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Contribution of inflammasome genetics in *Plasmodium vivax* malaria

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ABSTRACT

Recent reports showed that, in mice, symptomatic *Plasmodium* infection triggers NLRP3/NLRP12-dependent inflammasome formation and caspase-1 activation in monocytes. In humans, few works demonstrated that inflammasome is activated in malaria. As *Plasmodium vivax* is a potent inducer of inflammatory response we hypothesised that inflammasome genetics might affect *P. vivax* malaria clinical presentation. For this purpose, selected SNPs in inflammasome genes were analysed among patients with symptomatic *P. vivax* malaria.

157 Brazilian Amazon patients with *P. vivax* malaria were genotyped for 10 single nucleotide polymorphisms (SNPs) in inflammasome genes *NLRP1*, *NLRP3*, *AIM2*, *CARD8*, *IL1B*, *IL18* and *MEFV*. Effect of SNPs on hematologic and clinical parameters was analysed by multivariate analysis.

Our data suggested an important role of NLRP1 inflammasome receptor in shaping the clinical presentation of *P. vivax* malaria, in term of presence of fever, anaemia and thrombocytopenia. Moreover *IL1B* rs1143634 resulted significantly associated to patients' parasitaemia, while *IL18* rs5744256 plays a protective role against the development of anaemia.

Polymorphisms in inflammasome genes could affect one or other aspects of malaria pathogenesis. Moreover, these data reveal novel aspects of *P. vivax*/host interaction that involved NLRP1-inflammasome.

1. Background

Malaria remains a major public health problem worldwide, with 3.2 billion people at risk of being infected and developing the disease (WHO, 2015). Among the five *Plasmodium* species that cause malaria in humans, *Plasmodium falciparum* has been considered the main cause of severe and fatal disease, while *P. vivax* is a major cause of morbidity outside Africa and has also been associated with clinical severity and systemic inflammation (Barber et al., 2015).

A central mechanism for inflammation induction is the secretion of pro-inflammatory cytokines IL-1ß e IL-18 from innate immune cells. IL-1ß e IL-18 liberation depends on the activation of a cytoplasmic complex, known as inflammasome. Several intracellular Pattern Recognition Receptors (PRRs) belonging to NLR family (i.e.: NLRP1, NLRP3, NLRP12) and to other protein families (i.e.: AIM2 or pyrin/MEFV), are able to induce the inflammasome assembling in response to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Upon recognition of PAMPs and/or DAMPs, the receptor recruits the adaptor protein ASC and the cysteine-aspartic protease caspase-1, which is responsible for the processing of pro-IL-1ß and pro-IL-18 (Man and Kanneganti, 2015).

Recent reports showed that, in mice, symptomatic *Plasmodium* infection triggers NLRP3/NLRP12-dependent inflammasome formation and caspase-1 activation in monocytes, leading to dramatic IL-1ß secretion especially when exposed to a second microbial challenge (Ataide et al., 2014). Furthermore hemozoin and DNA activated inflammasome through NLRP3 and AIM2 receptors respectively, in murine infected erythrocytes (Kalantari et al., 2014).

In humans, few works demonstrated that inflammasome is activated in malaria. In *P. falciparum*, for example, the opsonization of malaria-infected erythrocytes activates the inflammasome and leads to FcR-mediated phagocytosis in macrophages (Zhou et al., 2012). On the other hand, the presence of inflammasome complexes was demonstrated in monocytes from malaria patients containing either NLRP3 or NLRP12 inflammasomes (Ataide et al., 2014).

Although multiple studies have revealed that malaria has been a major force of evolutionary selection on the human genome (reviewed in (Malaria Genomic Epidemiology, 2014) little is currently known about the effects of malaria on the evolution of the human immune genes, possibly because the phenotypic consequences are more subtle than those of the classic erythrocyte variants (Kwiatkowski, 2005).

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Table 1
Demographic, clinical and haematological data of Brazilian Amazon patients with *P. vivax* malaria.

CHARACTERISTICS	
Median age, years	34 (25–80)
Male/female, n	122/35
Previous malaria episodes[§], n	1.5 (1–40)
Days of acute illness, n	5 (3–7)
Presence of fever, yes/no	134/23
Parasitaemia, parasites/μL of blood	1117 (607.5–8238)
Leukocyte counts, $\times 10^3$/μl	5.4 (4.4–14.6)
Haematocrit, %	38.8 (35.6–50.6)
Haemoglobin level, g/dL	Male: 13.2 (12.1–16.9); Female: 12.3 (11.3–15.2)
Anaemia[¥], yes/no	Male: 52/70; Female:12/23
Platelet counts, $\times 10^3$/μl	95 (62–260)
Thrombocytopenia[‡], yes/no	23/134
Total score, high/low	93/64

Data are reported as median (interquartile range) except where otherwise specified.
[§] Self-reported number of lifetime malaria episodes.
[¥] Anaemia: haemoglobin <13 g/dL (Male) and <12 g/dL (Female).
[‡] Thrombocytopenia: platelet counts $<50 \times 10^3$/μL.

