

Background:

Multiple Sclerosis (MS) risk has been strongly associated with Human Leukocyte Antigen (HLA)-DRB1 allele groups and infection with Epstein-Barr Virus (EBV), in particular antibodies against viral capsid antigen (VCA) and EBV Nuclear Antigen-1 (EBNA-1). Additionally, a putative B-cell-epitope within the EBNA-1 (EBNA-1_(short)) protein at amino acid positions (aa) 401-411 has been previously identified as a target for antibodies that are specifically enriched in MS patients in discordant identical twins (n=12).

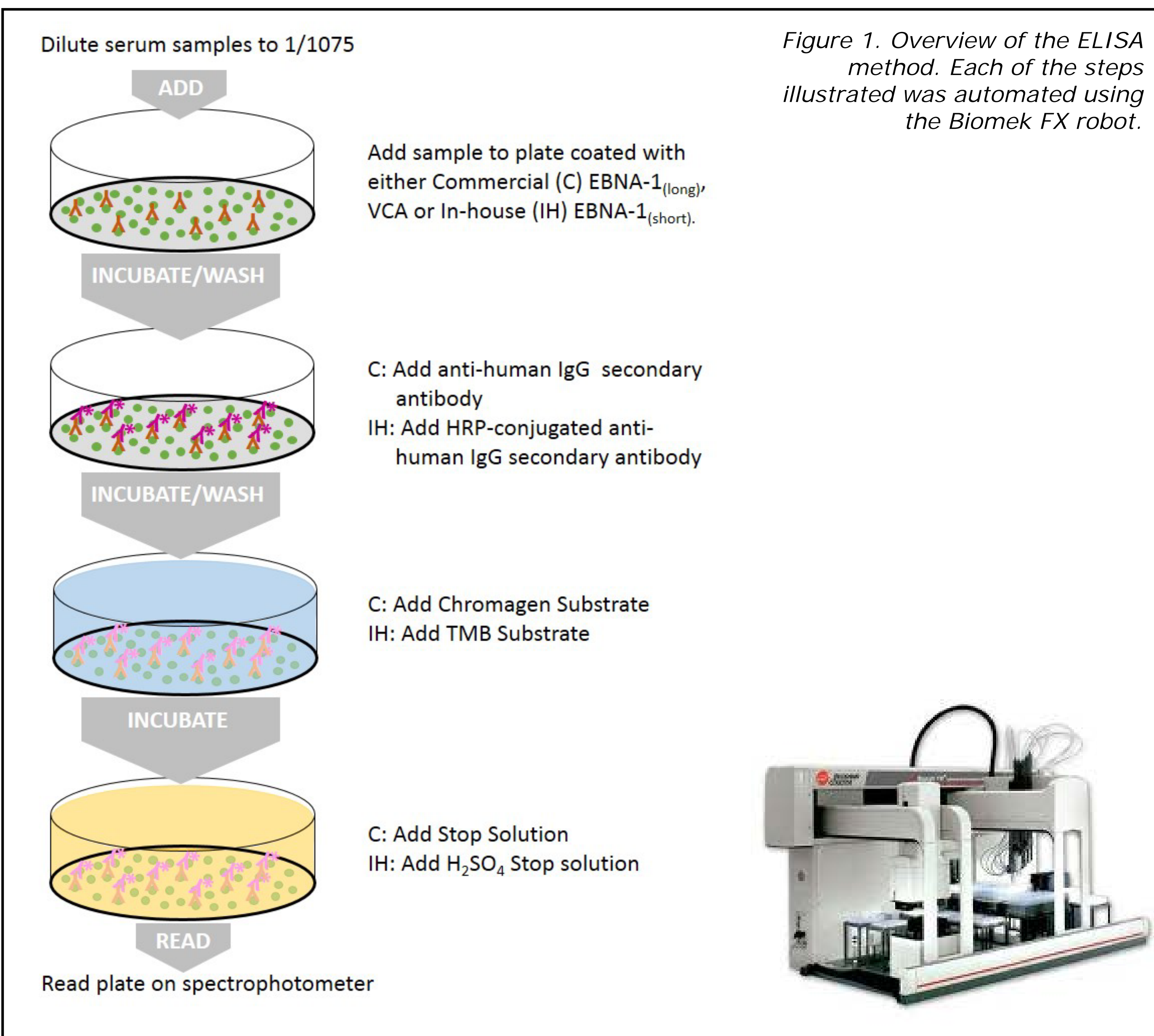
Objectives:

- Investigate antibody reactivity against EBNA-1_(short) as potential MS risk factor using a novel Enzyme-Linked Immunosorbent Assay (ELISA).
- Determine antibody reactivity against EBNA-1_(short) contribution to MS risk in combination with commercial anti-EBNA-1_(long) and anti-VCA ELISAs, genetic risk factors and gender.

