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# Human Leukocyte Antigen-G: A Promising Prognostic Marker of Disease Progression to Improve the Control of Human African Trypanosomiasis

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***Background.*** Human African trypanosomiasis (HAT) caused by *Trypanosoma brucei gambiense* can be diagnosed in the early hemolymphatic stage (stage 1 [S1]) or meningoencephalitic stage (stage 2 [S2]). Importantly, individuals harbouring high and specific antibody responses to *Tbg* antigens but negative parasitology are also diagnosed in the field (seropositive [SERO]). Whereas some develop the disease in the months following their initial diagnosis (SERO/HAT), others remain parasitologically negative for long periods (SERO) and are apparently able to control infection. Human leucocyte antigen (HLA)-G, an immunosuppressive molecule, could play a critical role in this variability of progression between infection and disease.

***Methods.*** Soluble HLA-G (sHLA-G) was measured in plasma for patients in the SERO (n = 65), SERO/HAT (n = 14), or HAT (n = 268) group and in cerebrospinal fluid for patients in S1 (n = 55), early S2 (n = 93), or late S2 (n = 110). Associations between these different statuses and the soluble level or genetic polymorphisms of HLA-G were explored.

***Results.*** Plasma sHLA-G levels were significantly higher in HAT ($P = 6 \times 10^{-7}$) and SERO/HAT ($P = .007$) than SERO patients. No difference was observed between the SERO/HAT and HAT groups. Within the HAT group, specific haplotypes (HG010102 and HG0103) displayed increased frequencies in S1 ($P = .013$) and late S2 ($P = .036$), respectively.

***Conclusions.*** These results strongly suggest the involvement of HLA-G in HAT disease progression. Importantly, high plasma sHLA-G levels in SERO patients could be predictive of subsequent disease development and could represent a serological marker to help guide therapeutic decision making. Further studies are necessary to assess the predictive nature of HLA-G and to estimate both sensitivity and specificity.

**Keywords.** HLA-G; human African trypanosomiasis; *Trypanosoma brucei gambiense*; susceptibility; genetic association.

More than 95% of human African trypanosomiasis (HAT) (sleeping sickness) cases are due to *Trypanosoma brucei gambiense*. The resulting chronic infection classically starts with an early hemolymphatic stage (stage 1 [S1]), during which trypanosomes proliferate in the blood and lymph associated with nonspecific symptoms [1]. This stage is followed by a meningoencephalitic stage (stage 2 [S2]), in which the parasite invades the central nervous system (CNS) and causes neurological disorders [2]. Without treatment, HAT has long been considered invariably fatal; however, spontaneous healing and asymptomatic carriers have been described [3]. Individuals exhibiting repeated high responses to both the card agglutination test for trypanosomiasis [4] and the specific *T. brucei gambiense* immune trypanolysis test [5] but remaining parasitologically negative (seropositive [SERO]) have been reported [6]. These observations suggest that some subjects can control trypanosome proliferation underlying the existence of a human trypanotolerance/resistance phenomenon, as demonstrated in some West African taurine breeds and inbred mouse models [7].

The variability of responses to *T. brucei gambiense* infection was formerly thought to be related to different genetic strains of parasites, although discrepant results exist [3, 8, 9]. Alternatively, trypanotolerance may result from specific features of the host's immune system, involving different profiles of inflammatory cytokines and chemokines in plasma and CSF [6, 10–12] and polymorphisms of genes encoding inflammatory mediators

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[13, 14]. *T. brucei* infection has been reported to reduce B lymphopoiesis, compromising host humoral immune competence and vaccine-induced memory responses [15, 16]. Altogether, these observations suggest that molecules regulating immunity play an important role in disease progression.

The human leukocyte antigen-G *(HLA-G)* gene encodes an immunomodulatory molecule that is a known ligand for inhibitory receptors present on the surface of immune cells of both innate and adaptive responses [17, 18]. HLA-G plays a crucial role during pregnancy in maternal-fetal tolerance. Increased HLA-G expression in pathological conditions can be either helpful or detrimental [19, 20]. Up-regulation of HLA-G has been observed in CSF in inflammatory CNS disorders [21]. However, the involvement of HLA-G in tropical parasitic infections has not yet been investigated extensively. The first association between soluble HLA-G (sHLA-G) levels and risk of malaria was described in 2014 [22]. A genetic association between polymorphisms at the *HLA-G* 3′ untranslated region (3′UTR) and HAT disease was also demonstrated, emphasizing the relevance of HLA-G in HAT susceptibility [23]. The current study was designed to further investigate the role of HLA-G, at both protein and genetic levels, in the onset and progression of HAT disease.

## METHODS

### Study Population and Phenotype Definition

The study took place in 3 close active HAT foci (Dubreka, Boffa, and Forecariah) located in the mangrove areas of coastal Guinea [24]. All subjects were identified between November 2007 and May 2011, according to the World Health Organization and NCP policies, as described elsewhere [25].

Patients were classified according to the results of serological and parasitological tests (see Supplementary Methods), as follows: (1) the seropositive group (SERO; n = 65) included individuals with positive results of the card agglutination test for trypanosomiasis (end titer 1/8 or more) and the trypanolysis test and negative parasitological results, in whole blood or lymphatic fluid samples at initial diagnosis and repeatedly during a 2-year follow-up; (2) the SERO/HAT group (n = 14) included seropositive individuals whose parasitological test results became positive during their follow-up; and (3) the HAT group included patients with positive serological and parasitological results. The HAT group was further subclassified into stages after CSF examination, as follows: S1 (n = 55) (white blood cell [WBC] count 0–5/μL and no trypanosome detection); early S2 (S2E; n = 93) (WBC count 6–20/μL with or without trypanosome detection or ≤5/μL with trypanosome detection); and late S2 (S2L; n = 110) (WBC count >20/μL, with or without trypanosomes in CSF) (Table 1). Patients in the HAT and SERO/HAT groups were treated according to NCP recommendations. The study sample is described in Table 1. Individuals from 3 foci were included. Although these foci were close to each other and similar in terms of environment, the distribution of the disease and of the age and sex ratios varied between foci owing to differences in human exposure to tsetse flies and intensity of transmission [25–27]. The 3 foci are very similar in terms of microbial exposure (tuberculosis, leprosy, and cholera remain present) and are highly endemic for malaria.

