RESEARCH ARTICLE

Persistence of full-length caspase-12 and its relation to malaria in West and Central African populations

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ABSTRACT. ***Background*. The full-length (L-) variant of caspase-12 is believed to predispose to sepsis. It has been replaced in the genome of most human populations by the (S-) variant, which leads to premature termination of translation. Strikingly, the L-allele is still widely prevalent in African populations, presumably due to a counterbalancing selective force specific to this continent, for which malaria is a prime candidate.** ***Methods*. We investigated associations between caspase-12 genotype and malarial parameters in three West-African populations, in studies encompassing immunological, clinical and obstetric data.** ***Results*. The caspase-12 L-allele was found at frequencies of 11-34%.** ***Plasmodium falciparum*-stimulated mononuclear cells from S/L heterozygote donors produced stronger interferon-γ and interleukin-10 responses than S/S homozygotes ($p = 0.011$ and $p = 0.023$ in uninfected and infected donors respectively). Nevertheless, we found no association between caspase-12 genotype and either the presentation of severe malaria or individual clinical parameters in sick children. Amongst pregnant women, the caspase-12 genotype did not influence peripheral or placental malaria infection, or basic obstetric parameters. Interestingly, perinatal mortality was more frequent in children of both S/S and L/L than S/L mothers, independent of placental *P. falciparum*-infection.** ***Conclusion*. We find little clinical or epidemiological evidence that malaria has contributed to the persistence of functional caspase-12 in Africa, suggesting either that alternative selective forces are at work or that genetic drift underlies its current global distribution.**

Keywords: caspase-12, *P. falciparum* malaria, genetic selection, cytokines, pregnancy

Caspase-12 is a member of the large family of cysteine proteases involved in apoptotic and inflammatory pathways [1], although it has limited proteolytic activity of its own [2]. Recently, caspase-12 has been implicated in the regulation of the pro-inflammatory cytokine IL-1β, through the inhibition of caspase-1-mediated cleavage of pro-IL-1β to IL-1β [3] and through the inhibition of NOD/RIP signalling [4]. Mice lacking the caspase-12 gene generated a stronger pro-inflammatory response than wild-type animals [5]. In man, two allelic forms of the caspase-12 locus exist, the long (L-) and short (S-) variants. The former encodes for a full-length protein, whereas the latter encodes for a product that is prematurely terminated during translation. The emergence of the S-variant represents a relatively recent event in human evolution, since this variant is not found in chimpanzee populations [6]. Clinical studies have found an association between the L-variant of caspase-12 and an increased risk of sepsis [7]. Whereas in Caucasian populations exclusively the S-variant of caspase-12 is found, in African populations the L-variant is still common [8]. The distinct global distribution of caspase-12 alleles on the one hand suggests selection of the inactive S-variant outside of Africa, presumably due to its protective role in sepsis [9]. On the other hand, maintenance of the functional L-variant in sub-Saharan Africa implies a

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comparative advantage of this allele against some major selective force specific to this continent.

Malaria remains responsible for high morbidity and mortality in large parts of the developing world, but nowhere more so than in sub-Saharan Africa, that suffers, by far, the highest transmission intensities. This burden, combined with its target of particularly young, pre-pubescent children, means that the disease has exerted a strong selective pressure on human populations for at least the last 10,000 years. Such selective pressure is visible today in the genetic make-up of humans from malaria-endemic areas, more specifically in the presence of allelic variants or other genetic polymorphisms that confer some measure of protection against malaria [10]. Indeed some of the oldest and best-studied human genetic variations, those of the haemoglobin genes, including the HbS and HbC variants and α-and β-thalassaemias, are found in African populations as a result of selection by malaria [11]. In addition to red blood cell phenotypes, malaria has also exerted strong selective pressure on genes involved in both the adaptive [12, 13] and innate immune response [14-18].

The strong selective pressure malaria is known to have exerted on other genes, particularly in African populations, suggests that it may also have played a role in the maintenance of full-length caspase-12 on this continent. In the present paper, we have combined caspase-12 genotype data from three separate studies investigating susceptibility to malaria in different African populations, and assessed associations with immunological, clinical and obstetrical data.

