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HUMAN POLYMORPHISMS IN PLACENTAL EXPRESSION GENES AND THEIR ASSOCIATION TO SUSCEPTIBILITY TO CONGENITAL INFECTION BY *Trypanosoma cruzi*.

Natalia Anahí Juiz[1], Nelly Melina Cayo[2], Marianela Burgos[3], Miriam Elena Salvo[3], Julio Ruben Nasser[4], Jacqueline Búa[5], Silvia Andrea Longhi[1], Alejandro Gabriel Schijman[1,*]

[1]Grupo de Biología Molecular de la Enfermedad de Chagas (LaBMECh), Instituto de Investigaciones en Ingeniería Genética y Biología Molecular ‘‘Dr. Héctor N. Torres’’ (INGEBI-CONICET), Buenos Aires, Argentina

[2]Instituto de Biología de la Altura, Universidad Nacional de Jujuy, Jujuy, Argentina

[3]Servicio de Obstetricia, Departamento Materno Infantil, Hospital Nacional “Profesor Alejandro Posadas”, Buenos Aires, Argentina

[4]Laboratorio de Química Biológica. Facultad de Ciencias Naturales. Universidad Nacional de Salta, Salta, Argentina

[5]Instituto Nacional de Parasitología ‘‘Dr. Mario Fatala Chaben’’, ANLIS, Buenos Aires, Argentina

[*]Corresponding author: Dr. Alejandro G. Schijman, Vuelta de Obligado 2490, Buenos Aires, Argentina (CP: 1428ADN), Tel. 54 11 4783 27871 ext 37, Fax. 54 11 4786 8578, schijman@dna.uba.ar

ABSTRACT

Background: It is currently unclear why only a proportion of children born to *T.cruzi* infected mothers acquire the infection. We have examined the association of 11 SNPs located in genes coding for placental expression enzymes as genetic markers of susceptibility to congenital *T.cruzi* infection (CI): rs2014683 and rs1048988 of *ALPP* gene, rs11244787 and rs1871054 of *ADAM12* gene, rs243866, rs243865, rs17859821, rs243864 and rs2285053 of *MMP2* gene and rs3918242 and rs2234681 of *MMP9* gene.

Methods: Two groups were compared: 101 CI and 116 non-infected children (NCI) born to mothers seropositive for *T.cruzi*. Novel High Resolution Melting and capillary electrophoresis genotyping techniques were designed.

Results: Logistic regression analysis shows that mutations in rs11244787, rs1871054 (*ADAM12*), rs243866, rs17859821 and rs2285053 (*MMP2*) are associated to susceptibility to CI. Multifactor dimensionality reduction analytical approach revealed that genotyping of rs11244787, rs1871054, rs243866, rs17859821 and rs243864 sites would be a good predictor of CI.

Conclusions: Our results also suggest an important role of human polymorphisms in proteins involved in extracellular matrix remodelling process and immune response in CI. To our knowledge, this is the first study demonstrating the association between mutations in placental expressed genes and susceptibility to CI.

INTRODUCTION

Vertical transmission of *Trypanosoma cruzi*, causative agent of Chagas disease, became more important as a result of vector and transfusion controls. The incidence of congenital *T.cruzi* infection (CI) is estimated at 8,000 cases annually in America [1]. Furthermore, due to migration movements, CI is being responsible for the urbanisation of Chagas disease and its perpetuation in non-endemic regions [2].

CI is a complex problem that involves multiple factors; such as the role of parasite diversity vs host genetic factors, as well as that of maternal and placental microbiomes and the microbiome acquisition by infant in the control of infection [3]. It has been reported that the parasite burden circulating in maternal blood increases the risk of transmission [3, 4, 5], which could be associated to maternal nutritional and immunological status or to the parasite strain. However, no clear association between *T.cruzi* genotypes and CI has been found yet. Indeed, all Discrete Typing Units (DTUs), with exception of DTU IV, have been detected in CI cases and prevalent DTUs in CI patients are in concordance with those predominant in the corresponding general population [6-8].

On the other hand, host genetics might play a role in the susceptibility to acquiring CI, leading to the existence of family clustering and the occurrence of congenital transmission of second generation [9, 10].

Different molecules expressed in placental tissues have been proposed to play a role in susceptibility to CI, namely Placental Alkaline Phosphatase (PLAP), receptor into placental syncitiotrophoblast cells [11,12], matrix-metalloproteinases (MMPs), especially MMP2 and MMP9, which degrade and remodel specific components of the extracellular matrix [13,14] and more recently, ADAM12 protein, another member of metalloproteinases family, associated with trophoblast mobility, chromosomal abnormalities and preeclampsia [15]. In this context, we have

selected certain polymorphisms in these genes based on bibliography that demonstrate effect in transcription, enzyme activity or association to disease. Thus, this study aimed to characterise the following SNPs in the above mentioned molecules: *ALPP*, PLAP coding gene (rs2014683 and rs1048988), *ADAM12* (rs11244787 and rs1871054), *MMP2* (rs243866, rs243865, rs17859821, rs243864 and rs2285053) and *MMP9* (rs3918242 and rs2234681), in samples of children born to mothers seropositive for *T.cruzi* infection, in order to find out the existence of associations between genotypes and/or haplotypes and occurrence of CI.

