

Haptoglobin polymorphism, iron metabolism and mortality in HIV infection

Joris R. Delanghe, Michel R. Langlois, Johan R. Boelaert*,
Jos Van Acker, Filip Van Wanzeele, Guido van der Groen[†],
Robert Hemmer[‡], Chris Verhofstede, Marc De Buyzere,
Dirk De Bacquer[§], Vic Arendt[‡] and Jean Plum

Background: Three phenotypes of the antioxidant protein haptoglobin are known: Hp 1-1, Hp 2-1 and Hp 2-2.

Objectives: To investigate the outcome of HIV infection according to haptoglobin type.

Design and methods: Haptoglobin phenotypes were determined using starch gel electrophoresis in serum obtained from 653 HIV-infected Caucasians in the AIDS reference centers of Gent (n = 184), Antwerp (n = 309), and Luxembourg (n = 160). Survival was compared between haptoglobin types using Kaplan–Meier curves. Plasma HIV-1 RNA was quantified by reverse transcriptase PCR. Serum iron, transferrin saturation, ferritin, and vitamin C were assayed to evaluate iron-driven oxidative stress in 184 HIV-infected patients and 204 controls.

Results: The haptoglobin type distribution amongst the patients (17.6% Hp 1-1, 49.9% Hp 2-1, 32.5% Hp 2-2) corresponded to that of the controls. Kaplan–Meier curves showed a higher mortality for the Hp 2-2 group ($P = 0.0001$; adjusted mortality risk ratio, 1.78; 95% confidence interval, 1.25–2.54). Median survival time was 11.0 years (Hp 1-1 and Hp 2-1) versus 7.33 years (Hp 2-2). Plasma HIV-1 RNA levels prior to antiviral therapy and their increase over 1 year were highest in Hp 2-2 patients ($P = 0.03$ and 0.003 , respectively). The Hp 2-2 type was associated with higher serum iron, transferrin saturation, and ferritin levels and with low vitamin C concentrations. Furthermore, ferritin concentrations were higher in HIV-infected patients than in controls ($P < 0.0001$).

Conclusion: HIV-infected patients carrying the Hp 2-2 phenotype show a worse prognosis, which is reflected by a more rapid rate of viral replication (in the absence of antiviral treatment). They also accumulate more iron and oxidize more vitamin C, suggesting that less efficient protection against haemoglobin/iron-driven oxidative stress may be a direct mechanism for stimulating viral replication.

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From the Department of Clinical Chemistry, Microbiology and Immunology, University Hospital, Gent, the *Unit for Renal and Infectious Diseases, General Hospital St Jan, Brugge, the [†]Institute of Tropical Medicine, Antwerp, the [‡]Department of Infectiology, Centre Hospitalier, Luxembourg, and the [§]Department of Public Health, University Hospital, Gent, Belgium.

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Requests for reprints to: Prof. Dr J. Delanghe, Department of Clinical Chemistry, Microbiology, and Immunology (1B2), University Hospital, De Pintelaan 185, B-9000 Gent, Belgium.

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Introduction

Following HIV infection, life expectancy shows a broad variation [1]. Possible causes to explain the large survival differences are to be found either in a different pathogenicity among the infecting HIV strains or in a more or less effective anti-HIV immune response [2]. Some attention has been paid to potentially relevant genetic factors of the host, such as MHC class I and II genes and genes coding for chemokine receptors [3]. Until now, a possible role of the genetic polymorphism of haptoglobin in determining the prognosis of HIV infection has not been examined. Haptoglobin is an acute phase plasma protein with haemoglobin-binding capacity [4]. Due to the existence of two different alleles (*Hp1* and *Hp2*), three main (pheno)types exist: Hp 1-1, Hp 2-1, and Hp 2-2 [4]. The main physiological function of haptoglobin is clearing free haemoglobin from the plasma. Following haemolysis, haptoglobin-haemoglobin complexes are formed, which are rapidly taken up by the hepatocytes [5]. Consequently, haptoglobin has been proposed to have an antioxidant function by preventing the haem iron-mediated generation of free radicals [6,7].