DONORS AND METHODS

Mali study

Blood samples for functional assays were collected in the Koro district of Mali as part of investigational studies into inter-ethnic differences in susceptibility to malaria. This study site has been described in detail elsewhere [19]. Briefly, it is a rural Sahelian area with intense malaria transmission exclusively during the rainy season (June-September). Samples for this study were collected both during the 2006 rainy season (September) and the 2007 dry season (April). The study included healthy Dogon or Fulani adult male volunteers and patients with symptoms of uncomplicated malaria. A thick blood film was made from each participant; slide-negative patients were excluded from the study. No positive blood films were recorded during the April 2007 inclusion. Approval for the study was provided by the institutional review boards of the University of Stockholm (National Ethical Committee no. 03 536) and the University of Bamako (N°0527/FMPOS).

Cameroon study

This was a hospital-based study, set up to investigate inflammatory parameters in relation to clinical severity of malaria, details of which are published elsewhere [20]. Briefly, the samples for this study were collected from children aged between eight months and 14 years (mean age 5.8) presenting at the outpatient clinic or admitted to the Central Hospital of Yaounde, Cameroon, with symptoms of malaria. Based on a positive thick blood smear for *Plasmodium falciparum* and a complete physical examination, patients were classified by clinical presentation according to the 2000 World Health Organization (WHO) standard [21] as uncomplicated or complicated/severe malaria. The number of patients categorised as suffering from mild and complicated/severe disease was 100 and 38, respectively. Clinical parameters at admission, including temperature, heart rate and respiratory rate were recorded, and venous whole blood was collected for a complete blood count. Informed consent was provided in all cases by a parent or guardian.

Ghana study

This study on the diagnosis and manifestation of placental malaria in pregnancy has been described in detail [22]. The study area is characterized by perennial and hyper- to holoendemic malaria transmission. Briefly, 889 women attending the Presbyterian Mission Hospital in Agogo, Ghana for delivery, were recruited after informed, written consent was obtained. Peripheral venous blood and, following delivery, placental intervillous blood were collected and examined for *P. falciparum* by microscopy, immuno-chromatographic test (ICT Malaria Pf/Pv, Becton Dickinson, Germany) and nested PCR assays [23]. Haemoglobin (Hb) was measured using a HemoCue photometer (Ångelholm, Sweden), and anaemia defined as Hb < 11 g/dL. Crude birth weight and gestational age were assessed within 24 hours of delivery. Low birth weight (LBW) was defined as < 2,500 g, and preterm delivery (PD) as gestational age < 37 weeks applying the Finnström score [24]. The study protocol was reviewed and approved by the Committee on Human Research Publications and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi.

Cytokine stimulation assay

In the Mali study, venous blood was collected from both healthy volunteers and patients into lithium heparin vacutainers. Further manipulations took place in Bandiagara, approximately three hours from the study sites; all samples were worked-up on the day of collection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll, washed 3 x in cold RPMI, counted and resuspended in complete culture medium [RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate, 50 μg/mL gentamicine and 10% pooled human AB^+ serum (Sanquin, Nijmegen, NL)], for a final concentration of 2.5 x 10^6/mL. PBMCs were transferred into 96-well, round-bottom plates and were immediately stimulated *in duplo* with cryopreserved NF54 strain *P. falciparum*-infected erythrocytes (PfRBC), uninfected erythrocytes (uRBC) at 5 x 10^6/mL final, or RPMI only. Cells were incubated for 21 hours at 37°C, following which cell supernatants were collected and stored at - 80°C for subsequent cytokine measurement by ELISA. Cell supernatants were analysed for cytokine production using commercially available ELISA kits according to the manufacturers' instructions: IFN-γ, IL-10 (Sanquin, Nijmegen, NL) and IL-1β (R&D, Abingdon, UK).

Caspase-12 genotyping

DNA was extracted from whole blood using either the Puregene isolation kit (Gentra Systems, Minneapolis, MN, USA) or the QIAmp blood mini-kit (Qiagen, Hilden, Germany). Primers flanking the T125C polymorphism region and the methodology were adapted as described by Saleh *et al.* [7]. Sequencing was performed on either a 48-capillary 3730 sequencer (Applied Biosystems) or a Lightcycler 480 (Roche). Genotypes were analysed using the software 4Peaks by A. Griekspoor and Tom Groothuis, (mekentosj.com).

