infection occurs through breastfeeding, possibly when HIV reaches the mucosa of the gastrointestinal tract [32] Since HLA-G is not expressed there, the lack of association

between HLA-G insertion polymorphism (and therefore reduced HLA-G expression) and post-natal transmission is not surprising. Since HLA-G is mainly expressed at the maternal-fetal interface, alteration of its expression levels could be crucial when transmission occurs transplacentally in utero or following placental microtransfusion during delivery [33].

In our study, we observed no association between *HLA-G* concordance for the 14bp insertion polymorphism and risk of perinatal HIV transmission. Given the peculiar expression of *HLA-G* at the maternal-fetal interface, it is likely that *HLA-G* genotype of both the children and their mothers is important for transmission but our study may not have had a large enough number of mothers to detect this. Mother-child HLA (in particular class I) concordance has been reported to increase the risk of vertical transmission of HIV-1 [34-36]. However, several papers reported no association between mother-to-child *HLA-G* concordance and the risk of vertical transmission of HIV-1 [20,37]. A study found a significant association between mother-child discordance in *HLA-G* codon 57 and 3743C>T SNP and a reduced risk of vertical transmission of HIV [38] but the sample size was small (34 mother-child pairs) and the study did not consider different routes of transmission. When *HLA-G* exon 2 and 3 variants were considered, no association was observed between mother-to-child *HLA-G* concordance and risk of HIV transmission in 194 mother-child pairs with known route of transmission [37].

The *HLA-G* 14 bp polymorphism has been reported to also affect HIV disease progression. In one study, *HLA-G* homozygous carriers of the 14bp deletion (D/D) had lower CD4 cell counts and higher levels of HIV-1 RNA in treatment-naïve HIV-infected and HIV-uninfected individuals in rural Zimbabwe, suggesting that high sHLA-G expression may impair the cytotoxic control of HIV [39]. In the current study, however, no statistically significant association between maternal *HLA-G* 14bp deletion/insertion genotype and maternal viral load or CD4 count was found, even when HIV-transmitting and non-transmitting mothers were considered separately.

In conclusion, we have found that the presence of the 14 bp insertion in the *HLA-G* gene is associated with protection towards *in utero* and intrapartum HIV infection in children from Zambia, providing further evidence of the importance of *HLA-G* in HIV infection. Further studies are needed to investigate the extent and the mechanisms of *HLA-G* involvement.

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Table 1
Baseline Characteristics of 329 infants with HLA-G genotype data and their mothers

Mothers Characteristics			
	Transmitter	Non-Transmitter	p-value
Maternal Age (years)			
Mean (SD)			
< 20	9 (10.6%)	22 (9.0%)	0.09
20–30	55 (64.7%)	186 (76.2%)	
> 30	21 (24.7%)	36 (14.8%)	
CD4 cell count (cells/mm ³)			
Median (Q1, Q3)			
< 350	72 (84.7%)	106 (43.4%)	<.0001
350	13 (15.3%)	138 (56.6%)	
Plasma viral load, copies/ml			
Median (Q1, Q3)			
50,000	62 (72.9%)	103 (42.2%)	<.0001
< 50,000	23 (27.1%)	141 (57.8%)	
Hemoglobin			
< 10	33 (38.8%)	66 (27.2%)	0.04
10	52 (61.2%)	177 (72.8%)	
Body Mass Index (kg/m ²)			
< 18.5	22 (26.2%)	45 (18.9%)	0.16
18.5	62 (73.8%)	193 (81.1%)	
Clinical Stage			
Symptomatic	46 (54.1%)	99 (40.6%)	0.03
Asymptomatic	39 (45.9%)	145 (59.4%)	
Infants Characteristics			
	Infected	Exposed uninfected	p-value
Infant Sex			
Male	46 (54.1%)	131 (53.7%)	0.95
Female	39 (45.9%)	113 (46.3%)	
Birth weight			
< 2500g	14 (17.1%)	28 (11.7%)	0.21
2500g	68 (82.9%)	212 (88.3%)	
Gestational Age (weeks)			
< 34	14 (16.9%)	37 (15.4%)	0.75
>34	69 (83.1%)	203 (84.6%)	

Table 2
Analysis of HIV vertical transmission according to infant and maternal HLA-G 14bp deletion/insertion polymorphism allele and genotype

Infants					
HLA-G	Infected	Exposed-uninfected	p-value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
Allele[†]	n= 170	n= 488			
I	44 (25.9%)	195 (40.0%)	0.001	0.53 (0.36-0.77)	0.53 (0.34-0.81)
D	126 (74.1%)	293 (60.0%)		1.0	1.0
Genotype	n= 85	n= 244			
I/I	6 (7.1%)	37 (15.2%)	0.003	0.30 (0.12-0.75)	0.29 (0.11-0.81)
I/D	32 (37.6%)	121 (49.6%)		0.48 (0.29-0.82)	0.51 (0.28-0.91)
D/D	47 (55.3%)	86 (35.2%)		1.0	1.0
Mothers					
HLA-G	Transmitter	Non-transmitter	p-value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)**
Allele[†]	n= 52	n= 146			
I	14 (26.9%)	57 (39.0%)	0.12	0.58 (0.29-1.16)	0.58 (0.27-1.26)
D	38 (73.1%)	89 (61.0%)		1.0	1.0
Genotype	n= 26	n= 73			
I/I	2 (7.7%)	12 (16.4%)	0.31	0.33 (0.07-1.70)	0.42 (0.07-2.40)
I/D	10 (38.5%)	33 (45.2%)		0.61 (0.23-1.58)	0.49 (0.17-1.46)
D/D	14 (53.8%)	28 (38.4%)		1.0	1.0

* Adjusted for maternal CD4 cell count and plasma viral load

** Adjusted for maternal CD4 cell count

[†] Each person carries two copies of alleles

Table 3
Analysis of HLA-G 14bp deletion/insertion polymorphism allele and genotype in infants according to the timing of HIV vertical transmission

HLA-G	Infants									
	Intra uterine (IU)	Infected Intra-partum (IP)	Post-partum (PP)	Exposed-uninfected (EU)	IU vs EU Unadjusted OR (95% CI)	p-value	IP vs EU Unadjusted OR (95% CI)	p-value	PP vs EU Unadjusted OR (95% CI)	p-value
Allele [†]	n= 46	n= 48	n= 76	n= 488						
I	11 (23.9%)	10 (20.8%)	23 (30.3%)	195 (40.0%)	0.47 (0.21-0.98)	0.039	0.40 (0.17-0.83)	0.012	0.65 (0.37-1.12)	0.128
D	35 (76.1%)	38 (79.5%)	53 (70.0%)	293 (60.0%)	1.0		1.0		1.0	
Genotype	n= 23	n= 24	n= 38	n= 244						
I/I	1 (4.4%)	1 (4.2%)	4 (10.5%)	37 (15.2%)	0.18 (0.01-1.28)	0.095	0.16 (0.01-1.09)	0.027	0.49 (0.11-1.62)	0.210
I/D	9 (39.1%)	8 (33.3%)	15 (39.5%)	121 (49.6%)	0.49 (0.18-1.31)		0.38 (0.13-1.01)		0.56 (0.25-1.24)	
D/D	13 (56.5%)	15 (62.5%)	19 (50.0%)	86 (35.2%)	1.0		1.0		1.0	

[†] Each person carries two copies of alleles