Several functional differences exist between the haptoglobin phenotypes [4,8]. Amongst them, Hp 2-2 is reported to have a less efficient haemoglobin-binding capacity and hence a weaker antioxidant function than Hp 1-1 and Hp 2-1. A lesser degree of clearance of free haemoglobin by Hp 2-2 may result in some retention of iron in Hp 2-2 subjects. Oxidative stress, induced by iron excess or other causes, is associated with vitamin C depletion [9-11]. Consequently, we recently found that vitamin C concentrations are lower in healthy individuals with an Hp 2-2 type compared with the other haptoglobin types [12].

In the present study, we investigated iron-driven oxidative stress in HIV-infected patients according to haptoglobin phenotype using serum concentrations of iron, iron-binding proteins (transferrin, ferritin), and vitamin C. Serum transferrin saturation as well as ferritin concentration reflect the degree of iron storage. Thus, iron overload is associated with high transferrin saturation and ferritin levels [10]. Since excess iron potentially enhances oxidative stress, activates HIV replication, and impairs already compromised immune defence mechanisms [13-15], we compared the plasma HIV-1 load and the outcome of HIV infection among patients with different haptoglobin phenotypes.

Materials and methods

Subjects

The study population comprised 653 Caucasian patients (545 men, mean \pm SD age 38 ± 11 years; 108

women, mean \pm SD age 37 ± 12 years) with proven HIV infection. Patients were treated in three different regional AIDS reference centers: Gent ($n = 184$), Antwerp ($n = 309$), and Luxembourg ($n = 160$). Median follow-up time was 48 months. Blood sampling was performed during a period of clinical stability. Concomitantly, a group of 204 healthy age-matched Caucasian controls from the same region (108 men mean \pm SD age 38 ± 12 years; 96 women, mean \pm SD age 40 ± 11 years) were studied. Patients and controls taking iron/vitamin C preparations and patients recently transfused (≤ 2 months prior to sampling) were excluded. All subjects gave informed consent to participate in the study, which was approved by the ethical committee of the University Hospital of Gent.

Biochemical assays

The haptoglobin phenotype of the subjects was determined using starch gel electrophoresis of haemoglobin-supplemented serum followed by peroxidase staining [16]. Further assays were performed on serum samples from patients and controls in the subgroup from Gent. Serum haptoglobin, transferrin, and ferritin concentrations were measured using fixed-time immunonephelometry with a BN II nephelometer (Behringwerke AG, Marburg, Germany) according to the International Federation of Clinical Chemistry standards [17,18]. Serum iron was assayed spectrophotometrically using commercial reagents with a Hitachi 747 analyser (Boehringer Mannheim, Mannheim, Germany). Serum concentrations of total vitamin C were measured with a Hitachi 911 analyser (Boehringer) using the method described by Beutler [19]. Blood was obtained by venipuncture between 0800 and 1000 h after overnight fasting during the last follow-up visit (August-October 1996). To avoid pre-analytical vitamin C degradation *in vitro*, blood was sampled in small test tubes (4 ml) containing commercial clotting activators (Terumo, Haasrode, Belgium), allowed to clot for 15 min, and centrifuged ($10\,000\text{ g}$ for 10 min) at 4°C . The supernatant serum was immediately put into the Hitachi 911 analyser, which was operated in emergency testing mode. Intra-individual variation of serum vitamin C was $< 5\%$.

Determination of CD4+ cell counts

In all HIV-infected patients, peripheral blood was simultaneously collected into tubes containing $\text{K}_2\text{-EDTA}$ anticoagulant. The CD4+ T-cell subsets were analysed by flow cytometry [20], making use of the TriTEST reagent and a FACSort flow cytometer (both from Becton Dickinson, Mountain View, California, USA). The fractional rate of peripheral CD4+ T-cell loss was calculated and compared according to the haptoglobin type of the patients.