Statistics

Data were analysed in SPSS; differences in genotype distributions between populations were assessed using the χ^2-test or Fisher's exact test. Differences in clinical parameters between groups were analysed using the Kruskal-Wallis test. Differences in cytokine responses to PfRBC between groups were analysed using the Mann-Whitney test after subtracting the response to uRBC; negative values were set to zero. P-values of < 0.05 were considered statistically significant in all analyses.

RESULTS

Distribution of caspase-12 alleles in African populations

Table 1 presents the varying caspase-12 genotype- and allele-frequencies amongst the African populations studied. Frequencies of the L-allele as high as 34% were observed. None of the populations studied showed evidence of violation of the Hardy-Weinberg equilibrium. Caspase-12 genotype-frequencies differed significantly ($p < 0.05$) between all populations studied except those from Cameroon and Dogon ($p = 0.13$); similarly, allele-frequencies differed between all populations except those from Ghana and Dogon ($p = 0.12$).

Mali study: cytokine profile amongst caspase-12 genotypes

During the malaria transmission season, parasite prevalence differed between caspase-12 genotypes within the Fulani ethnic group [S/S, 13/31 (41.9%); S/L, 11/26 (42.3%); L/L, 10/11 (90.9%); $p = 0.012$], but not within the sympatric Dogon [S/S, 20/42 (47.6%); S/L, 9/15 (60.0%); $p = 0.41$]. Irrespective of ethnicity, no effect of the caspase-12 genotype was found on clinical parameters, including parasite density or haemoglobin (Hb) levels (data not shown).

Ex vivo stimulation assays were performed with PBMCs from 33 uninfected and 31 *P. falciparum*-infected donors in September 2006, and a further 38 uninfected volunteers in April 2007. Cytokine production by volunteer peripheral blood mononuclear cells (PBMCs) stimulated *ex vivo* with *P. falciparum*-infected erythrocytes (PfRBC) is presented in *figure 1*. Interferon-γ (IFN-γ) and interleukin-10 (IL-10) responses to uninfected-red blood cells (uRBC) or to medium alone were generally below the detection limit of the assay (IFN-γ, 3.1 pg/mL; IL-10, 4.7 pg/mL). IFN-γ, but not IL-10, responses to PfRBC were significantly stronger in uninfected than in infected volunteers ($p < 0.001$ and $p = 0.29$, respectively), and therefore cytokine responses were analysed separately by infection status; cytokine responses did not differ between uninfected volunteers in the rainy and dry season. IFN-γ responses to PfRBC were stronger in caspase-12 S/L carriers than in S/S carriers for the infected group ($p = 0.011$), but not for the uninfected group, although the pattern remained similar *(figure 1)*. IL-10 responses were higher in S/L than in S/S carriers among uninfected donors ($p = 0.023$) but not among infected donors, although again the pattern remained similar. Furthermore, these patterns were observed individually in both Fulani and Dogon populations and during both the transmission- and non-transmission seasons (data not shown). Cytokine data were only available for three infected L/L carriers; both IFN-γ and IL-10 appeared slightly lower than in S/L carriers. We also analysed the IFN-γ /IL-10 ratio as an estimate of the overall pro/anti-inflammatory balance. This ratio was lower in S/L than in S/S carriers for both uninfected and infected donors, although not significantly so. Interleukin-1β (IL-1β) production was also assessed, but responses to PfRBC were barely measurable above the detection limit of the assay (40 pg/mL IL-1β) (data not shown).

Cameroon study: clinical presentation amongst caspase-12 genotypes

Table 2 presents the distribution of caspase-12 genotypes amongst Cameroonian children with uncomplicated or severe malaria. Amongst the 38 patients with severe malaria, most had symptoms of cerebral malaria [31 were in coma (Blantyre score < 3) and 10 suffered repeated convulsions], nine were in severe respiratory distress and

Table 1
Distribution of caspase-12 genotypes and allele-frequencies in the populations studied

Population	N	Caspase-12 genotype[a]			Allele frequency	
		S/S[b]	S/L[b]	L/L[b]	S	L
Cameroon	138	81 (58.7%)	53 (38.4%)	4 (2.9%)	0.78	0.22
Ghana	885	699 (79.0%)	172 (19.4%)	14 (1.6%)	0.89	0.11
Mali Dogon	81	56 (69.1%)	25 (30.9%)	0 (0%)	0.85	0.15
Mali Fulani	91	42 (46.2%)	37 (40.7%)	12 (13.2%)	0.66	0.34

[a] All populations are in Hardy-Weinberg equilibrium.
[b] S: truncated variant; L: full-length variant. Data represent n (%).