Materials and Methods:

Serum samples of MS patients (n=426) and healthy controls (n=186) were collected in Western Australia. All samples were HLA-DRB1 genotyped using sequence based methods. Commercial ELISAs were used to determine levels of immunoglobulin gamma (IgG) antibodies against full length EBNA-1_(long) and VCA of EBV. An in-house ELISA was developed, specific to the EBNA-1_(short) peptide (PPPGRFFHPVGEAD), based on MS EBNA-1 sequencing. The ELISA protocol is summarized in Figure 1.

Statistical analysis was performed using Pearson's Chi-squared tests or Fisher's exact tests as appropriate. ELISA optical density (OD) values were analysed on log (base 10) scale to normalize data, and using multiple linear regression models (ANOVA). Logistic regression was used to analyse joint influence of risk factors and to create a predictive risk model.



Results:

The MS cohort was universally EBV positive, and showed more individuals reactive to all three EBV targets compared to healthy controls ($p < 10^{-10}$). MS patients had significantly higher antibody titres across all ELISAs, when compared with controls ($p < 10^{-15}$). Within the MS cohort, females had higher anti-VCA IgG titres than males ($p = 0.0001$; Figure 2).

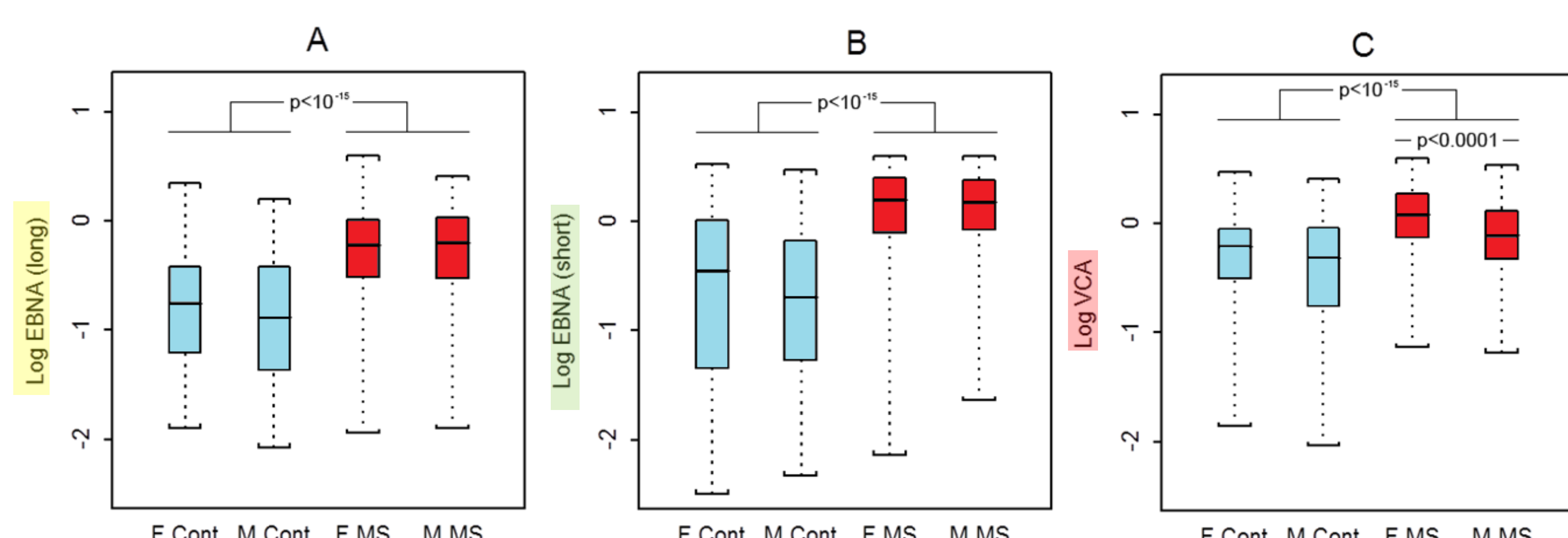


Fig 2. ELISA results for MS patients (red) and healthy controls (blue) stratified by gender (F: female; M: male).

For MS cases and healthy controls, older males had increases anti-EBNA-1_(long) and anti-VCA antibody titres ($p = 0.0009$ and $p = 0.0001$ respectively), while only anti-EBNA-1_(long) antibody titres were increased in younger females ($p = 0.02$).