Notwithstanding, the last few years have seen a rapid growth in the number of reported genetic associations with susceptibility and resistance to clinical malaria, many of which involve immunity and inflammation. In this context, genetic variants in host innate immune genes (i.e., *CRP*, *MBL2*, *NOS2*, *IFNAR1*) and adhesion molecules (*THBS1* and *ESEL*) have been described as predisposing factors for the outcome of *P. falciparum* malaria (Kanchan et al., 2015a, 2015b), while antioxidant enzymes (i.e.: *GSTT*, *GSTM*, *GSTP*, *SOD*, *CAT*) were evaluated as risk factors for *P. vivax* malaria (Andrade et al., 2010; Fernandes et al., 2015).

As *P. vivax* is a potent inducer of inflammatory response (reviewed in (Anstey et al., 2009) we hypothesised that inflammasome genetics might affect *P. vivax* malaria clinical presentation. To our knowledge, inflammasome genes have not yet been evaluated in malaria. For this purpose, selected single nucleotide polymorphisms (SNPs) in inflammasome genes were analysed among Brazilian Amazon patients with symptomatic *P. vivax* malaria.

2. Material and methods

2.1. Subjects

A total of 157 patients with *P. vivax* malaria (122 males/35 females) were recruited for the study after written informed consent, as specified by the Brazilian National Commission for Ethics on Research (Protocol CEPSH/CPqRR/03/2008). Antimalarial and supportive therapies were given according to standard protocols. The study included patients with symptomatic *P. vivax* malaria, and all volunteers were negative for *P. falciparum* and/or *P. malariae* by the means of microscopy and polymerase chain reaction (PCR). Clinical and demographical data were acquired through a standardized questionnaire, and the haematological profiles were assessed by automated complete blood cell counts carried out at local health facilities. Table 1 summarizes demographic, epidemiological, parasitological and haematological data of *P. vivax* infected-volunteers. For statistical purposes, we used a previously validated semi-quantitative clinical assessment to enable numerical comparisons (Campos et al., 2013). Briefly, scores of 0 or 1 were assigned to clinical and hematologic parameters reported as absent (or within reference ranges) or present (or outside reference ranges), respectively; the sum of scores provided patient's final clinical score (scores range from 0 to 7). We grouped 0–3 score as "low" and 4–7 as "high" total clinical score.

2.2. Single nucleotide polymorphisms selection and genotyping

10 SNPs in inflammasome genes *NLRP1*, *NLRP3*, *MEFV*, *CARD8*, *IL1B*, and *IL18* were selected based on functional effect, minor allele frequency (MAF) and/or previously reported association with human disorders (Levandowski et al., 2013; Verma et al., 2012) (Frayling et al., 2007; Hitomi et al., 2009; Pontillo et al., 2011, 2012a, 2012b, 2013). SNPs genotyping was performed using commercially available TaqMan assays (Applied Biosystems/AB) using StepOne Real-Time platform (AB). Allelic discrimination was performed using the StepOne software (AB).

2.3. Data analysis

Effect of SNPs on hematologic and clinical parameters, including haematocrit, haemoglobin, leukocyte and platelet levels, parasitaemia, fever, presence of severe thrombocytopenia and anaemia as well as the total clinical score was analysed by multivariate association based

Table 2
Frequency of studied polymorphisms in Brazilian Amazon cohort of patients with *P. vivax* malaria.