SUBJECTS AND METHODS

Ethics Statement. Research protocols followed the tenets of the Declaration of Helsinki and Guidelines according to Resolution N°1480/11 of the "Ministerio de Salud" from Argentina and were approved by the Local Medical Ethics Committees named Comité Provincial de Bioética de Jujuy and Comité de Bioética "*Dr. Vicente Federico del Giúdice*" – Hospital Nacional Prof. Alejandro Posadas. All mothers of enrolled children provided informed consent on their behalf before blood collection and after the purposes of the study were explained.

Subjects and Samples. A case-control study has been carried out including 217 children born to seropositive mothers for *T.cruzi* infected in endemic areas of Argentina (Provinces of Salta, Jujuy, Misiones, Chaco and Santiago del Estero), Bolivia (Departments of Cochabamba, Santa Cruz and Potosí) and Paraguay. They were classified into two groups: 101 CI and 116 non-infected (NCI) children. CI was considered in individuals born in non-endemic areas, who did not received transfusions nor travelled to an endemic region before sample collection. Diagnosis of CI was carried out by the respective health centres based on routine assays, micromethod or microhematocrite in neonates and babies younger than six months old and by two concordant serological assays after nine months of age.

The study was performed on frozen DNA samples stored at -20ºC extracted from peripheral blood that was conserved in 1 volume of Guanidine Hidrochloride 6M, EDTA 0.2 M buffer, pH 8.00 (GE) from theNational Institute of Parasitology Dr. Mario Fatala Chaben (N=80) and from LabMECh, INGEBI-CONICET (N=72) (mean age: 5.74 years old; range: 9 months-17 years old). Additionally, peripheral blood samples containing GE were collected newborns delivered atPablo Soria Hospital from Jujuy (N=55) and "Profesor Alejandro Posadas National Hospital" from Buenos Aires (N=10) and DNA was extracted using High Pure PCR Template Preparation Kit (*Roche, Germany*). Quantification of all DNA samples was carried out using a NanoDrop 1000 spectrophotometer (*Thermo Scientific, USA*). PCR targeted to rs2014683 *locus* (see Genotyping), which showed the lowest sensitivity among all studied *loci*, was used as DNA integrity control. Only DNA samples rendering Cts ≤ 30 for this target were used in this study.

SNP selection. The entries of SNPs for the genes studied are in the public NCBI Single Nucleotide Polymorphism database. SNPs were chosen according to the following selection criteria: minor allele frequency (MAF) ≥10%, validated by 1,000 Genomes and with previous evidence that sequence variants have a demonstrated effect in transcription, enzyme activity or an association to disease.

Genotyping. Specific primers were designed flanking the selected SNPs sites intended for High Resolution Melting (HRM) analysis. Amplification followed by HRM was carried out on a Rotor-Gene® thermal cycler (*RG6000, Corbett Research, UK*). Each reaction consisted in 1X Type-it HRM PCR Kit (*Qiagen, Germany*), 0.35 µM of primer forward and reverse and RNase-free water in a final volume of 15 µl, using 30 ng of genomic DNA. Reactions were incubated at 95ºC for 5 min and then subjected to an initial step of 95ºC for 10 s, a SNP specific length of incubation time and annealing temperature and final step of 72ºC for 10 s. Optical measurements in HRM channel were recorded during the extension step. After completion of PCR cycles, melting-curve data were generated by increasing the temperature with a SNP specific ramp at 0.1ºC s^{-1}. Supplementary Table

1 shows primer sequences, annealing length of incubation time and temperatures, and ramps for each SNP. HRM curve analysis was performed using the software and the HRM algorithm provided in the Rotor-Gene 1.7.27 version. Normalisation regions and a confidence threshold of 90% were applied, and profiles giving an identity of less than 90% were considered as distinct profiles. Identification of the HRM curves for each amplicon was assessed by sequencing. Accordingly, DNA products were purified using the QIAquick PCR purification kit (*Qiagen, Germany*) following manufactures instructions, prior to nucleotide sequencing analysis.

In the case of *MMP9* polymorphism corresponding to the microsatellite rs2234681 ($(CA)_{14-24}$ repeats), capillary electrophoresis was performed after PCR, using a forward labelled and a reverse unlabeled primer sequences (Supplementary Table 1), taken from Metzger *et al*, 2012 [16]. Peak analysis was done applying Peak Scanner Software v1.0 (*Applied Biosystems*) and alleles were grouped as low (L) when the number of CA repeats was less than 21 and as high (H) when the number of CA repeats was 21 or more [17].

Statistical analysis. Frequencies and their association with CI, odd ratios (OR) and 95% confidence intervals ($CI_{95\%}$) were calculated using PLINK V1.07 software [18]. The genetic effect of each polymorphism in CI was assessed by logistic regression model with cases or control as the dependent variables. Hardy–Weinberg equilibrium (HWE) was determined by Haploview 4.2 software [19] and a p-value <0.05 was considered as evidence of deviation from HWE. Implementing the same software, pairwise linkage disequilibrium (LD), square correlation coefficient and haplotypes were estimated.

To identify and characterise genes polymorphisms interactions, data mining MDR approach [20] (version 3.0) was employed. The accuracy of each model was evaluated by a Bayes classifier in the context of 10-fold cross-validation. Statistical significance was evaluated using a 1,000-fold permutation test. Best models were defined by 1) Balanced Testing Accuracy (BTA): classification accuracy, calculated from the testing set, 2) Cross-Validation Consistency (CVC): a measure of the

number of times of 10 divisions of the dataset that the best model is extracted, and 3) significance level.