### sHLA-G Quantification

The sHLA-G level in plasma and CSF samples was quantified using a validated enzyme-linked immunosobent assay method with MEM-G/9 [28], which recognizes the most abundant soluble isoforms (shed sHLA-G1 and soluble HLA-G5) and anti-human β2-microglobulin as capture and detection antibodies, respectively (see Supplementary Methods).

### Single-Nucleotide Variation Discovery

*HLA-G* nucleotide sequence variation was evaluated in 51 unrelated and healthy Guineans from the same ethnic group. A 4508–base pair fragment was sequenced, including all variants known to be relevant for the transcriptional and posttranscriptional control of *HLA-G* expression (see Supplementary Methods). A total of 86 variable sites, including 83 single-nucleotide variations (SNVs) and 3 insertions/deletions (indels), were identified (Supplementary Table 1). All of these variants have already been reported in previous studies [29–31].

### Tagging and Genotyping

The Tagger program implemented in Haploview was used to select the subset of markers that efficiently tagged all common *HLA-G* variations (minor allele frequency, ≥0.05) in the sample of 51 Guineans ($r^2 \geq 0.70$). A subset of 25 tag markers was selected with a mean $r^2$ of 0.94 between tagged and tag marker sets. They were genotyped in 309 individuals of the HAT cohort using Sequenom MassARRAY technology (functional genomics platform, Bordeaux, France). The genotype frequencies observed at 5 variable sites (rs115056017, rs114440137, rs149298227, rs114982518, and rs114115214) did not conform with the Hardy-Weinberg equilibrium assumptions ($P = 1.8 \times 10^{-41}$–$1.4 \times 10^{-13}$) and were therefore removed from subsequent analyses. The genotype data of the remaining 20 tag markers were then used to impute the missing genotypes for all *HLA-G* variants previously identified by sequencing, as described below.

### Genotype Imputation

The genotype data of *HLA-G* variants not genotyped in the 309 individuals of the HAT cohort were imputed using MACH 1.0 with default settings and 500 rounds. The *HLA-G* haplotype data of the 51 unrelated healthy Guineans and the 1000 Genome African panel were used as reference haplotypes in the genotype imputation procedure (see Supplementary Methods for a description of haplotype reconstruction and genotype imputation). Very high-quality scores (mean [standard deviation], 0.98 [0.03]) and $r^2$ values (0.92 [0.16]) were obtained for 59 variable sites (Supplementary Table 1), considered of sufficient

**Table 1. Characteristics of Study Participants**

| Phenotypic Group | Sex % (No.) | Age, Mean (SEM), y | Focus, (%)[a] | Exposure Time, Mean (SEM), y[b] | sHLA-G in Plasma, Mean (SEM), ng/mL | sHLA-G in CSF, Mean (SEM), ng/mL | Genetic Data Available, No. |
|---|---|---|---|---|---|---|---|
| SERO (n = 65) | Female: 44.61 (29); Male: 53.85 (35) | 37.68 (1.93) (n = 63) | Boffa: 47.69 (31); Dubreka: 35.38 (23); Forecariah: 16.92 (11) | 32.49 (2.09) (n = 60) | 13.24 (1.97) (n = 65) | NA | 62 |
| SERO/HAT (n = 14) | Female: 50 (7); male: 50 (7) | 24.21 (4.97) (n = 14) | Boffa: 14.29 (2); Dubreka: 42.86 (6); Forecariah: 42.86 (6) | 19.00 (3.72) (n = 13) | 24.05 (5.44) (n = 14) | NA | 14 |
| HAT (n = 268)[c] | Female: 41.79 (112); male: 58.21 (156) | 27.93 (0.98) (n = 267) | Boffa: 48.88 (131); Dubreka: 37.31 (100); Forecariah: 13.81 (37) | 25.13 (1.02) (n = 229) | 38.32 (2.32) (n = 218) | 10.06 (0.72) (n = 218) | 247 |
| S1 (n = 55) | Female: 36.36 (20); male: 63.64 (35) | 28.69 (2.14) (n = 55) | Boffa: 78.18 (43); Dubreka: 14.54 (8); Forecariah: 7.27 (4) | 28.33 (2.21) (n = 48) | 39.55 (5.70) (n = 47) | 8.32 (1.21) (n = 48) | 47 |
| S2 (n = 205)[d] | Female: 43.41 (89); male: 56.58 (116) | 28.09 (1.14) (n = 204) | Boffa: 42.93 (88); Dubreka: 42.93 (88); Forecariah: 14.15 (29) | 24.62 (1.16) (n = 176) | 37.98 (2.51) (n = 179) | 10.53 (0.86) (n = 167) | 192 |
| S2E (n = 93) | Female: 58.06 (54); male: 41.93 (39) | 27.99 (1.61) (n = 93) | Boffa: 49.46 (46); Dubreka: 38.7 (36); Forecariah: 11.83 (11) | 25.88 (1.55) (n = 83) | 37.54 (3.43) (n = 84) | 8.39 (0.91) (n = 79) | 90 |
| S2L (n = 110) | Female: 31.82 (35); male: 68.18 (75) | 28.53 (1.60) (n = 109) | Boffa: 38.18 (42); Dubreka: 45.45 (50); Forecariah: 16.36 (18) | 23.81 (1.72) (n = 91) | 38.64 (3.71) (n = 93) | 12.47 (1.41) (n = 86) | 101 |
| All (n = 333) | Female: 42.34 (141); male: 57.36 (191) | 29.8 (0.90) (n = 330) | Boffa: 48.65 (162); Dubreka: 36.94 (123); Forecariah: 14.41 (48) | 26.66 (0.93) (n = 289) | 32.56 (1.94) (n = 297) | 10.02 (0.72) (n = 219) | 309 |

Abbreviations: CSF, cerebrospinal fluid; HAT, human African trypanosomiasis; NA, not available; S1, stage 1; S2, stage 2; S2E, early S2; S2L, late S2; SEM, standard error of the mean; SERO, seropositive; sHLA-G, soluble human leukocyte antigen-G.