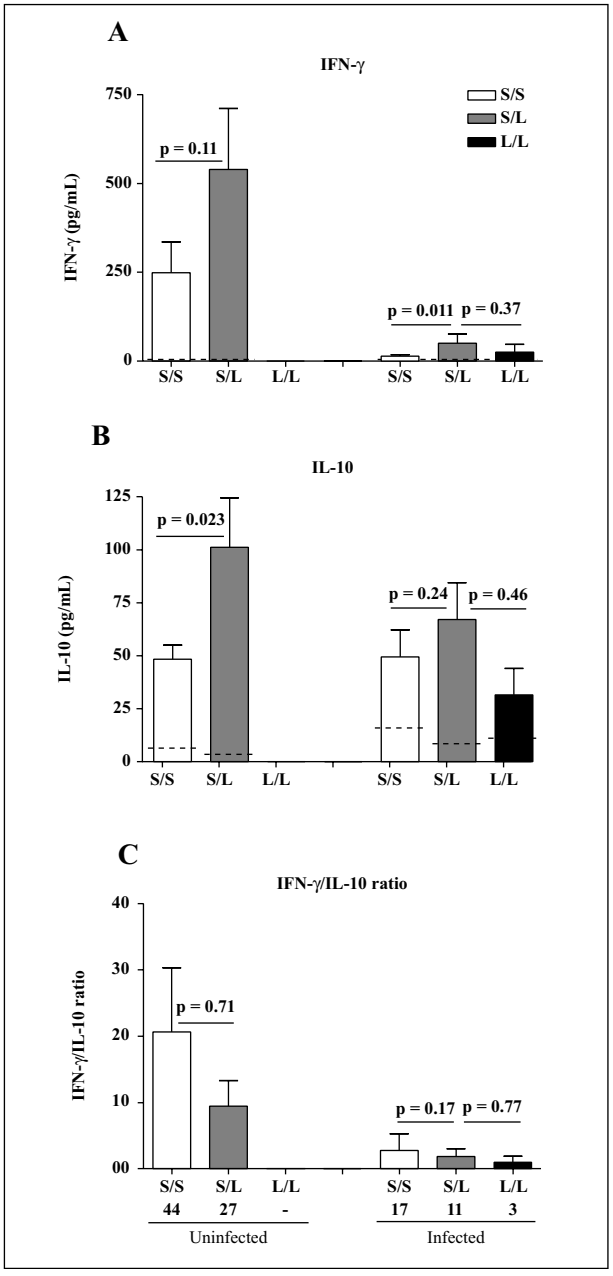

Figure 1

Peripheral blood mononuclear cells from uninfected (left) and *P. falciparum*-infected Malian donors (right) were stimulated for 21 hours with 5 x 10^6 PfRBC/mL and cytokine production in the supernatant was measured by ELISA [**A**) IFN-γ; **B**) IL-10; **C**) IFN-γ/IL-10 ratio]. Donors were stratified by caspase-12 genotype and data represent the mean + SEM for each group of volunteers (number for whom cytokine data were available is indicated under each bar). Dashed lines indicate mean responses to uninfected red blood cells. P-values for differences between caspase-12 genotypes by Mann-Whitney.

one had severe anaemia (Hb < 5g/dL); not unexpectedly, a number of patients presented with two or more symptoms of severe malaria. However, no association was found between the caspase-12 genotype and disease severity overall (p = 0.86), or between caspase-12 genotype and individual manifestations of severe malaria *(table 2)*. Furthermore, there was no obvious effect of the caspase-12 genotype on individual clinical parameters among either uncomplicated or severe cases *(table 2)*.