Information gain (IG) was also evaluated by the MDR method. IG selects significant combinations of variables on the basis of entropy [21]: positive entropy values indicate synergistic interaction while negative entropy values indicate redundancy.

RESULTS

Identification and genotyping of SNPs

Eleven SNPs were selected according to the attributes mentioned in Material and Methods: rs2014683 and rs1048988 (*ALPP*), rs11244787 and rs1871054 (*ADAM12*), rs243866, rs243865, rs17859821, rs243864 and rs2285053 (*MMP2*) and rs3918242 and rs2234681 (*MMP-9*). Their characterisation was done using two different approaches, depending on the characteristics of the SNP: i) HRM analysis for polymorphisms corresponding to one nucleotide substitution and ii) Capillary Electrophoresis for microsatellite polymorphism.

HRM method. We successfully identified polymorphisms in rs2014683 (*ALPP*), rs11244787 and rs1871054 (*ADAM12*), rs243866, rs243865, rs17859821, rs243864, and rs2285053 (*MMP2*) and rs3918242 (*MMP9*), in the studied population(Figure 1 and Supplementary Figure 1), whereas rs1048988 (*ALPP)* resulted in a non-polymorphic *locus*, being the ancestral allele G the only one found; therefore this locus was no relevant in this study.

Figure 1A shows an example of fluorescence decay with temperature increment of the studied *ADAM12* SNPs. In rs11244787 the fluorescence for homozygote GG decays at a higher temperature than for homozygote AA. The heterozygote GA formed after denaturation and rehybridisation generates homoduplexes as well as heteroduplexes, showing a different curve behaviour. Curves were normalised respect to the genotype with lowest melting temperature (Figure 1B); typical sequencing electropherograms for each genotype are shown in Figure 1C.

Capillary electrophoresis. rs2234681 was analysed using capillary electrophoresis with labelled primers because neither HRM nor standard sequencing were suitable for this type of polymorphism (data not shown). Figure 1D shows examples of samples corresponding to individuals carrying both alleles of low CA copy numbers (LL), both alleles of high copy numbers (HH, single peak to the right), and the last electrophoresis image shows a heterozygote sample (HL) in which two peaks are distinguished (Figure 1D).

Genotype and Allele Frequencies Distribution.

Genotypic distributions of all studied polymorphic *loci* were in conformity with HWE. In 8 out of 9 SNPs analysed by HRM the three genotypes were detected: A_1A_1, A_1A_2 and A_2A_2, where A_1 is the allele with the minor frequency and A_2 is the most represented allele. rs3918242 was the only site in which the homozygote A_1A_1 was not detected among the samples analysed (Table 1 and Supplementary Figure 1).

Allele and genotype distributions of rs2014683 (*ALPP*), rs243865 and rs243864 (*MMP2*), rs3918242 and rs2234681 (*MMP9*) were similar among CI and NCI ($p > 0.05$) (Table 1).

In allelic and genotype comparisons, both *ADAM12* polymorphisms (rs11244787 and rs1871054) resulted significant between groups ($p^a=0.002$ and $p^a=0.005$, respectively; $p^g=0.019$ in both cases). Moreover, the 1,000 permutation test was also significant for these two polymorphic *loci* ($p=0.011$ and $p=0.002$, respectively). In addition, three *MMP2* studied SNPs (rs243866, rs17859821 and rs2285053) had allele frequencies with noticeable statically differences between CI and NCI ($p^a=2E^{-12}$, $p^a=0.005$ and $p^a=0.02$, respectively) (Table 1).

Each genotype was regressed in a logistic model, assuming dominant (major homozygotes versus heterozygotes plus minor homozygotes) and recessive (major homozygotes plus heterozygotes versus minor homozygotes) models of inheritance, with gender as covariate.

Logistic regression analysis using dominant model revealed that individuals carrying one or two copies of the MAF, guanidine, in rs11244787 (*ADAM12*) were more represented in CI than in NCI

group (60.4%, 40.2%, respectively; p_{ad}=0.008). On the contrary, genotypes with almost one copy of the T allele in rs1871054 (*ADAM12*) had a lower frequency in CI than in NCI cases (53.5%, 74.1%, respectively; p_{ad}=0.002). The OR_{ad} and their $CI_{95\%}$ for this model showed that mutation A>G in rs11244787 might be associated to CI (OR_{ad}: 2.085, $CI_{95\%}$: 1.212-3.589), whereas C>T transition in rs1871054 might have a protective role (OR_{ad}: 0.401, $CI_{95\%}$: 0.227-0.709) (Table 1). Under this dominant model, rs243866 (p_{ad}<0.001) and rs17859821 (p_{ad}=0.004) in *MMP2* were also differentially represented. For both SNPs, the genotypes with the MAF, adenine, were more represented in CI their frequencies were 46.5% and 1.7% in rs243866 and 44.6% and 25.9% in rs17859821, for CI and NCI groups, respectively. Therefore, carrying A in these positions might be associated to an increment of susceptibility to CI (rs243866, OR_{ad}: 49.61, $CI_{95\%}$: 11.62-211.8; rs17859821, OR_{ad}: 2.304, $CI_{95\%}$: 1.301-4.08).

After applying an adjustment assessed by logistic regression under a recessive model, we observed that the likelihood of rs2285053 TT allelotype in *MMP2* carriers developing CI was almost 5 times greater than in CC and CT allelotype carriers (p_{ad}=0.048; OR_{ad}: 4.903, $CI_{95\%}$: 1.017-23.65) (Table 1).