[a] Three HAT mangrove foci in littoral Guinea were considered for this study.

[b] Residence time was defined as the time the individual resided within the HAT focus before diagnosis.

[c] Undetermined S1 or S2 stage for 2 patients with HAT and CSF sHLA-G levels for 6 subjects who developed HAT but were determined to be SERO at the first diagnosis were added in HAT group.

[d] Two patients in the HAT S2 group had an undetermined early or late S2 phenotype.

quality to be confidently included in the genetic association analysis, together with the 20 genotyped tag markers.

### Statistical Analysis

sHLA-G was used both as a quantitative and categorical variable (low, medium, or high), based on the tertiles of the distribution. We used multiple logistic regression to compare the probability of different outcomes (groups or stages) according to the level of sHLA-G and other explanatory variables (age, sex, focus, exposure time, and genetic polymorphisms). When groups (SERO, SERO/HAT, HAT) or stages (S1, S2E, S2L) were used as outcomes, multinomial logistic regressions were performed. However, when the outcome variable was dichotomic (S1 vs S2), a binomial logistic regression was applied. When sHLA-G level was used quantitatively as a dependant variable (or outcome), linear regression was applied.

Multiple logistic regression and linear regression were performed to compare sHLA-G level and parasitological status with the other explanatory variables (age, sex, focus, exposure time). Single-marker and haplotype association tests were carried out under an additive model (Supplementary Methods). All analyses were firstly adjusted on sex, age (in years), HAT foci (Boffa, Dubreka, and Forecariah) and exposure time (in years) (Table 1). All variables were initially introduced into the multivariate model. The final model was selected through a backward procedure, and only covariates with a $P$ value <.05 were retained.

## RESULTS

### sHLA-G Plasma Level According to HAT Group and Stage

The study population and sHLA-G levels are fully described in Table 1. Significantly higher levels of plasma sHLA-G were observed in the HAT ($P = 1.22 \times 10^{-6}$) and SERO/HAT ($P = .027$) groups than in the SERO group (Figure 1*A*). No significant difference in plasma sHLA-G levels was observed between HAT stages (Figure 1*B*). To assess the prognostic value of HLA-G levels in HAT progression, multinomial logistic regression was performed, including age and focus covariates (Table 2). Individuals with medium or high sHLA-G levels had a higher risk of an HAT diagnosis (odds ratio [OR], 2.35 [$P = .014$] and 16.33 [$P = 6 \times 10^{-7}$], respectively) than those with low sHLA-G levels (Table 2). Moreover, seropositive subjects with a high sHLA-G level had nearly 10-fold higher odds of developing HAT (SERO/HAT) than individuals who did not develop the disease (SERO) (OR, 9.87; $P = .007$) (Table 2). The same patterns of results were observed when sHLA-G was used as a quantitative variable. High levels of sHLA-G were also significantly associated with the presence of parasites in plasma (OR, 1.04; 95% confidence interval [CI], 1.02–1.06; $P = 1.3 \times 10^{-6}$), although parasite numbers do not influence sHLA-G level (Figure 1*C*). Age and focus were also associated with parasitological status (data not shown).

### sHLA-G Level in CSF According to HAT Stage

There was no significant difference between CSF sHLA-G levels between S1 and S2 (Table 1, Figure 1*D*, and data not shown). No significant association between sHLA-G and the probability of being diagnosed in S2L was observed when sHLA-G was used as a categorical variable (data not shown). However, among patients in the S2 subgroup, high CSF levels of sHLA-G were associated with a higher probability of developing S2L disease (OR, 1.04; $P = .035$) when sHLA-G was used as a quantitative variable. In contrast, CSF levels of sHLA-G in S1 were not associated with the probability of developing S2E (OR, 0.99; $P = .80$) or S2L (OR, 1.03; $P = .14$) disease (Table 3). Sex and HAT focus were also associated with disease stage (Table 3). High levels of sHLA-G were significantly associated with the presence of parasites (independently of cell count) (OR, 1.05; 95% CI, 1.02–1.08; $P = .003$) (Figure 1*E*). Sex was also associated with parasitological status (data not shown).

### HLA-G Polymorphism and Haplotype Association According to HAT Group and Stage

Twenty *HLA-G* tag markers (17 SNVs, 3 indels) were genotyped in 309 individuals of the HAT cohort. Moreover, the genotype data of 59 additional variants were imputed with sufficiently high accuracy ($r^2 > 0.30$) in the same individuals based on the sequencing data of a reference panel comprising 51 unrelated healthy Guineans and 691 other Africans from the 1000 Genome panel (see Supplementary Methods). Overall, 79 variants (73 SNVs, 6 indels) were tested for their association with both HAT status and stage (Supplementary Table 2). Single-marker association analyses revealed no significant association with either HAT group or stage after correction for multiple testing ($P < .003$) (data not shown).

Strong and significant linkage disequilibrium was observed in the 79 variants tested, which were all included in a single block of strong linkage disequilibrium encompassing the entire *HLA-G* gene (Supplementary Figure 1). A total of 24 extended haplotypes (upstream regulatory, coding, and untranslated region segments) were reconstructed in the study sample, with 10 occurring at a frequency above 2% (Table 4; Supplementary Tables 3–5). No significant association was observed between these different haplotypes and HAT groups (SERO, HAT) and stages (S1, S2E, S2L) after correction for multiple testing (Bonferroni-corrected significance threshold, $P = .007$) (data not shown).