Ghana study: pregnancy outcome amongst caspase-12 genotypes

Table 3 shows the distribution of caspase-12 genotypes amongst 885 women attending antenatal clinics at the Agogo District Hospital, Ghana for whom genetic data was available. Overall, neither the prevalence of *P. falciparum* by PCR nor parasite density was found to vary significantly between caspase-12 genotypes *(table 3)*. In a sub-group analysis of primigravidae (n = 315), the prevalence of peripheral (but not placental) parasitaemia by PCR was slightly higher in S/L than S/S mothers (70.8 *versus* 56.9%, p = 0.04), and this association remained stable adjusting for age, presence of pyrimethamine in plasma, and wet season (adjusted odds ratio, 1.89; 95%CI, 1.03-3.46; p = 0.04). Peripheral parasite densities in these S/L mothers were, if anything, lower (2.6 *versus* 3.1 log10/μL, p = 0.09).

A mother's caspase-12 genotype status was not associated with fever, preterm delivery, or LBW *(table 3)*. In L/L mothers, anaemia appeared to occur at a reduced rate, but this marginal association (p = 0.04) was lost after stratification for malaria. Also, stratification into infected and non-infected women did not change the observed absence of an effect of the caspase-12 genotype on maternal and foetal morbidity (data not shown).

Interestingly, the incidence of perinatal mortality was lower in infants of S/L mothers (0/172) than in either S/S mothers (19/699, p = 0.02) or L/L mothers (2/14, p = 0.005). This effect could not be ascribed to malaria however, since the majority of all such cases occurred in mothers without placental parasitaemia *(table 3)*. Although the precise cause of death could not always be defined, LBW appeared to contribute in a majority (58%) of recorded cases. Furthermore, perinatal mortality was associated with a history of complicated labour (47% of cases), often requiring caesarean section (47% of cases). Finally, 52% of cases involved either still-birth or poor delivery outcome (10-minute Apgar score < 7).

DISCUSSION

The striking global distribution of caspase-12 alleles begs the question of what evolutionary pressures have shaped this pattern. Humans are the only mammals, apart from rabbits and cows, to have lost a functional caspase-12 gene, indicating relatively recent and species-specific selective pressure [6]. The proposed evolutionary benefit of an untranslated caspase-12 gene lies in protection against sepsis [7], although to our knowledge no further studies have yet confirmed this. In any case, estimates of the genetic age of the caspase-12 S-variant suggest fixating pressure starting as late as 60 thousand years ago [9]. In line with this previous report, we confirm the presence of the L-variant caspase-12 in African populations, at allele frequencies of up to 34%. Whatever the selective force that has eliminated the full-length L-variant outside Africa, within Africa, it must have been counter-balanced by a factor acting with an opposite effect, in order to explain the continued high frequencies of this allele in populations on this continent. Malaria, given its strong

Table 2
Cameroon study: clinical presentation amongst caspase-12 genotypes

	N	Caspase-12 genotype			P-value
		S/S	S/L	L/L	
Overall genotype frequency, n (%)	138	81 (58.7%)	53 (38.4%)	4 (2.9%)	
Clinical presentation					
Uncomplicated malaria, n (%)	100	60 (74.1%)	37 (69.8%)	3 (75.0%)	
Severe malaria (all forms), n (%)	38	21 (25.9%)	16 (30.2%)	1 (25.0%)	0.86[a]
Individual forms of severe malaria[b]					
- Coma (Blantyre score < 3), n (%)	31	16 (76.2%)	14 (87.5%)	1 (100%)	0.66[c]
- Repeated convulsions, n (%)	10	6 (28.6%)	4 (25.0%)	0 (0.0%)	0.80[c]
- Respiratory distress, n (%)	9	6 (28.6%)	3 (18.8%)	0 (0.0%)	0.80[c]
- Severe anaemia (haemoglobin < 5 g/dL), n (%)	1	1 (4.8%)	0 (0.0%)	0 (0.0%)	0.70[c]
Clinical parameters- uncomplicated cases (n = 100)					
Gender, male[d]		36 (60%)	19 (53%)	0 (0%)	0.11
Age, years[e]		4.8 ± 3.5	5.2 ± 3.8	6.3 ± 5.0	0.79
Temperature, °C[e]		39.2 ± 1.1	38.3 ± 6.7	39.2 ± 0.6	0.89
Parasitaemia, log10/μL[e]		4.4 ± 0.9	4.5 ± 1.1	4.7 ± 1.8	0.61
Haemoglobin, g/dL[e]		8.9 ± 2.0	9.0 ± 1.7	7.3 ± 3.4	0.45
Leukocytes, x 106/mL[e]		8.7 ± 3.9	9.8 ± 5.2	8.4 ± 1.2	0.60
Heart rate, /min[e]		126 ± 22	127 ± 23	127 ± 11	0.92
Respiratory rate, /min[e]		37 ± 9	39 ± 14	36 ± 7	0.99
Clinical parameters - severe cases (n = 38)					
Gender, male[d]		11 (55%)	7 (47%)	1 (100%)	0.56
Age, years[e]		3.3 ± 2.2	3.3 ± 2.2	7.8	0.31
Temperature, °C[e]		39.1 ± 1.2	38.7 ± 1.2	39.0	0.57
Parasitaemia, log10/μL[e]		4.4 ± 1.3	4.6 ± 1.0	5.1	0.89
Haemoglobin, g/dL[e]		8.7 ± 1.9	9.3 ± 1.5	9.4	0.52
Leukocytes, x 106/mL[e]		11.2 ± 5.2	14.3 ± 7.7	11.9	0.37
Heart rate, /min[e]		137 ± 27	130 ± 44	160	0.57
Respiratory rate, /min[e]		43 ± 13	45 ± 18	32	0.69