Haplotype Analysis across the *MMP2* region

Haplotype analysis of SNPs corresponding to *ALPP*, *ADAM12* and *MMP9* genes did not showLD, whereas SNPs across *MMP2* did and so haplotype analysis was performed for this gene. In this case, the five SNPs in *MMP2* could be allocated to one block of correlated SNPs showing certain levels of LD (D´=69) (Figure 2). Comparing CI and NCI groups, several haplotypes showed significant differences: GTGGC ($p=5E^{-10}$), ATGGC ($p=4E-^{9}$), GCGGC ($p=1E^{-4}$), ACGGC ($p=2E^{-4}$), GCGTT (p=0.044) and GCATC (p=0.021) (Table 2). Moreover, differences in the first four haplotypes were still significant after 10,000 permutation test with p-values ≤ 0.001; haplotypes ATGGC (CI: 0.162; NCI: 0.008) and ACGGC (CI: 0.059; NCI: 0) showed higher frequencies in CI than in NCI, whereas frequencies of haplotypes GTGGC (CI: 0.02; NCI: 0.218) and GCGGC (CI: 0.013; NCI: 0.102) were higher within the NCI group (Table 2).

Gene-Gene Interaction Analysis

Potential interaction of the examined gene polymorphisms was explored by means of a MDR analytical approach.. Four models were obtained; namely a five-*loci* model rs11244787/rs1871054/rs243866/rs17859821/rs243864 (two SNPs corresponding to *ADAM12* and three to *MMP2* gene) with a BTA=0.7934 and a CVC=9; a three-*loci* model rs1871054/rs243866/rs243864 with BTA=0.7805 and CVC=8; a two-*loci* model rs243866/ rs243864 with BTA=0.7102 and also a CVC=8; and a single-*locus* model rs243866 that had a BTA=0.7241 and a CVC=10. All these models were statistically significant by 1,000-fold permutation test ($p < 0.001$) (Table 3). On the basis of BTA and CVC parameters (OR=17.03, $IC_{95\%}$: 1.81-159.96) we selected the five-*loci* as the best model for its ability to classify and predict disease status reducing the genotypes predictors from ten dimensions to a new one-dimensional multilocus genotype variable (Table 3).

Furthermore, Information gain (IG) was evaluated by the MDR method, to look for the strongest interaction effect between SNPs, as explained in Subjects and Methods. The strongest synergistic interaction was found between rs17859821/ rs2285053 and rs243866/ rs243864, with IG values of 4.57% and 2.16%, respectively, indicating a synergistic effect (Figure 3). Finally, the SNP with the strongest individual main effect was rs243866 (IG=24.14%).

DISCUSSION

In this study, we have examined the association of 11 SNPs located in 4 genes coding for placental expression enzymes, as genetic markers of susceptibility to CI, namely: rs2014683 and rs1048988 of *ALPP*, rs11244787 and rs1871054 of *ADAM12*, rs243866, rs243865, rs17859821, rs243864 and rs2285053 of *MMP2* and rs3918242 and rs2234681 of *MMP9*.

Sample size in our present study is limited as congenital cases have low frequencies, taking into account that CI rate is proximally 4.7% [22], and the difficulties in follow-up to confirm CI; therefore it will require confirmation in larger independent cohorts. Since this is an association

study, we cannot rule out the presence of possible LD with other neighbouring genes that might explain the significant association with CI. Moreover, prospective studies must be carried out before clinical application.

Placental factors

MMPs: MMP proteins play an important role in placental infections. *MMP2* rs243866, rs17859821 and rs2285053 SNPs had different allelic frequencies in CI and NCI children. On the basis of our results of logistic regression, we can conclude that for rs243866 and rs17859821 one or two copies of the mutant allele (A for both) are enough to increase risk of CI, while in the case of rs2285053 two copies of T allele are required to confer susceptibility to acquire CI. The last mutation disrupts a Sp1-type promoter site (CCACC box), displaying a strikingly lower promoter activity, associated to several diseases [23, 24]. rs243866 is another functional polymorphism in *MMP2*, located immediately 5' to a half-palindromic potential estrogen receptor binding site [25]. In estrogen receptor-positive MCF-7 cells the A allele reduces transcription activity and although it is not involved in any known transcription factor binding site s, it has been found to improve the survival of systolic heart failure [26, 27]. Therefore, rs243866 and rs2285053 mutant alleles decrease *MMP2* transcription activity. As host defence against pathogens requires a well regulated inflammatory response, these proteases not only participate in infection processes by degrading extracellular matrix molecules but also cryptic epitopes, regulating inflammatory and immune responses [28]. Taken this into account, a decrease in *MMP2* transcription may produce a reduction in the immune response which might favours susceptibility to infection. Moreover, we showed that there are statistically significant differences in the distribution of some *MMP2* haplogroups between CI and NCI.

Among polymorphisms situated in the *MMP9* promoter region we have selected two described as functional [29]: rs2234681, a microsatellite repeat of $(CA)_n$ and rs3918242. The first one is a multiallelic polymorphism. The length of the CA repeat is closely related to transcriptional activity,

because it increases the binding affinity of the nuclear protein(s) with the promoter [30-32]. This polymorphism has been associated to cardiac [33] and kidney problems [34].

The rs3918242 SNP is a substitution C>T [35]. *In vitro* studies have shown that this mutation results in loss of a recognition site for a transcriptional repressor nuclear protein in this promoter region. It has been shown to be associated in preeclampsia [36], gastric cancer [37] and other large number of diseases; however, we did not observe an association with CI.