However, suggestive associations (uncorrected threshold, $P = .05$) were identified between some haplotypes and different disease stages (Table 5). *HLA-G* lineage HG010102 was found to be overrepresented in the S1 group compared with the S2E (OR, 0.52; $P = .020$), S2L (0.50; $P = .016$), and pooled S2 groups (0.51; 95% CI, .30–.87; $P = .013$), like the corresponding 010102 5′ upstream regulatory (or promoter) region (5′URR) and 3′ UTR-2 haplotypes (Table 5). Moreover, *HLA-G* lineage HG0103 [32] was overrepresented in the S2L compared with the S2E group (OR, 1.92; $P = .036$), like the corresponding

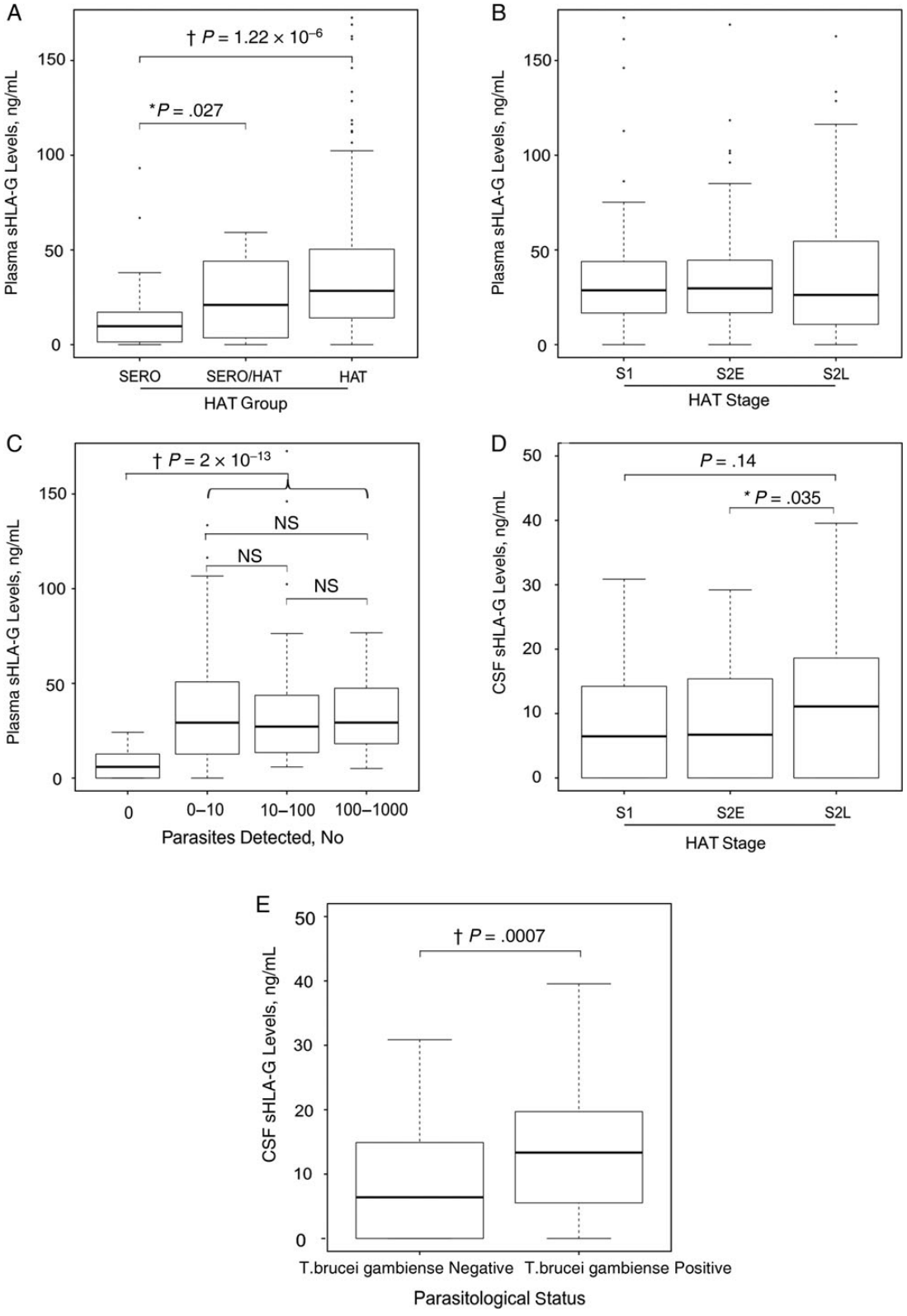


**Figure 1.** Plasma and cerebrospinal fluid (CSF) soluble HLA-G (sHLA-G) profiles by human African trypanosomiasis (HAT) stages and parasitological status. *A,* Plasma levels in seropositive (SERO), SERO/HAT, and HAT groups. *B,* Plasma levels in stage 1 (S1), early stage 2 (S2E), and late stage 2 (S2L) groups. (*C*) Plasma levels in different classes of parasite numbers. *D,* CSF levels in S1, S2E, and S2L groups. *E,* CSF levels in trypanosome-negative and positive detection group. *$P < .05$; †$P < .001$ ($P$ values based on multinomial logistic regression for associations between explanatory variables [sHLA-G level, sex, age, HAT, focus, and exposure time] and HAT group or stage and linear regression for analysis of associations between explanatory variables [parasitological status or parasite count in plasma or CSF, sex, age, HAT, foci, and exposure time] and sHLA-G level with logarithmic transformation). Abbreviations: NS, not significant; *T. brucei gambiense, Trypanosoma brucei gambiense.*