[a] P-value by the χ^2-test, for the difference in prevalence of severe disease between caspase-12 genotypes (within the total population).
[b] Patients could present with more than one symptom of severe malaria.
[c] P-values by the χ^2-test, for the difference in prevalence of specific symptoms between caspase-12 genotypes (within the severe disease population).
[d] Data represent number of cases (%), p-values by χ^2-test. Data on the gender of 1 uncomplicated and 2 complicated cases were unavailable.
[e] Data represent mean ± standard deviation, p-values by Kruskal-Wallis test.

selective effect, specifically in Africa, would appear to be a prime candidate for this role.

However, in none of the three studies did we find any convincing evidence for an effect of the full-length caspase-12 L-allele on either the prevalence or density of parasitaemia or the clinical severity of malaria. Furthermore, caspase-12 genotypes did not directly affect most individual obstetric parameters, including prevalence or density of placental parasitaemia, fever, preterm delivery or LBW. Nor was there any robust effect on Hb levels or anaemia in delivering women (or either of the other two study populations).

One limitation that must be borne in mind when considering these findings is the low number of data on L/L homozygotes, limiting the characterisation of their phenotype. However, although L/L homozygotes may arguably show a more pronounced phenotype than heterozygous individuals, as for any recessive genetic trait this difference would not be expected to be greater than the observed (lack of) difference between S/L heterozygotes and S/S homozygotes, since both L/L homozygotes and S/L heterozygotes express some full-length protein whereas S/S homozygotes do not express any caspase-12 protein at all.

The full-length caspase-12 protein has been proposed to reduce pro-inflammatory cytokine production through inhibition of caspase-1 mediated activation of IL-1β [3]. To test this hypothesis, we measured cytokine responses to *P. falciparum*-infected erythrocytes by PBMCs from healthy and parasitaemic African volunteers. Although IL-1β production was too weak to draw meaningful conclusions, we were able to measure robust IFN-γ responses. IFN-γ is induced downstream of IL-18, which itself requires activation by caspase-1 in a manner similar to IL-1β [25, 26]. Remarkably, IFN-γ production in caspase-12 S/L-heterozygotes tended to be stronger than in S/S individuals. However, S/L donors also elicited stronger anti-inflammatory IL-10 responses than S/S homozygotes and, overall, the IFN-γ/IL-10 ratio was lower in heterozygotes. For diseases in which inflammation contributes significantly to pathology, as is the case for various presentations of severe malaria [27], a relatively stronger anti-inflammatory balance may actually provide a survival advantage. IL-10 production has been shown to reduce susceptibility to severe malaria in animal models [28, 29], but in humans the role of IL-10 is less well established. Clinical and *post-mortem* studies have shown elevated levels of both pro-inflammatory cytokines and IL-10 in most forms of