HIV-1 RNA assay

The HIV-1 RNA-specific sequences were quantified

using the Amplicor HIV Monitor reverse transcriptase PCR assay (Roche Diagnostic Systems, Inc., Branchburg, New Jersey, USA) [21] in K_2 -EDTA-plasma samples. Reverse transcriptase PCR was performed on the first plasma sample available prior to antiviral therapy in patients from Gent ($n = 75$), and was monitored during 1995 in patients from Gent ($n = 52$) and Luxembourg ($n = 58$) who were not receiving protease inhibitors. The assay made use of *rTth* DNA polymerase (from *Thermus thermophilus*) and a quantification standard (QS) RNA with primer binding sites identical to those on the HIV-1 target. A 142 base-pair sequence in the *gag* gene of HIV-1 was recognized by a pair of primers (SK462/SK431) and amplified over 30 cycles. The HIV-1 and QS amplicons were quantified in separate wells of a microwell plate coated with HIV-1-specific (SK102) and QS-specific oligonucleotide probes, respectively, making use of an avidin-horseradish peroxidase conjugate followed by a colorimetric reaction for horseradish peroxidase [21].

Statistical analysis

Results are given as means \pm SD. The validity of the Hardy-Weinberg equilibrium was tested using the χ^2 test. Statistical survival analysis was performed by Kaplan-Meier estimates and log-rank testing. Comparisons of biochemical parameters and viral loads among haptoglobin type groups were carried out using the Kruskal-Wallis test. Comparison of data between patients and controls was performed using the Mann-Whitney U test. Correlations between data were examined using regression analysis. Statistical significance was considered at the level of $P < 0.05$.

Results

Haptoglobin phenotypes in controls and patients

The haptoglobin phenotype distribution in the control population (16.7% Hp 1-1, 48.0% Hp 2-1, 35.3% Hp 2-2) resulted in an *Hp1* allele frequency of 0.407 and was in agreement with the Hardy-Weinberg equilibrium. In the HIV-infected population, 115 patients (17.6%; 99 men and 16 women) showed an Hp 1-1 type, 326 (49.9%; 272 men and 54 women) showed an Hp 2-1 type, and 212 (32.5%; 174 men and 38 women) showed an Hp 2-2 type. This corresponded to a relative *Hp1* allele frequency of 0.426, which was in agreement with the Hardy-Weinberg equilibrium and with results obtained in healthy controls.

Survival after HIV infection

The Kaplan-Meier curves for 653 HIV-infected patients showed a significantly higher mortality for the

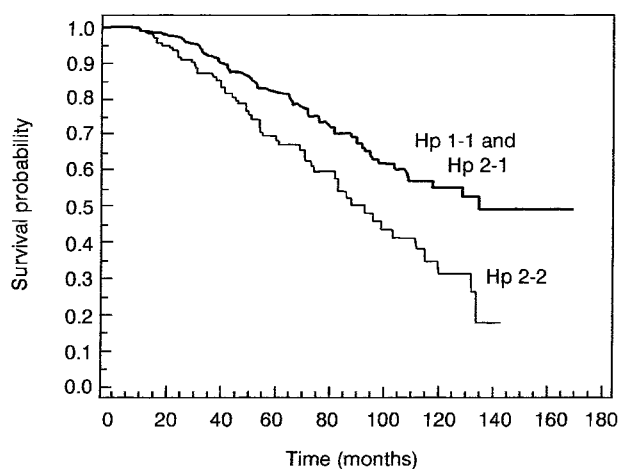


Fig. 1. Kaplan-Meier plot comparing survival following HIV infection in Hp 2-2 patients ($n = 212$) versus patients carrying another haptoglobin phenotype ($n = 441$; $P = 0.0001$).

Hp 2-2 group than for the other haptoglobin types (log-rank test for homogeneity of survivor function, $P = 0.0001$; Fig. 1). The median survival time was 11.0 years for Hp 1-1 and Hp 2-1 patients versus 7.33 years for Hp 2-2 patients. The mortality rate was 37.7 per 1000 patient-years for the Hp 1-1 group, 45.1 per 1000 patient-years for the Hp 2-1 group, and 79.2 per 1000 patient-years for the Hp 2-2 group, corresponding to a risk ratio of 1.67 (95% confidence interval, 1.13–2.48). Median age of patients at diagnosis of HIV infection did not differ according to haptoglobin type: 37.9 years (Hp 1-1, Hp 2-1) versus 37.7 years (Hp 2-2). After adjustment for age and sex, an adjusted risk ratio of 1.78 (95% confidence interval, 1.25–2.54) was calculated.