**Table 2. Association Between Human African Trypanosomiasis Group and Soluble Human Leucocyte Antigen-G Level in Plasma**

| Comparison | OR (SE)[a] | 95% CI | *P* Value |
|---|---|---|---|
| HAT vs SERO | | | |
| sHLA-G in plasma (categorical)[b] | | | |
| Low | . . . | . . . | . . . |
| Medium | 2.35 (0.35) | 1.19–4.65 | .014[c] |
| High | 16.33 (0.56) | 5.45–48.91 | $6.06 \times 10^{-7}$[c] |
| Age | 0.97 (0.01) | .95–.99 | .004[c] |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 0.73 (0.35) | .37–1.47 | .38 |
| Forecariah | 0.56 (0.47) | .22–1.40 | .21 |
| Sex | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |
| SERO/HAT vs SERO | | | |
| sHLA-G in plasma (categorical) | | | |
| Low | . . . | . . . | . . . |
| Medium | 1.6 (0.76) | .36–7.10 | .54 |
| High | 9.87 (0.85) | 1.87–52.16 | .007[c] |
| Age | 0.94 (0.02) | .90–.99 | .01[c] |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 3.14 (0.88) | .56–17.73 | .19 |
| Forecariah | 8.3 (0.92) | 1.36–50.80 | .02[c] |
| Sex | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |
| HAT vs SERO/HAT | | | |
| sHLA-G in plasma (categorical) | | | |
| Low | . . . | . . . | . . . |
| Medium | 1.47 (0.73) | .35–6.12 | .59 |
| High | 1.65 (0.69) | .43–6.34 | .46 |
| Age | 1.03 (0.02) | .99–1.07 | .19 |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 0.23 (0.84) | .04–1.21 | .08 |
| Forecariah | 0.07 (0.86) | .01–.36 | .002[c] |
| Sex | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |

Abbreviations: CI, confidence interval; HAT, human African trypanosomiasis; NS, not significant; OR, odds ratio; SE, standard error; SERO, seropositive; sHLA-G, soluble human leukocyte antigen-G.

[a] Multinomial logistic regression was applied to investigate the association between explanatory variables (sHLA-G groups in plasma, sex, age, HAT focus, and exposure time) and status of HAT disease (SERO, SERO/HAT, and HAT groups).

[b] Medium and high levels of sHLA-G ([13.3–32.6] and [32.6–172.6] ng/mL, respectively) were compared with low levels (0–13.3 ng/mL), the reference group.

[c] Significant at $P < .05$.

**Table 3. Association Between Human African Trypanosomiasis Stage and Soluble Human Leucocyte Antigen-G Level in Cerebrospinal Fluid**

| Comparison | OR (SE)[a] | 95% CI | *P* Value |
|---|---|---|---|
| S2E vs S1 | | | |
| sHLA-G in CSF (continuous) | 0.99 (0.02) | .95–1.04 | .80 |
| Sex | 0.41 (0.39) | .19–.89 | .02[b] |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 4.79 (0.48) | 1.87–12.30 | .001[b] |
| Forecariah | 4.09 (0.83) | .80–20.81 | .09 |
| Age | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |
| S2L vs S1 | | | |
| sHLA-G in CSF (continuous) | 1.03 (0.02) | .99–1.08 | .14 |
| Sex | 1.64 (0.41) | .74–3.67 | .22 |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 5.12 (0.48) | 2.00–13.10 | $6.54 \times 10^{-4}$[b] |
| Forecariah | 6.64 (0.8) | 1.39–31.82 | .018[b] |
| Age | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |
| S2L vs S2E | | | |
| sHLA-G in CSF (continuous) | 1.04 (0.02) | 1.00–1.07 | .035[b] |
| Sex | 3.99 (0.34) | 2.05–7.77 | $4.8 \times 10^{-5}$[b] |
| Focus | | | |
| Boffa | . . . | . . . | . . . |
| Dubreka | 1.07 (0.36) | .53–2.15 | .85 |
| Forecariah | 1.62 (0.54) | .56–4.67 | .37 |
| Age | . . . | . . . | NS |
| Exposure time | . . . | . . . | NS |

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; HAT, human African trypanosomiasis; NS, not significant; OR, odds ratio; S1, stage 1; S2E, early stage 2; S2L, late stage 2; SE, standard error; sHLA-G, soluble human leukocyte antigen-G.

[a] Multinomial logistic regression was applied to investigate the association between explanatory variables (sHLA-G in CSF, sex, age, HAT foci, and exposure time) and stages of HAT disease (S1, S2E, and S2L groups).

[b] Significant at $P < .05$.

0103 5′URR, G*01:01:02:01 coding region and 3′UTR-5 haplotypes (Table 5; Supplementary Table 6).

The same analyses were performed by including the CSF sHLA-G level as another explanatory variable. A higher sHLA-G level in CSF and an overrepresentation of the HG0103 haplotype (like the corresponding 5′URR, 3′UTR, and coding region) were found in the S2L compared with the S2E group (uncorrected threshold, $P = .05$), suggesting an independent effect of these variables (Supplementary Table 7).

Finally, we analyzed associations between single markers and *HLA-G* haplotypes with sHLA-G level. No significant association was found with either sHLA-G plasma or CSF level after Bonferroni correction (data not shown).

## DISCUSSION

In this study, we monitored the level of sHLA-G and *HLA-G* polymorphisms in individuals displaying contrasted clinical *T. brucei gambiense* infections outcomes. Plasma sHLA-G levels were significantly higher in the HAT than in the SERO group, who controlled *T. brucei gambiense* infection over long periods. Furthermore, high plasma sHLA-G levels in SERO patients were associated with an increased risk of developing the disease during their follow-up, whereas no difference was observed between the SERO/HAT and HAT patient groups. These results are consistent with the hypothesis that inhibited immunity is associated with progression toward disease after infection. HLA-G is known to exert immunosuppressive effects through