ADAM12: ADAM12 is a membrane bound zinc-dependant protease of the ADAM protein family, involved in cell-cell and cell-matrix interactions, playing a role in inflammatory and immune reactions, fertilisation, muscle development and neurogenesis. Plasma concentrations of ADAM12 have been found to be altered in several pregnancy related disorders, and so it has been considered as a useful biomarker in trisomy 21 and trisomy 18, preeclampsia [38] and ectopic pregnancy [39]. Moreover, this protein functions by a mechanism independent of its protease activity to facilitate the activation of transforming growth factor β (TGF-β) signalling that concludes with transcriptional activation. TGF-β has already been implicated in *T.cruzi* host mammalian cells invasion [41], as its presence in infected tissues serves to enhance invasion directly by increasing susceptibility to infection and indirectly by counteracting the effects of antiparasitic cytokines [42]. For this gene, two alternatively spliced transcripts are known, a short secreted and a long membrane-bound form. Our research reveals an important association between the tested SNPs in *ADAM12* and CI as rs11244787 and rs1871054 differ between groups comparing genotype, allelic frequencies and considering a logistic regression under a dominant model. In particular, the protective effect of allele T in rs1871054 was also significant using a recessive model, but the susceptibility effect of rs11244787 was not found under this model. These SNPs have been previously reported as risk markers in other diseases, such as the rs11244787 variant that confers susceptibility to Alzheimer's disease [43, 44] and the rs1871054 variation associated with risk of knee osteoarthritis [45].

PLAP: On the basis of studies demonstrating that PLAP activity is decreased in syncitiotrophoblast of chagasic placentas [46] and in HEp2 cells co-cultivated with *T.cruzi* [47], *ALPP* was selected as gene candidate. Our results did not show an association between *ALPP* polymorphisms and CI. However, some authors [48, 49] have demonstrated that the allelic variants in rs1048988 differed in mRNA levels, being C allele significantly less expressed than G allele. It has been also suggested that it is probably a result of LD with the sequence variation rs2014683 in *ALPP* promoter, which has been shown to have allele-specific binding patterns to placental nuclear proteins. However, LD between these SNPs is not supported by our results, because rs2014683 is polymorphic (MAF=0.263, Table 1) whereas rs1048988 is not.

Gene-gene interaction

Recent studies [20] have proposed that gene–gene interactions may represent an ubiquitous component of the genetic architecture of common diseases. We used MDR analytical approach to identify the best SNP-SNP interaction model with significant differences between CI and NCI groups. This analysis revealed that genotyping of rs11244787, rs1871054, rs243866, rs17859821 and rs243864 sites would be a good predictor of CI.

SNP genotyping techniques

This study shows that the HRM approach allows sensitive, specific, cost-effective and rapid detection of single base polymorphisms in amplified DNA sequences. Moreover, HRM assay can be used efficiently in genotyping samples in association studies, alternatively to PCR-RFLP, technique used until now for genotyping the selected SNPs. Although HRM analysis has proved useful for microsatellite genotyping [50, 51], its implementation for genotyping the rs2234681 microsatellite in the 5´-flanking region of *MMP9* was not possible; probably due to the high number of alleles (eleven) that made resolution of melting curves difficult. Thus, capillary electrophoresis

was showed to be an interesting alternative to polyacrylamide–urea gel electrophoresis followed by silver staining visualisation used in other studies [16].

Final Remarks

Carlier and Truyens have recently proposed to consider CI as an ecological model of multiple and complex interactions between parasites, pregnant women, placenta and foetuses [3]. Some relevant questions remain unsolved, such as those regarding the role of parasite diversity, host genetic and immune responses, as well as that of maternal microbiomes in the establishment of CI.

To our knowledge, the present study is a first approach towards demonstration of an association between mutations in human placental expressed genes and susceptibility to infection.

Supplementary Data

Supplementary Data are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). It consists of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