Viral load

Plasma HIV-1 RNA levels obtained prior to any anti-retroviral therapy showed significant differences ($P = 0.03$) according to the haptoglobin type of the patients: $3.75 \pm 1.01 \log_{10}$ RNA copies/ml (Hp 1-1, $n = 14$), $4.64 \pm 0.73 \log_{10}$ copies/ml (Hp 2-1, $n = 35$), and $5.26 \pm 0.82 \log_{10}$ copies/ml (Hp 2-2, $n = 26$). HIV-1 RNA load monitored during 1995, prior to the utilization of protease inhibitors, showed a more pronounced increase in Hp 2-2 patients ($0.52 \pm 0.38 \log_{10}$ RNA copies/ml; $n = 39$) compared with Hp 1-1 and Hp 2-1 patients ($0.28 \pm 0.30 \log_{10}$ RNA copies/ml per year; $n = 71$; $P = 0.003$; Fig. 2).

Evolution of CD4+ T-cell counts

The evolution of the peripheral blood CD4+ T-cell count during HIV infection did not differ significantly ($P = 0.919$) between the haptoglobin phenotypes: the fractional rates of CD4+ T-cell loss were 0.010 ± 0.031 per day (Hp 1-1), 0.007 ± 0.016 per day (Hp 2-1), and 0.010 ± 0.027 per day (Hp 2-2).

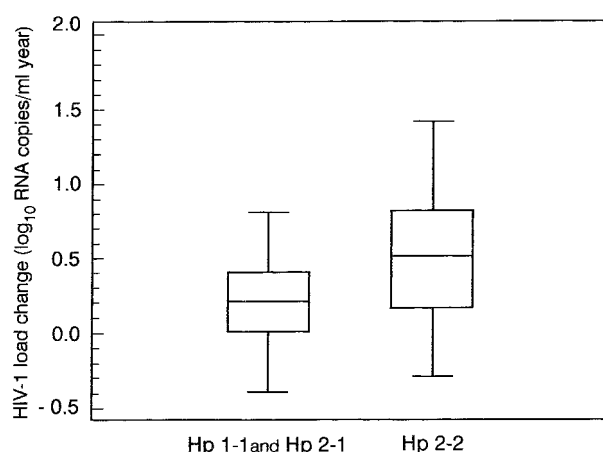


Fig. 2. Box-and-whisker plot comparing the change in plasma HIV-1 RNA load over 1 year according to haptoglobin phenotype in 110 HIV-infected patients (39 Hp 2-2 types versus 71 other types). The boxes show medians and quartiles; the whiskers are 10th and 90th percentiles ($P = 0.003$).

Iron status

For the various haptoglobin types, the serum haptoglobin concentration measured in clinically stable HIV-infected patients corresponds with the haptoglobin type-adjusted reference values in Caucasians [4], as found in the control group. Serum iron concentration and transferrin saturation differed significantly between haptoglobin types, showing the highest levels in HIV-infected patients carrying the Hp 2-2 phenotype (Table 1). Similar results were obtained in the control population (Table 2). In all HIV-infected patients,

serum ferritin concentrations were significantly increased compared with those of healthy controls ($P < 0.0001$). Serum ferritin levels were also dependent on the haptoglobin type of both patients and controls ($P = 0.006$ and 0.003 , respectively) and were found to be highest in the Hp 2-2 subpopulations. For all parameters mentioned above, the observed differences were statistically significant in both men and women.

Vitamin C status

The concentration of total vitamin C in serum of HIV-infected patients was dependent on their haptoglobin type ($P = 0.009$), showing lower levels in Hp 2-2 patients (Table 1). Similar associations were obtained in the control population (Table 2) [12]. In HIV-infected patients, serum vitamin C concentrations were lower than those in controls ($P = 0.002$). These differences and associations were statistically significant in both men and women.