**Table 4. Human Leukocyte Antigen-G Extended Haplotypes, Lineage Structure, and Frequencies in the Guinean Sample (n = 309)**

| *HLA-G* Lineage[a] | 5′URR Haplotype[b] | Coding Haplotype[c] | 3′UTR Haplotype[d] | Frequency | Extended Haplotype[e] |
|---|---|---|---|---|---|
| HG0104 | 0104a | G01:04:01 | UTR-3 | 0.259 | G0104a/G01:04:01/UTR-3 |
| HG010101a | 010101a | G01:01:01:01 | UTR-1 | 0.191 | G010101a/G01:01:01:01/UTR-1 |
| | 010101d | G01:01:01:01new | UTR-1 | 0.021 | G010101a/G01:01:01:01new/UTR-1 |
| HG010102 | 010102a | G01:01:02:01 | UTR-2 | 0.137 | G010102a/G01:01:02:01/UTR-2 |
| | 010102a | G01:05N | UTR-2 | 0.057 | G010102a/G01:05N/UTR-2 |
| | 010102a | G01:01:02:01new | UTR-2 | 0.048 | G010102a/G01:01:02:01new/UTR-2 |
| HG0103 | 0103e | G01:03:01:02 | UTR-5 | 0.11 | G0103e/G01:03:01:02/UTR-5 |
| | 0103d | G01:03:01:02 | UTR-5 | 0.040 | G0103d/G01:03:01:02/UTR-5 |
| HG010101b | 010101f | G01:01:01:04 | UTR-6 | 0.048 | G010101f/G01:01:01:04/UTR-6 |
| HG010101c | 010101b | G01:01:01:05 | UTR-4 | 0.031 | G010101b/G01:01:01:05/UTR-4 |

Abbreviations: HLA-G, human leukocyte antigen-G; URR, upstream regulatory (or promoter) region; UTR, untranslated region.

[a] *HLA-G* lineages were named according to a previous study [32].

[b] *HLA-G* 5′URR haplotypes were named according to nomenclature used in previous studies [29, 32].

[c] *HLA-G* coding haplotypes were named according to nomenclature used in previous studies [29, 32], based on the International Immunogenetics Database (www.ebi.ac.uk/ipd/imgt/hla).

[d] *HLA-G* 3′UTR haplotypes were named according to nomenclature used in previous studies [29, 31, 32].

[e] *HLA-G* extended haplotypes were named according to nomenclature used in a previous study [29]; only extended haplotypes with frequencies ≥0.02 are listed.

its receptors, inhibiting lymphocyte differentiation, proliferation, cytolysis, cytokine secretion, immunoglobulin production and expansion of suppressor T-cell populations [20].

**Table 5. Association Between Human African Trypanosomiasis Stage and Human Leukocyte Antigen-G Haplotype**

| Comparison | OR (SE)[a] | 95% CI | *P* Value |
|---|---|---|---|
| S2E vs S1 | | | |
| *HLA-G* lineage | | | |
| HG010102[b] | 0.52 (0.29) | .29–.92 | .020[c] |
| 5′URR | | | |
| 010102a[b] | 0.52 (0.29) | .29–.92 | .02[c] |
| 3′UTR | | | |
| UTR-2[b] | 0.54 (0.29) | .30–.95 | .033[c] |
| S2L vs S1 | | | |
| *HLA-G* lineage | | | |
| HG010102[b] | 0.50 (0.29) | .28–.88 | .016[c] |
| 5′URR | | | |
| 010102a[b] | 0.50 (0.29) | .28–.88 | .016[c] |
| 3′UTR | | | |
| UTR-2[b] | 0.51 (0.29) | .29–.91 | .021[c] |
| S2L vs S2E | | | |
| *HLA-G* lineage | | | |
| HG0103[d] | 1.92 (0.31) | 1.04–3.52 | .036[c] |
| 5′URR | | | |
| 0103e[d] | 1.92 (0.31) | 1.04–3.52 | .036[c] |
| 3′UTR | | | |
| UTR-5[d] | 1.94 (0.31) | 1.06–3.57 | .030[c] |
| Coding region | | | |
| G*01:03:01:02[d] | 1.92 (0.31) | 1.04–3.5 | .036[c] |

Abbreviations: CI, confidence interval; HAT, human African trypanosomiasis; HLA-G, human leukocyte antigen-G; OR, odds ratio; S1, stage 1; S2E, early stage 2; S2L, late stage 2; SE, standard error; URR, upstream regulatory (or promoter) region; UTR, untranslated region.

[a] Multinomial logistic regression was applied to investigate the association between explanatory variables (HLA-G haplotypes, sex, age, HAT foci, and exposure time) and stages of HAT disease (S1, S2E, and S2L groups). Explanatory variables with or without significant *P* values are presented in Supplementary Table 6.

[b] HAT focus was also associated with disease stage (Supplementary Table 6).

[c] Significant at $P < .05$.

[d] Sex was also associated with disease stage (Supplementary Table 6).

These results are in agreement with the higher levels of interleukin 10 (IL-10) previously observed in HAT and SERO/HAT compared with SERO individuals [6, 10]. A correlation between sHLA-G level and IL-10 expression has already been observed in vivo [33]. As for HLA-G, IL-10 exhibits immunosuppressive properties inhibiting both T-helper 1 and T-helper 2 pathways [33]. In contrast, higher levels of inflammatory interleukin 8 and tumor necrosis factor α were observed in the SERO group than in the SERO/HAT or HAT group [6]. Tumor necrosis factor α was previously shown to be induced ex vivo by *T. brucei gambiense* in human macrophages [34] and was described as having a role in controlling parasite growth through the induction of cytotoxic molecules [35]. Hence, our results are in line with sHLA-G-mediated inhibition of the host immune response associated with greater susceptibility to *T. brucei gambiense* infections. Alternatively, one could hypothesize that sHLA-G is simply induced by trypanosome molecules, but this does not fit with the absence of correlation between plasma sHLA-G levels and parasite density observed in the blood of patients with HAT.