Individuals with high risk HLA-DRB1 alleles (DRB1*08, *15, *16) had significantly higher antibody titres against EBNA-1_(long) and EBNA-1_(short) in comparison to low-risk carriers ($p < 0.0001$; Figure 3D & E), but not significantly different anti-VCA titres (Figure 3F).

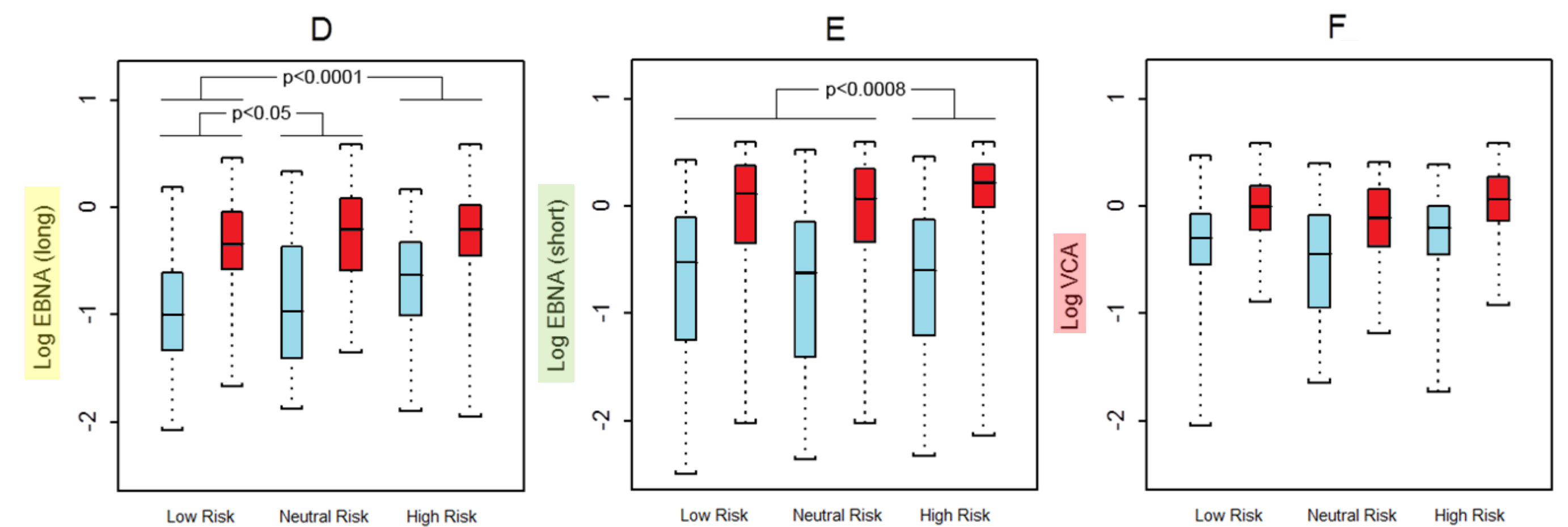


Fig 3. ELISA results for MS patients (red) and healthy controls (blue) stratified according to HLA-DRB1 allele variation.

When combined into a case-control logistic regression, HLA-DR high and low risk groups and gender are significantly associated with MS risk. Interestingly, with the addition of independently significant anti-EBV antibody titres, the high risk HLA-DR risk group becomes non-significant (Table 1), while low risk HLA-DR groups and gender significance are preserved.

Risk Factor	Model 1		Model 2		Model 3	
	Odds	P value	Odds	P value	Odds	P value
HLA-DRB1 High risk group	2.40	0.008	1.40	0.26	1.26	0.47
HLA-DRB1 Low risk group	0.43	0.0013	0.42	0.0061	0.38	0.003
Gender (Female)	3.47	1.7×10^{-7}	2.63	3.5×10^{-5}	2.73	4.0×10^{-5}
EBNA-1 _(long)			6.76	1.8×10^{-14}	3.17	3.0×10^{-5}
VCA			4.96	6.3×10^{-7}	4.30	2.3×10^{-9}
EBNA-1 _(short)					3.47	1.7×10^{-9}

Table 1. Analysis of genetic and serological Multiple Sclerosis risk factors using progressive logistic regressions. EBV values are logged optical density values.

The improvements in predictive ability of the successive models in Table 1 are evident from the receiver-operating characteristic (ROC) curves shown in Figure 4. Combining the independently significant anti-EBNA-1_(long), VCA and EBNA-1_(short) ELISA results with gender and genetic risk factors provided an MS risk model in which cases and controls could be classified with an odds ratio of 21.9 (sensitivity 92%, specificity of 64% corresponding to a logistic score of ≥ 0).