Discussion

The observed haptoglobin phenotype frequencies amongst HIV-infected patients corresponded with those of the reference population (*Hp1* allele frequency, 0.407) or other data obtained in Western Europe [4]. This suggests that the distribution of haptoglobin phenotypes in HIV-infected patients is comparable to that of the reference population, sug-

Table 1. Serum concentrations (mean \pm SD) of haptoglobin, iron, transferrin, ferritin, and vitamin C according to the haptoglobin type of 184 HIV-infected patients, measured in a period of clinical stability.

	Hp 1-1 (n = 28)	Hp 2-1 (n = 101)	Hp 2-2 (n = 55)	<i>P</i> *
Men/women	23/5	85/16	46/9	
Age at HIV diagnosis (years)	37 \pm 8	38 \pm 10	36 \pm 11	NS
Haptoglobin (g/l)	1.32 \pm 0.45	1.14 \pm 0.78	0.80 \pm 0.46	0.004
Iron (μ mol/l)	16.1 \pm 5.9	18.3 \pm 7.0	20.6 \pm 7.7	0.04
Transferrin (g/l)	2.74 \pm 0.59	2.79 \pm 0.39	2.69 \pm 0.52	NS
Transferrin saturation (%)	29 \pm 10	32 \pm 13	38 \pm 14	0.03
Ferritin (μ g/l)	92 \pm 89	154 \pm 115	270 \pm 193	0.006
Vitamin C (μ mol/l)	58.3 \pm 13.6	54.3 \pm 12.5	47.1 \pm 10.8	0.009

*Kruskal-Wallis. NS, Not significant.

Table 2. Serum concentrations (mean \pm SD) of haptoglobin, iron, transferrin, ferritin, and vitamin C according to the haptoglobin type of the control population (n = 204).

	Hp 1-1 (n = 34)	Hp 2-1 (n = 98)	Hp 2-2 (n = 72)	<i>P</i> *
Men/women	18/16	52/46	38/34	
Age (years)	38 \pm 15	39 \pm 11	39 \pm 12	NS
Haptoglobin (g/l)	1.26 \pm 0.43	1.08 \pm 0.50	0.84 \pm 0.42	0.005
Iron (μ mol/l)	16.6 \pm 6.1	18.6 \pm 7.7	21.3 \pm 8.6	0.04
Transferrin (g/l)	3.03 \pm 0.41	3.01 \pm 0.49	2.98 \pm 0.51	NS
Transferrin saturation (%)	27 \pm 9	30 \pm 10	35 \pm 12	0.03
Ferritin (μ g/l)	51 \pm 35	64 \pm 45	85 \pm 59	0.003
Vitamin C (μ mol/l)	61.5 \pm 16.5	63.7 \pm 10.8	49.9 \pm 8.5	0.01

*Kruskal-Wallis. NS, Not significant.

gesting equal probabilities of becoming infected by HIV in the three haptoglobin phenotypes.

Our study demonstrated for the first time that the outcome of HIV infection greatly differs according to the various haptoglobin phenotypes. Patients with Hp 2-2 had an increased rate of mortality compared with those bearing Hp 1-1 or Hp 2-1. The difference in median survival time between Hp 2-2 and other patients (3.67 years) was not only statistically significant but it also carried clinical significance.

The *Hp* allele frequencies are known to present marked geographical differences [4], with a minimal *Hp1* allele frequency in south-east Asia (0.10) and a maximal frequency in the indigenous population of South America (0.80). These geographical data should be taken into consideration when evaluating the course of HIV infections in different ethnic groups. For this reason, only Caucasians were included in the present study cohort.

The viral loads measured in our study were comparable to those reported in previous studies [22–24]. Prior to antiviral therapy, HIV-1 load was related to the haptoglobin phenotype, the highest number of HIV-1 RNA copies being found in plasma of Hp 2-2 patients. Furthermore, the increase in HIV-1 RNA load over 1 year was more pronounced in patients with Hp 2-2 compared with those with other haptoglobin types. Because HIV-1 RNA load reflects the overall replication of the virus *in vivo* [25], these findings suggest the presence of a higher viral replication rate in Hp 2-2 patients. Both the baseline level of HIV-1 RNA and the change in HIV-1 RNA load have been shown to be accurate predictors of survival independent of CD4+ T-cell count [25]. In our study cohort, the rate of loss of CD4+ T cells was comparable amongst the three haptoglobin phenotypes. We therefore suggest that the worse prognosis of Hp 2-2 patients can be explained by a higher rate of viral replication in this subgroup of patients.