Table 3
Ghana study: pregnancy outcome amongst caspase-12 genotypes

	Caspase-12 genotype			P-value			
	S/S	**S/L**	**L/L**	**Overall[a]**	**S/S *vs* S/L[b]**	**S/S *vs* L/L[b]**	**S/L *vs* L/L[b]**
Overall genotype frequency, n (%)	699 (79.0%)	172 (19.4%)	14 (1.6%)				
Data for all 885 women							
Age of mother in years (mean)	26.3 ± 6.2	26.4 ± 6.8	27.8 ± 4.9	0.57			
Primigravidae, n (%)	246 (35.5%)	65 (38.2%)	4 (28.6%)	0.68			
Maternal fever, n (%)	22 (3.2%)	6 (3.5%)	0 (0.0%)	0.77			
Maternal anaemia, n (%)	262 (37.5%)	58 (33.7%)	1 (7.1%)	**0.048**	0.36	**0.020**	**0.04**
Perinatal mortality[c], n (%)	19 (2.7%)	0 (0.0%)	2 (14.3%)	**0.001**	**0.020**	0.06	**0.005**
- amongst infected[d] (n = 516), n (%)	9 (2.2%)	0 (0.0%)	0 (0.0%)	0.28	0.22	1.0	-
- amongst uninfected (n = 368), n (%)	9 (3.1%)	0 (0.0%)	2 (33.3%)	**< 0.001**	0.22	**0.017**	**0.006**
Parasite prevalence (by PCR)							
- peripheral venous blood, n (%)	363 (51.9%)	92 (53.5%)	6 (42.9%)	0.73			
- placental intervillous blood, n (%)	405 (57.9%)	104 (60.5%)	8 (57.1%)	0.82			
Geometric mean parasite density							
- peripheral blood, log10/µL	2.79	2.69	2.24	0.58			
- placental blood, log10/100 fields	1.91	1.81	2.43	0.36			
Data of live, singleton deliveries							
Preterm delivery[e], n (%)	119/647 (18.4%)	31/168 (18.5%)	4/12 (33.3%)	0.42			
Low birth weight[f], n (%)	105/653 (16.1%)	27/169 (16.0%)	1/12 (8.3%)	0.77			

[a] χ^2-test or Kruskal Wallis test, ns – not significant ($p > 0.1$).
[b] χ^2-test or Fisher's exact test.
[c] Child born dead or died within 24 hours.
[d] As defined by placental *P. falciparum* infection detected by PCR. Data on placental infection were unavailable for one delivery.
[e] Data available for n = 827.
[f] Data available for n = 834.

severe malaria [30-32]. However, the ratios of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6) to IL-10 are usually increased in severe malaria patients [30, 31, 33, 34], suggesting that enhanced anti-inflammatory responses protect against severe manifestations of malaria. In particular, high IL-10 levels appear to attenuate severe anaemia [33, 35]. It remains unclear however, by what pathway a full-length caspase-12 might lead to stronger IL-10 production. Finally, the association observed between maternal caspase-12 genotype and infant perinatal mortality risk, although potentially coincidental, remains intriguing. This effect could not be ascribed to malaria, or to other infections specifically. Rather, perinatal death was associated with LBW and complicated labour requiring caesarean section, possibly implying a role of caspase-12 in the physiology of pregnancy. Nevertheless, this finding may represent a balanced evolutionary force acting on caspase-12 alleles. Bearing in mind sampling error, the observed relative selection against both homozygote states predicts an equilibrium frequency of the L-allele of $2.7/(2.7 + 14.3) = 0.16$ by standard population genetics theory, which remarkably similar to the observed frequency of 0.11 in this population. This alone, of course, could not explain the disappearance of the L-allele in Europe and Asia, which would then presumably be due to the superimposed effects of other infections on those continents *(e.g.* plague, influenza).

In summary, we aimed to investigate the evolutionary pressures that maintain a full-length caspase-12 allele on the African continent, with an emphasis on malaria infection. No major effects of the caspase-12 genotype on clinical or parasitological parameters were apparent, although functional studies assessing cytokine stimulation by *P. falciparum*-infected erythrocytes showed a slightly more anti-inflammatory profile in the presence of functional caspase-12. These data, obtained in cross-sectional settings, should be followed by larger prospective studies, in order to fully understand the impact of caspase-12 polymorphisms on malaria. Coincidentally, we found indications of an effect of the caspase-12 genotype on perinatal mortality, which could itself represent an important selective pressure. Alternatively, genetic drift or other (parasitic) infections that could have led to the balanced evolution of caspase-12 in Africa should be considered.

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