Gene	SNP ID	Alleles/ Genotypes	Frequency	H-W p-value
NLRP1	rs12150220	A	0.41	0.185
		T	0.59	
		A/A	0.38	
		A/T	0.43	
		T/T	0.19	
	rs11651270	C	0.48	0.247
		T	0.52	
		C/C	0.25	
		C/T	0.45	
		T/T	0.30	
	rs2670660	A	0.46	0.743
		G	0.54	
		A/A	0.28	
		A/G	0.52	
		G/G	0.20	
NLRP3	rs35829419	A	0.02	1.0
		C	0.98	
		A/A	0	
		A/C	0.03	
		C/C	0.97	
	rs10754558	C	0.62	0.862
		G	0.38	
		C/C	0.39	
		C/G	0.46	
		G/G	0.15	
MEFV	rs224204	A	0.12	1.0
		G	0.88	
		A/A	0	
		A/G	0.24	
		G/G	0.76	
CARD8	rs2043211	A	0.70	0.556
		T	0.30	
		A/A	0.50	
		A/T	0.40	
		T/T	0.10	
	rs6509365	A	0.63	0.303
		G	0.37	
		A/A	0.37	
		A/G	0.51	
		G/G	0.12	
IL1B	rs1143634	A	0.22	0.218
		G	0.78	
		A/A	0.06	
		A/G	0.30	
		G/G	0.64	
IL18	rs5744256	A	0.83	0.767
		G	0.17	
		A/A	0.69	
		A/G	0.29	
		G/G	0.02	

Gene symbol, Single nucleotide polymorphism (SNP) identification (ID), allele and genotype frequencies and p-value for Hardy–Weinberg (H–W) equilibrium test are reported.

Table 3
Association results for polymorphisms in inflammasome genes and clinical presentation in *P. vivax* malaria patients.

Gene	SNP ID	Fever (Yes/No)	PRS (Log)	WBC (Log)	HTC (Log)	HB (Log)	Anaemia (Yes/No)	Platelets (n/μL)	TCP (Yes/No)	Severity (High/Low)
NLRP1	rs12150220	0.019	0.897	0.243	0.081	0.169	0.291	**4.5 exp.-4**	0.189	**0.001**
	rs2670660	0.174	0.262	0.172	0.099	0.103	0.027	0.324	0.008	0.024
	rs11651270	0.030	0.402	0.800	0.320	0.590	0.272	0.642	0.672	0.164
NLRP3	rs35829419	0.108	0.468	0.083	0.559	0.730	0.634	0.308	0.369	0.812
	rs10754558	0.708	0.063	0.616	0.842	0.997	0.542	0.620	0.254	0.958
MEFV	rs224204	0.379	0.344	0.900	0.343	0.371	0.059	0.378	0.056	0.935
CARD8	rs2043211	0.092	0.195	0.928	0.149	0.136	0.056	0.291	0.025	0.121
	rs6509365	0.030	0.175	0.202	0.419	0.756	0.594	0.377	0.308	0.522
IL1B	rs1143634	0.295	**5.2 exp.-4**	0.803	0.387	0.339	0.260	0.519	0.161	0.499
IL18	rs5744256	0.200	0.515	0.348	**0.004**	**2 exp.-4**	0.082	0.929	0.249	0.810

p-values (adjusted for sex, age and number of malaria episodes) according to general linear model (GLM) analysis are reported for studied SNPs and clinical variables as well as for malaria severity (based on "total score"). p-values <0.05 are underlined; p-values <0.005 are in bold characters.
SNP ID: Single nucleotide polymorphism identification number; PRS: parasitaemia; WBC: white blood cells (leukocytes); HB: haemoglobin level; HTC: haematocrit; TCP: thrombocytopenia.

on general linear model (GLM) adjusted for variables (sex, age, number of malaria episodes) using R-project package "SNP-assoc" version 1.5–2 (Gonzalez et al., 2007). The Haploview software (Barrett, 2009) was used to investigate the association and linkage disequilibrium (LD) pattern and for deriving the haplotypes. A multiple testing correction (Bonferroni) would require a significance threshold of $p = 0.005$.

3. Results

The frequency of studied polymorphisms in our patients is reported in Table 2. The distribution of genotypes was consistent with Hardy–Weinberg equilibrium for all SNPs (Table 2). Multivariate analysis results are summarized in Table 3.

Polymorphisms in candidate genes, such as *NLRP3* and *MEFV*, previously shown as important players in malaria (Dutra et al., 2014; Fumagalli et al., 2009; Kalantari et al., 2014), was not associated to malaria presentation in our cohort (Table 3).

Un-expectantly, SNPs in *NLRP1* resulted differently distributed in *P. vivax* malaria patients according to several clinical parameters (fever, anaemia, platelets counts and thrombocytopenia) (Table 3).

It was possible to observe a significant lower level of platelets in patients harbouring the minor allele T of the rs12150220 (A/T + T/T: 4.92 n/μL vs A/A: 5.02 n/μL; $p_{adj} = 4.5$ exp.-4) (Table 4).