The fact that high sHLA-G plasma levels were associated with a markedly increased risk of subsequent disease development in the SERO group further argues for an early role of sHLA-G in the regulation of protective versus deleterious immune responses against *T. brucei gambiense* . More importantly, this result indicates that sHLA-G in combination with other *T. brucei gambiense* serological tools (eg, the trypanolysis test) may provide useful prognostic tools to identify those individuals who may progress to disease or be a risk to the community by harboring circulating trypanosomes. Such tools would be essential for an effective control and elimination program. Further studies on larger cohorts from other endemic areas are nevertheless required to better characterize the role of *HLA-G* in HAT progression and determine its specificity and sensitivity as a

prognostic tool, because it is known that other infections, such as malaria, are associated with increased sHLA-G levels [22, 36]. Nevertheless, coinfection studies with human trypanosomes are scarce, but these coinfections are thought to be frequent [37]. Animal models showed that helminth–*T. brucei*, and *Plasmodium berghei*–*T. brucei* coinfection could modulate the host's immune response [38] and increase parasitemia in the host [39]. However, their impact on HLA-G expression is unknown but needs to be explored.

Although no significant associations were found between the plasma sHLA-G level and the different disease stages, different profiles of sHLA-G expression in CSF were associated with disease stages, particularly between early and late meningoencephalitic stages. Elevated levels of cytokines with both inflammatory (interleukin 8 and 6) and anti-inflammatory (IL-10) properties were previously reported in CSF of patients with late-stage disease [6, 11, 12]. Late-stage HAT develops when trypanosomes cross the blood–brain barrier, inducing a neuroinflammatory process associated with leukocyte infiltration [40]. Up-regulation of sHLA-G observed in CSF under pathological conditions is produced by macrophages, microglia, endothelial cells, and monocytes [21]. In the CSF, HLA-G up-regulation may have 2 distinct and opposite effects in HAT diseases. On the one hand, HLA-G could facilitate parasite proliferation by inhibiting immune cells, but it may also play a protective role by down-regulating the inflammatory response caused by parasite invasion and thus preventing organ dysfunction.

In this study, no significant associations were found between *HLA-G* polymorphisms and different stages and statuses of disease after correction for multiple testing. However, suggestive associations were observed that deserve further investigation to determine whether they reflect statistical artifacts or true biological findings. The HG010102 *HLA-G* lineage was found to be associated with a lower risk of developing the meningoencephalitic stage of the disease, suggesting a protective role in CNS invasion by parasites. Moreover, the HG0103 *HLA-G* lineage was associated with an increased risk of developing S2L compared with S1 and S2E HAT disease states. Therefore, HG0103 may be involved in the advanced invasion of CNS by parasites responsible for the disease's neurological disorders. In a previous study, UTR-2, and to a lesser extent UTR-5, were found to be significantly overtransmitted to affected offspring in an HAT family-based association analysis [14]. The present results strengthen the relevance of UTR-2 and UTR-5 in HAT disease progression.

Although the genetic study was not conclusive, probably due to lack of statistical power, the HG010102 lineage overrepresented in the S1 group and the HG0103 lineage overrepresented in the S2L group could explain the different levels of sHLA-G in CSF measured in different HAT stages. Nevertheless, *HLA-G* haplotypes may also encode HLA-G proteins (soluble or surface-bound) differently from those detected by our experimental method focusing on sHLA-G1 and G5 isoforms. We also found other variables to be associated with the distribution of the disease, such as focus, sex, and age. These associations are mainly due to differences in bite exposure profiles between and within foci and individual recruitments during the medical survey [25–27].

The present study strongly supports the notion that sHLA-G protein levels and possibly *HLA-G* genetic polymorphisms are implicated in HAT progression. More importantly, the sHLA-G plasma level seems to be a prognostic marker of disease progression in parasitologically unconfirmed seropositive individuals, who are currently sent home untreated in most low-endemic areas, owing to the low positive predictive value of field-applicable serological tests and the long duration and toxicity of available treatments. sHLA-G detection is therefore a promising tool to assist treatment decisions for such individuals and could help improve control of the human reservoir of trypanosomes, on the road to HAT elimination. The possibility of HLA-G involvement in tropical parasitic diseases, as suggested by several recent studies, needs to be explored.

## Supplementary Data

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

## Notes

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## References

1. Blum J, Schmid C, Burri C. Clinical aspects of 2541 patients with second stage human African trypanosomiasis. Acta Trop **2006**; 97:55–64.
2. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet Lond Engl **2010**; 375:148–59.
3. Jamonneau V, Ilboudo H, Kaboré J, et al. Untreated human infections by *Trypanosoma brucei gambiense* are not 100% fatal. PLoS Negl Trop Dis **2012**; 6:e1691.
4. Magnus E, Vervoort T, Van Meirvenne N. A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of *T. b. gambiense* trypanosomiasis. Ann Soc Belg Méd Trop **1978**; 58:169–76.
5. Jamonneau V, Bucheton B, Kaboré J, et al. Revisiting the immune trypanolysis test to optimise epidemiological surveillance and control of sleeping sickness in West Africa. PLoS Negl Trop Dis **2010**; 4:e917.
6. Ilboudo H, Bras-Gonçalves R, Camara M, et al. Unravelling human trypanotolerance: IL8 is associated with infection control whereas IL10 and TNFα are associated with subsequent disease development. PLoS Pathog **2014**; 10:e1004469.