NOTES

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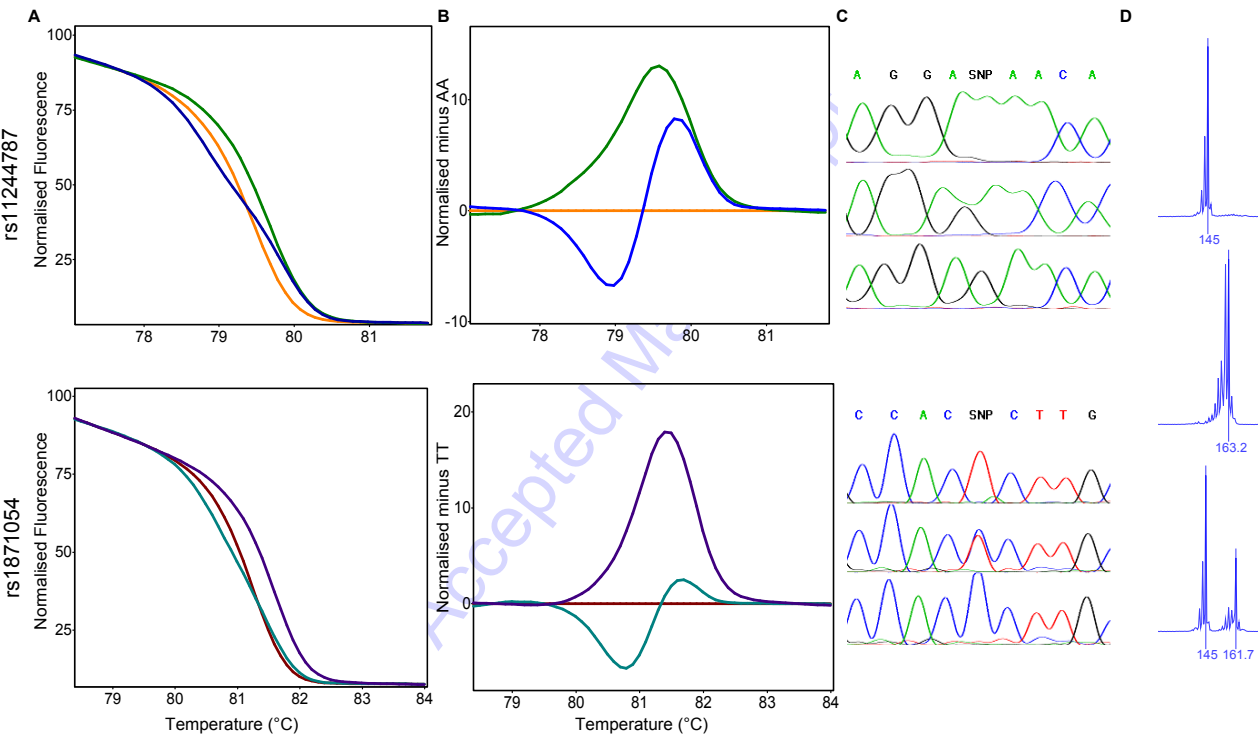
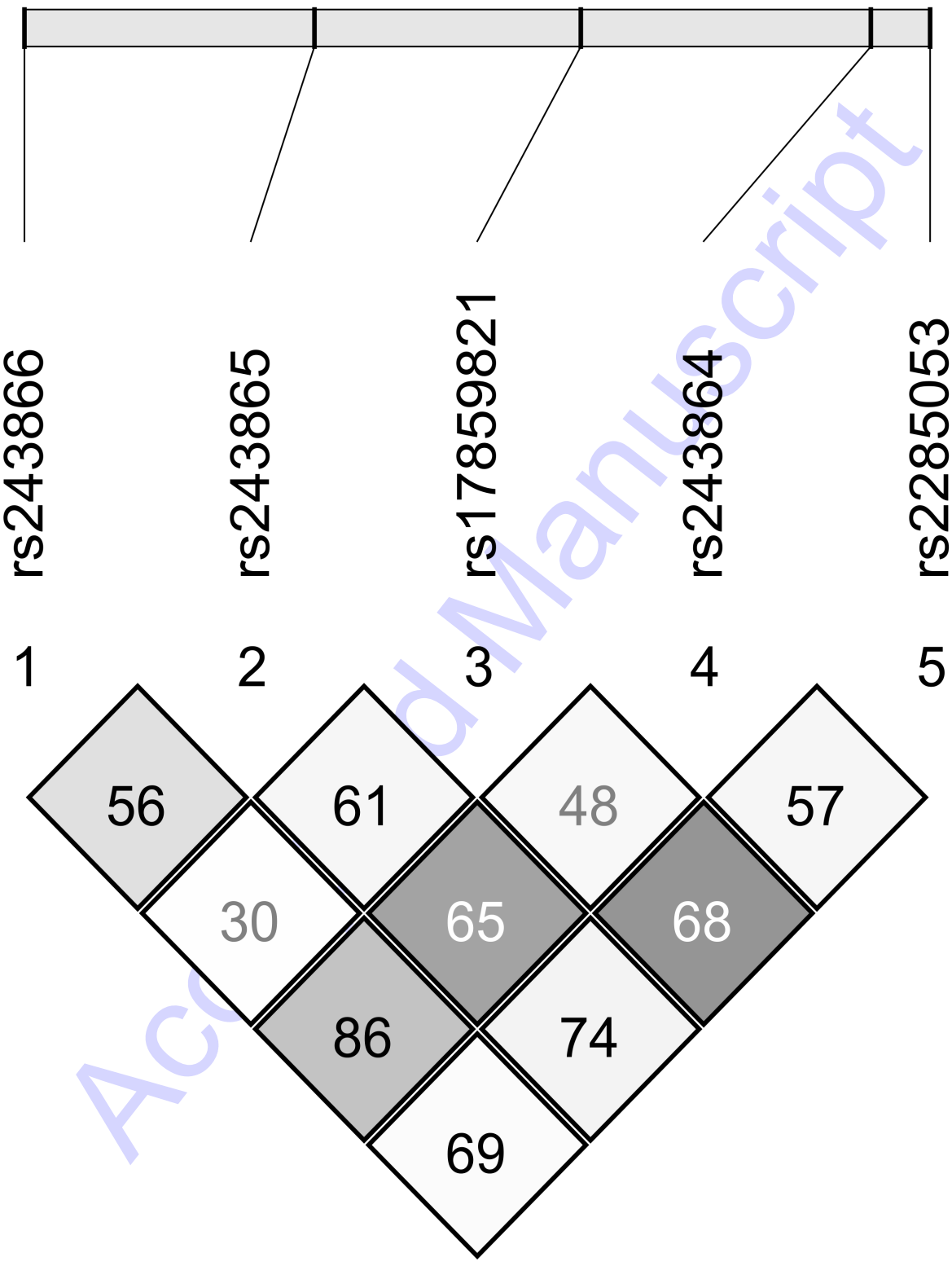
Figure 1. Examples of runs corresponding to *ADAM12* SNPs (rs11244787 and rs1871054). Panel A and B show normalized fluorescence respect to sample concentration or genotype vs Temperature (ºC), respectively. Panel C shows typical amplicon sequencing for each genotype. Panel D corresponds to examples of capillary electrophoresis peaks corresponding to individuals carrying both alleles of CA low copy number of repetitions, LL (top), both high CA copy number, HH (middle), and heterozygote, HL (bottom) in rs2234681 *loci*.