Serum haptoglobin concentrations obtained in clinically stable HIV-infected patients were comparable with the haptoglobin type-dependent reference values, which is in agreement with the data of Grunfeld *et al.* [26]. The lowest haptoglobin concentrations were found in serum from Hp 2-2 patients. Due to the difference in molecular mass between the haptoglobin types (170–900 kDa for Hp 2-2 versus 86–300 kDa for Hp 2-1 and only 86 kDa for Hp 1-1) [4], the type-related difference in serum haptoglobin concentration on a molar basis was even more pronounced [8]. As a consequence, quantitative binding of free haemoglobin is less in Hp 2-2 plasma than in Hp 1-1 or Hp 2-1 plasma.

A less efficient removal of free haemoglobin from the plasma results in some degree of haem iron accumula-

tion, as shown by the association of Hp 2-2 with higher serum iron, higher transferrin saturation, and higher serum ferritin level observed in both the healthy control population and the HIV-infected group. This excess iron in turn induces an iron-driven peroxidation of vitamin C [12,27]. Indeed, in the present cohort of HIV-infected patients, lower serum vitamin C concentrations were found in the Hp 2-2 group than in the Hp 1-1 and Hp 2-1 groups. Similar associations were obtained in controls and were reported in a previous study [12]. Hp 2-2 subjects have lower haptoglobin concentrations and hence a less efficient haemoglobin-binding capacity [4], which may favour an iron-mediated vitamin C depletion. Vitamin C is a powerful antioxidant (free radical scavenger) [28], but paradoxically has a pro-oxidant activity in the presence of Fe^{3+} [27,29]. Iron is trapped within ferritin as Fe^{3+} [10], and vitamin C enters the pores of the ferritin protein where it converts Fe^{3+} to Fe^{2+} , itself becoming oxidized [29]. Fe^{2+} then leaks out of the ferritin protein and generates free radicals (Fenton reaction) [6,29]. Vitamin C depletion has also been described in other pathological conditions associated with iron overload, such as thalassaemia and haemochromatosis [11].

Oxidative stress induced by reactive oxygen radicals stimulates HIV replication through the activation of the nuclear transcription factor NF- κ B, and contributes to the development of cell damage and immunodeficiency [30–32]. Iron excess has been proposed to be potentially deleterious in HIV infection, where it may increase oxidative stress and activate the replication of HIV [14]. On the other hand, vitamin C suppresses HIV reverse transcriptase activity and viral replication in chronically HIV-infected cells [33,34]. Therefore, the combination of higher iron-driven oxidative stress and vitamin C depletion may contribute to the increased HIV replication observed in Hp 2-2 patients. However, this is still a matter of controversy as there is also some evidence that low vitamin C concentrations may increase HIV replication and that the inhibitory effect is observed at much higher concentrations of vitamin C [35]. Moreover, there might be an influence by the cell type itself on the effect of vitamin C on HIV replication [35]. Demonstrable effects of haptoglobin types on viral replication, free radical scavengers, and lipid-soluble antioxidants are needed to confirm our findings.

In conclusion, the Hp 2-2 phenotype provides evidence for a genetic predisposition to higher oxidative stress, which hastens the development of death in HIV-infected patients. Iron accumulation, vitamin C oxidation, and viral replication are more pronounced in Hp 2-2 patients, probably due to a less efficient protection against haemoglobin/iron-driven oxidative stress. Further studies are needed to establish the requirement and the possible benefit of vitamin C supplementation

to HIV-infected patients carrying the Hp 2-2 type. More importantly, we suggest that the latter subpopulation should be targeted for strategies aiming at limiting iron accumulation as well as oxidative stress [14,36–38].

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