When total clinical score was taken in account ("high" versus "low"), *NLRP1* rs12150220 appeared to be significantly more frequent in individuals with a high clinical score (0.73) compared to a low one (0.55) ($p_{adj} = 0.001$; $OR_{adj} = 3.89$) (Table 4).

These findings suggest the contribution of missense variation *NLRP1* rs12150220 (L155H) in severity of *P. vivax* malaria.

We would like to briefly mention other association results with $p < 0.05$, even if they did not pass Bonferroni correction, maybe due to the limited size of analysed cohort, as in our opinion corroborate fully statistically significant data. *NLRP1* rs1215022 and rs11651270 SNPs are more frequent in patients with fever (n = 134) versus individuals reporting no fever episodes (n = 23) ($p_{adj} = 0.019$ and $OR_{adj} = 4.68$; and $p_{adj} = 0.030$ and $OR_{adj} = 7.74$, respectively) (Supplementary File 1). Moreover *NLRP1* promoter variant rs2670660 resulted more frequent in anaemic patients ($p_{adj} = 0.027$; $OR_{adj} = 2.67$) as well as in individuals with thrombocytopenia ($p_{adj} = 0.008$; $OR_{adj} = 4.54$) (Supplementary File 1).

Polymorphisms in inflammasome-related cytokines IL-1ß and IL-18 resulted significantly associated to clinical parameters in our cohort.

Malaria patients carrying *IL1B* rs1143634 minor A allele presented a lower parasite load than G/G ones (A/A + A/G: 2.89 log (parasite/μl) versus G/G: 3.2; $p_{adj} = 5.2$ exp.-4), emphasizing the contribution of this inflammasome-dependent cytokine in controlling parasitaemia (Table 5).

Table 4
Detailed results for significantly associated *NLRP1* SNPs.

SNP	Clinical variable							
		Individuals (frequency)	**me ± se**	**dif**	**Lower**	**upper**	**p**	**p_{adj}**
	Platelets counts (103/μL)							
rs12150220	A/A	0.38	120.484 ± 7.990	Ref.			3.9 exp–4	4.5 exp–4
	A/T–T/T	0.62	94.629 ± 04.892	−25.855	−43.179	−8.532		
		Individuals (frequency)		**OR**	**95% CI**		**p**	**p_{adj}**
	Total clinical score	**High**	**Low**					
rs12150220	A/A	0.27	0.45	Ref			0.022	0.001
	A/T–T/T	0.73	0.55	3.89	1.63	9.30		

Genotypes distribution according to inheritance model (dominant, over-dominant), p-value, p-values adjusted for sex, age and number of malaria episodes, as well as Odds Ratio (OR) and 95% confidence intervals (CI), or difference (dif) and 95% confidence intervals (lower; upper) are reported for NLRP1 SNPs associated with malaria clinical variables. me±se: mean and standard error. Ref.: reference genotype.

Table 5
Detailed results for significantly associated *IL1B* and *IL18* SNPs.

Gene/SNP	Clinical variable							
IL1B		Individuals (frequency)	me ± se	dif	Lower	Upper	p	p_{adj}
	Parasitaemia (log)							
rs1143634	G/G	0.73	3.20 ± 0.07	Ref.			0.009	5.2 exp.-4
	A/G-A/A	0.27	2.89 ± 0.09	−0.51	−0.78	−0.24		
IL18								
	Haemoglobin level (log)							
rs5744256	A/A	0.69	2.53 ± 0.01	Ref.			0.003	1.9 exp.-4
	A/G	0.29	2.58 ± 0.02	0.06	0.01	0.12		
	G/G	0.02	2.31 ± 0.34	−0.37	−0.58	−0.16		
	Haematocrit (log)							
rs5744256	A/A-A/G	0.98	3.64 ± 0.01	Ref.			0.005	0.004
	G/G	0.02	3.39 ± 0.35	−0.26	−0.43	−0.08		

Genotypes distribution according to inheritance model (co-dominant, dominant or recessive), p-value, p-values adjusted for sex, age and number of malaria episodes, as well as difference (dif) and 95% confidence intervals (lower; upper) are reported for inflammasome SNPs associated with discrete/continue variables. me ± se: mean and standard error; Ref.: reference genotype.