Figure 2. Linkage disequilibrium (LD) pattern of the genomic region in MMP2 gene between SNPs rs243866, rs243865, rs17859821, rs243864 and rs2285053 in the population from this study using Haploview. The positions of the tested SNPs are indicated above Haploview output. The square correlation coefficient (r^2) between specific pair of *MMP2* SNPs is indicated by the colour scheme, where white corresponds to $r^2=0$ and shades in grey to $0<r^2<1$. Numbers in boxes are LD relationships, based on D′ values (normalized linkage disequilibrium measure or D) multiplied by 100; D′ is calculated as D divided by the theoretical maximum for the observed allele frequencies. Values approaching zero indicate absence of LD, and those approaching 100 indicate complete LD.

Figure 3. Interaction map among SNPs in *ALPP, ADAM12, MMP2* and *MMP9* genes and CI. IGs values of individual attribute, main effects, are represented in nodes whereas IGs values of each pair of attributes or interaction effects are shown between nodes.





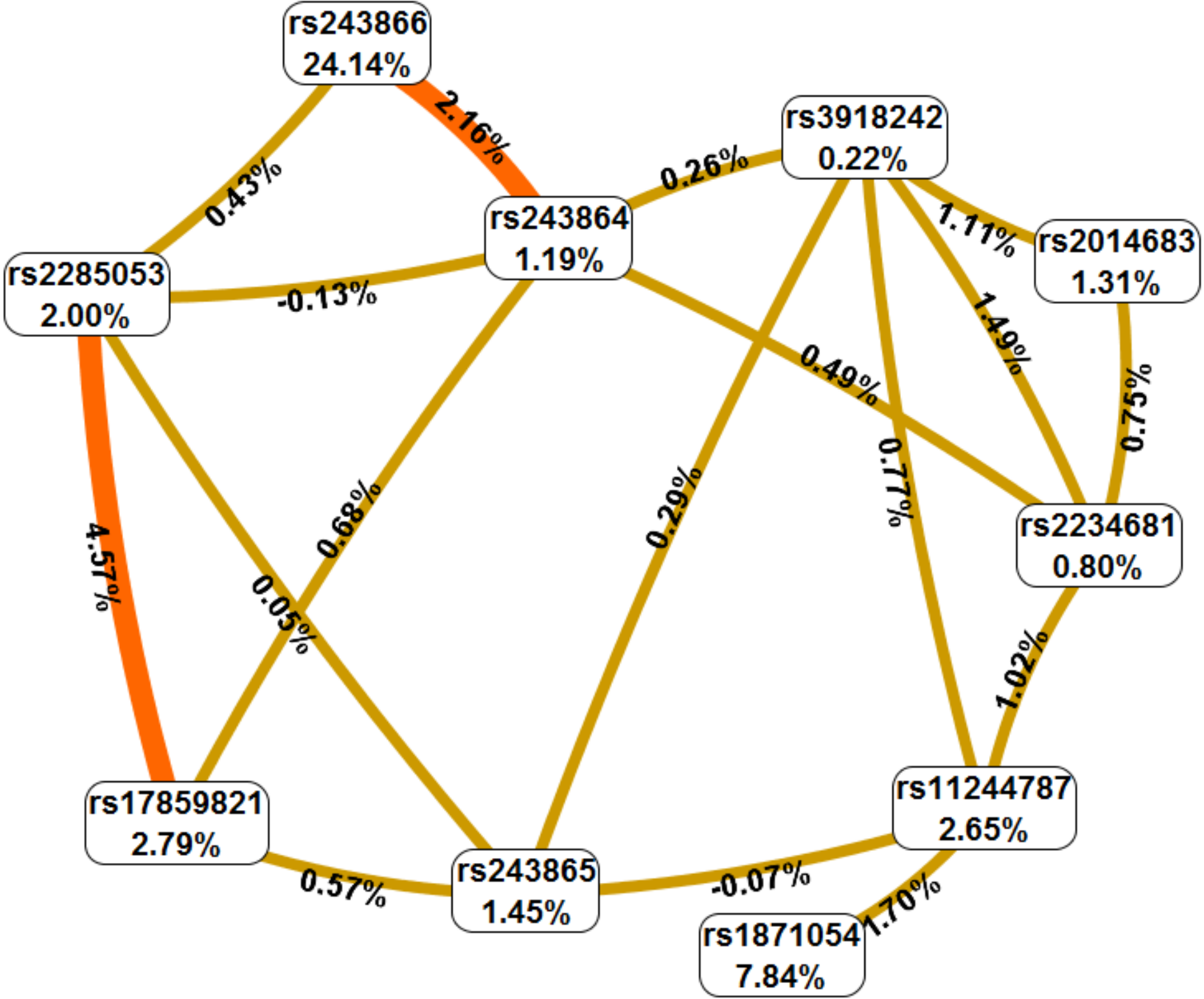

rs243866
24.14%
rs3918242
0.22%
rs243864
1.19%
rs2014683
1.31%
rs2285053
2.00%
rs2234681
0.80%
rs17859821
2.79%
rs11244787
2.65%
rs243865
1.45%
rs1871054
7.84%
2.16%
0.26%
0.43%
1.11%
-0.13%
1.49%
0.49%
0.75%
0.77%
0.68%
0.29%
4.57%
0.05%
1.02%
0.57%
-0.07%
1.70%

Table 1. Genotype and Allele Frequencies Distribution.