7. Berthier D, Brenière SF, Bras-Gonçalves R, et al. Tolerance to trypanosomatids: a threat, or a key for disease elimination? Trends Parasitol **2016**; 32:157–68.
8. Kaboré J, Koffi M, Bucheton B, et al. First evidence that parasite infecting apparent aparasitemic serological suspects in human African trypanosomiasis are *Trypanosoma brucei gambiense* and are similar to those found in patients. Infect Genet Evol **2011**; 11:1250–5.
9. Jamonneau V, Ravel S, Garcia A, et al. Characterization of *Trypanosoma brucei s.l.* infecting asymptomatic sleeping-sickness patients in Côte d'Ivoire: a new genetic group? Ann Trop Med Parasitol **2004**; 98:329–37.
10. Courtin D, Jamonneau V, Mathieu J-F, et al. Comparison of cytokine plasma levels in human African trypanosomiasis. Trop Med Int Health **2006**; 11:647–53.
11. Lejon V, Lardon J, Kenis G, et al. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of *Trypanosoma brucei gambiense* sleeping sickness patients before and after treatment. Trans R Soc Trop Med Hyg **2002**; 96:329–33.
12. Courtioux B, Boda C, Vatunga G, et al. A link between chemokine levels and disease severity in human African trypanosomiasis. Int J Parasitol **2006**; 36:1057–65.
13. Courtin D, Argiro L, Jamonneau V, et al. Interest of tumor necrosis factor-alpha-308 G/A and interleukin-10 -592 C/A polymorphisms in human African trypanosomiasis. Infect Genet Evol J **2006**; 6:123–9.
14. Courtin D, Milet J, Jamonneau V, et al. Association between human African trypanosomiasis and the IL6 gene in a Congolese population. Infect Genet Evol J **2007**; 7:60–8.
15. Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S. Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog **2008**; 4:e1000078.
16. Bockstal V, Guirnalda P, Caljon G, et al. *T. brucei* infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. PLoS Pathog **2011**; 7:e1002089.
17. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. J Immunol **2001**; 166:5018–26.
18. Bahri R, Hirsch F, Josse A, et al. Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. J Immunol **2006**; 176:1331–9.
19. Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, Rey D, Moreau P. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association. Cell Mol Life Sci **2011**; 68:369–95.
20. Amiot L, Vu N, Samson M. Immunomodulatory properties of HLA-G in infectious diseases. J Immunol Res **2014**; 2014:298569.
21. Wiendl H, Feger U, Mittelbronn M, et al. Expression of the immune-tolerogenic major histocompatibility molecule HLA-G in multiple sclerosis: implications for CNS immunity. Brain J Neurol **2005**; 128:2689–704.
22. Sadissou I, d'Almeida T, Cottrell G, et al. High plasma levels of HLA-G are associated with low birth weight and with an increased risk of malaria in infancy. Malar J **2014**; 13:312.
23. Courtin D, Milet J, Sabbagh A, et al. HLA-G 3' UTR-2 haplotype is associated with human African trypanosomiasis susceptibility. Infect Genet Evol **2013**; 17:1–7.
24. Camara M, Kaba D, KagbaDouno M, Sanon JR, Ouendeno FF, Solano P. Human African trypanosomiasis in the mangrove forest in Guinea: epidemiological and clinical features in two adjacent outbreak areas [in French]. Med Trop **2005**; 65:155–61.
25. Ilboudo H, Jamonneau V, Camara M, et al. Diversity of response to *Trypanosoma brucei gambiense* infections in the Forecariah mangrove focus (Guinea): perspectives for a better control of sleeping sickness. Microbes Infect **2011**; 13:943–52.
26. Courtin F, Jamonneau V, Camara M, et al. A geographical approach to identify sleeping sickness risk factors in a mangrove ecosystem. Trop Med Int Health **2010**; 15:881–9.
27. Kagbadouno MS, Camara M, Rouamba J, et al. Epidemiology of sleeping sickness in Boffa (Guinea): where are the trypanosomes? PLoS Negl Trop Dis **2012**; 6: e1949.
28. Rebmann V, Lemaoult J, Rouas-Freiss N, Carosella ED, Grosse-Wilde H. Report of the Wet Workshop for Quantification of Soluble HLA-G in Essen, 2004. Hum Immunol **2005**; 66:853–63.
29. Castelli EC, Ramalho J, Porto IOP, et al. Insights into HLA-G genetics provided by worldwide haplotype diversity. Front Immunol **2014**; 5:476.
30. Gineau L, Luisi P, Castelli EC, et al. Balancing immunity and tolerance: genetic footprint of natural selection in the transcriptional regulatory region of HLA-G. Genes Immun **2015**; 16:57–70.
31. Sabbagh A, Luisi P, Castelli EC, et al. Worldwide genetic variation at the 3' untranslated region of the HLA-G gene: balancing selection influencing genetic diversity. Genes Immun **2014**; 15:95–106.
32. Castelli EC, Mendes-Junior CT, Veiga-Castelli LC, Roger M, Moreau P, Donadi EA. A comprehensive study of polymorphic sites along the HLA-G gene: implication for gene regulation and evolution. Mol Biol Evol **2011**; 28:3069–86.
33. Rizzo R, Hviid TVF, Stignani M, et al. The HLA-G genotype is associated with IL-10 levels in activated PBMCs. Immunogenetics **2005**; 57:172–81.
34. Daulouède S, Bouteille B, Moynet D, et al. Human macrophage tumor necrosis factor (TNF)-alpha production induced by *Trypanosoma brucei gambiense* and the role of TNF-alpha in parasite control. J Infect Dis **2001**; 183:988–91.
35. Stijlemans B, Guilliams M, Raes G, Beschin A, Magez S, De Baetselier P. African trypanosomosis: from immune escape and immunopathology to immune intervention. Vet Parasitol **2007**; 148:3–13.
36. d'Almeida TC, Sadissou I, Cottrell G, et al. Evolution of the levels of human leukocyte antigen G (HLA-G) in Beninese infant during the first year of life in a malaria endemic area: using latent class analysis. Malar J **2016**; 15:78.
37. Kagira JM, Maina N, Njenga J, Karanja SM, Karori SM, Ngotho JM. Prevalence and types of coinfections in sleeping sickness patients in Kenya (2000/2009). J Trop Med **2011**; 2011:248914.
38. Chiejina SN, Wakelin D, Goyal PK. Trypanosome-induced modulation of responses to concurrent helminth infection. Res Vet Sci **2003**; 74:47–53.
39. Ademola IO, Odeniran PO. Co-infection with *Plasmodium berghei* and *Trypanosoma brucei* increases severity of malaria and trypanosomiasis in mice. Acta Trop **2016**; 159:29–35.
40. Kennedy PGE. The continuing problem of human African trypanosomiasis (sleeping sickness). Ann Neurol **2008**; 64:116–26.