Lower haemoglobin levels were found in *IL18* rs5744256 G/G individuals (log(Hb) = 2.305) compared to A/A (log(Hb) = 2.526) and A/G individuals (log(Hb) = 2.578) (p_{adj} = 1.9 exp.-4) (Table 4). The same *IL18* SNP was significantly associated with low haematocrit values according to a recessive inheritance model (p_{adj} = 0.004) (Table 4), suggesting that this 3′UTR variant, which lead to a reduced serum level of IL-18 (Frayling et al., 2007), could contribute to the development of anaemia in malaria patients. Accordingly, *IL18* rs5744256_A > G resulted less frequent in the anaemic patients (male: Hb < 13.00 g/dL; female: Hb < 12.00 g/dL) as compared to non-anaemic ones (0.21 versus 0.37; OR = 0.46), however once again this association could not be considered statistically significant after Bonferroni correction (p_{adj} = 0.037) (Supplementary File 1).

Finally, linkage disequilibrium was tested in the studied population and the 2 *CARD8* SNPs (rs2043211, rs6509365) resulted in LD (D′ = 0.94; r^2 = 0.64) (Supplementary File 2), however the derived 4 haplotypes (A–A, T–G, A–G, T–A) did not result significantly associated with malaria clinical presentation (p > 0.05).

4. Discussion

In murine models of malaria infection NLRP3-inflammasome has been pointed out as the complex responsible for inflammation in response to *Plasmodium* spp. hemozoin (Kalantari et al., 2014) moreover haemolysis in term of increasing extracellular heme also activates NLRP3-mediated IL-1ß secretion (Dutra et al., 2014). However, unexpectantly, *NLRP3* variants did not associate with any analysed characteristics of clinical malaria.

Similarly, the other strong candidate gene, *MEFV*, which has been previously pointed out as a protector factor against *P falciparum* malaria (Fumagalli et al., 2009) did not resulted significantly associated to *P. vivax* malaria in our cohort.

However our data suggested an important role of another inflammasome receptor, the NLRP1, in shaping the clinical presentation of *P. vivax* malaria, in term of presence of thrombocytopenia, a relevant complication in *P. vivax* malaria (Lacerda et al., 2011) and severity of the disease. Up to date, NLRP1 has not been associated to disease characterized by episodes of fever as other inflammasome-related NLRs, nor with platelet disorder. Moreover information about PAMPs and/or DAMPs able to activate NLRP1 is still lacking, suggesting that during *P. vivax* infection still unknown molecular patterns could appear and induce inflammasome activation through this receptor. Recent reports indicate that NLRP1 could play a key role in endothelium, and especially in endothelial disorders (Bleda et al., 2014, 2015). As *P. vivax* targets endothelium and induces vascular damage (Barber et al., 2015), we can hypothesise that individual carrying *NLRP1* gain-of-function SNPs could be more susceptible to develop *P. vivax*-related vascular disorder. On the other hand, the association of *NLRP1* SNPs with cytopenia (platelets and, even if not fully significant, red blood cells) recalls the work by (Masters et al. (2012)) where NLRP1-inflammasome activation has been associated to pyroptosis cell death in haematopoietic progenitor cells in mice. Deeper investigations are needed to elucidate the role of NLRP1 in *P vivax* malaria.

Cyclic inflammation is a common hallmark of malaria and it is related to high levels of pro-inflammatory cytokines (Anstey et al., 2009), however it is still unclear whether the production of cytokine is a benefit for immune response against *Plasmodium*. Our results suggested that IL-1ß seemed to be important in controlling parasitaemia. For what concerns IL-18, it was recently shown in a murine model of malaria that augmented levels of IL-18 positively correlated with *Plasmodium* parasitaemia and with the severity of the infection (Basir et al., 2012). In our cohort, the *IL18* loss-of-function variation rs5744256 (Frayling et al., 2007) was associated to reduced haemoglobin level and haematocrit. These results partially confirmed previously published data about the association between *IL18* promoter variants and severe malarial anaemia in children (Anyona et al., 2011).

5. Conclusions

Despite the limited size of studied cohort, our results showed a significant association between inflammasome genetics and clinical presentation of *P. vivax* malaria, suggesting that polymorphisms in inflammasome genes could affect one or another aspect of malaria pathogenesis. Moreover, these data confirm previously reported results about the key role of NLRP3-inflammasome in mouse model of malaria, and reveal novel aspects of *P. vivax*/host interaction that might be deeper investigated in term of *NLRP1*/platelets and/or endothelium interplay.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.02.038.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MLSS and TNS participated to the statistical analysis and to the manuscript draught; ECR and PB carried out the genotyping experiments; CFAB and LHC participated in the design of the study and drafted the manuscript; MVL and CJF F recruited malaria patients; AP conceived

and coordinated the study, analyses the data and wrote the final manuscript. All authors read and approved the final manuscript.

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