SNP ID (*GENE*)	A1	A2	MAF	AF (A_1/A_2)		p^a	GF ($A_1A_1/A_1A_2/A_2A_2$)		p^g	DOMINANT MODEL		RECESSIVE MODEL	
				CI	NCI		CI	NCI		p_{ad}	OR_{ad} (L95- U95)	p_{ad}	OR_{ad} (L95- U95)
rs2014683 (*ALPP)*	G	A	0.263	45/157	69/163	0.078	4/37/60	12/45/59	NA	0.208	0.707 (0.413-1.212)	0.083	0.357 (0.112-1.146)
rs11244787 *(ADAM12*)	G	A	0.332	82/120	62/170	0.002**	21/40/40	13/36/67	0.019*	0.008**	2.085 (1.212-3.589)	0.056	2.08 (0.982-4.407)
rs1871054 (*ADAM12*)	T	C	0.412	60/142	119/113	5,2E-03**	6/48/47	33/53/30	0.019*	0.002**	0.401 (0.227-0.709)	0.086*	0.159 (0.063-0.398)
rs243866 (*MMP2*)	A	G	0.127	53/149	2/230	2,3E-12*	6/41/54	0/2/114	NA	1E-04**	49.61 (11.62-211.8)	0.998	1,97E+12 (NA)
rs243865 (*MMP2*)	T	C	0.279	52/150	69/163	0.354	9/34/58	7/55/54	0.115	0.111	0.646 (0.377-1.105)	0.421	1.523 (0.546-4.25)
rs17859821 (*MMP2*)	A	G	0.191	50/152	33/199	0.005**	5/40/56	3/27/86	NA	0.004**	2.304 (1.301-4.08)	0.365	1.962 (0.457-8.422)
rs243864 (*MMP2*)	G	T	0.318	58/144	80/152	0.198	10/38/53	11/58/47	0.169	0.079	0.617 (0.360-1.057)	0.917	1.049 (0.426-2.583)
rs2285053 (*MMP2*)	T	C	0.173	44/158	31/201	0.021*	8/28/65	2/27/87	NA	0.089	1.662 (0.926-2.983)	0.048*	4.903 (1.017-23.65)
rs3918242 (*MMP9*)	T	C	0.088	17/185	21/211	0.815	1/15/85	2/17/97	NA	0.915	0.961 (0.465-1.986)	0.648	0.57 (0.051-6.381)
rs2234681 (*MMP9*)	H	L	0.376	68/134	95/137	0.118	16/36/49	28/39/49	0.306	0.354	0.776 (0.454-1.327)	0.132	0.592 (0.299-1.171)

Absolute allele (AF) and genotype (GF) frequencies in CI and NCI groups;A_1: allele of minor frequency and A_2 allele of major frequency; χ^2 Test and Fisher's exact test p-values comparing genotypes (p^g) and alleles (p^a) for each SNP analyzed; adjusted p-values (p_{ad}) by using a logistic regression model (dominant or recessive); adjusted Odds ratios (OR_{ad}) and their lower (L95) and upper (U95) limits of the 95% confidence intervals; NA: not applicable (frequencies less than 5 are observed).

* $p<0.05$

** $p<0.01$

Table 2. Distribution of 5-loci *MMP2* haplotypes in CI and NCI groups.

Haplotype	HF_{TOTAL}	CI, NCI	P	p_{per}
GTGGC	0.126	0.020, 0.218	$5E^{-10}$*	<0.001
ATGGC	0.080	0.162, 0.008	$4E^{-9}$*	<0.001
GCGGC	0.060	0.013, 0.102	$1E^{-4}$*	<0.001
ACGGC	0.027	0.059, 0.000	$2E^{-4}$*	0.001
GCGTT	0.043	0.065, 0.025	0.044*	0.123
GCATC	0.038	0.060, 0.018	0.021*	0.256
GCGTC	0.413	0.379, 0.442	0.183	0.802
GTGTC	0.056	0.042, 0.068	0.246	0.913
GCATT	0.114	0.131, 0.099	0.299	0.963

rs243866/rs243865/rs17859821/rs243864/ rs2285053 haplotype total frequencies (HF_{TOTAL}); haplotype frequencies in CI and NCI groups; p-values corresponding to Fisher´s exact test (p) and 10,000-Permutation test (p_{per}).

Table 3. Gene-Gene Interaction findings using multifactor dimensionality reduction (MDR) analytical approach.

Best combination of each model	BTA	CVC	p	Testing OR (L95- U95)
rs243866	0.7241	10	<0.0001	49.61 (0.50-4889)
rs243866,rs243864	0.7102	8	< 0.0001	7.57 (0.94-61.04)
rs1871054,rs243866,rs243864	0.7805	8	< 0.0001	53.89 (18.40-157.83)
rs11244787,rs1871054,rs243866,rs17859821,rs243864	0.7934	9	0.006	17.03 (1.81-159.96)

BTA: Balanced Testing accuracy; CVC: Cross-validation consistency; OR: Odds ratios; Lower (L95) and upper (U95) limits of the 95% OR confidence interval.