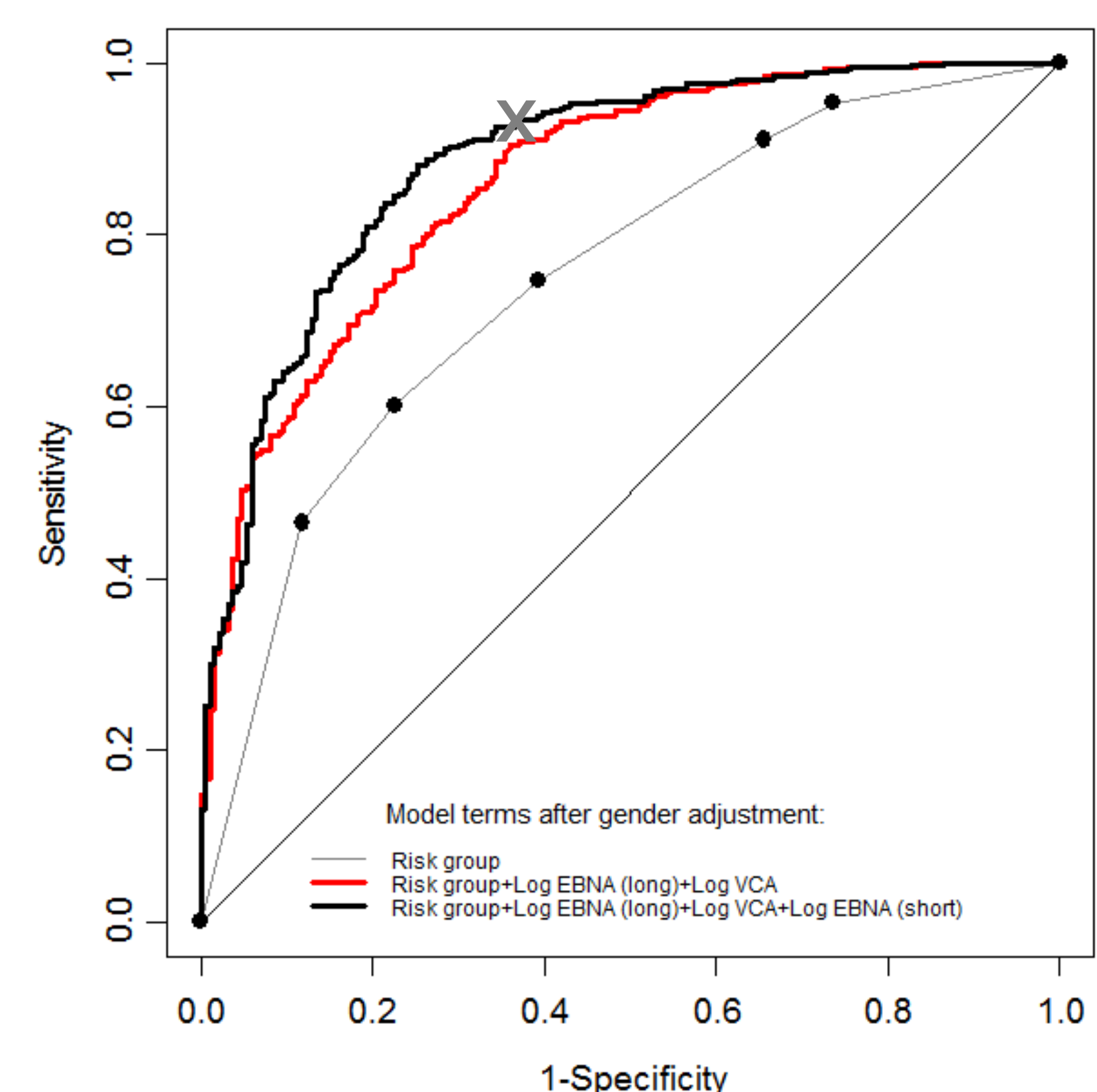


Fig 4. Receiver operating characteristic curve for logistic scores including HLA-DRB1 risk alleles, gender and ELISA values.

Discussion and Conclusions:

Our data showed the strong independent effect of high-risk HLA-DRB1 alleles was substantially abrogated after incorporation of anti-EBNA-1_(long) antibody levels, suggesting these risk factors may share a common pathway in disease susceptibility. Additionally, unlike EBNA-1_(long), EBNA-1_(short) was not influenced by genotype, gender or age. This may explain the disparity in identical twins.

The addition of the novel in-house EBNA-1_(short) ELISA results improved the predictive value independently to the other parameters, however this model would benefit from validation with another cohort.

Including serological data into an MS risk model supports the pathogenic importance of EBV in MS development, and potential for diagnostic and therapeutic targeting.