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ITN protection, MSP1 antibody levels and malaria episodes in young children of rural Burkina Faso

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ABSTRACT

Malaria blood-stage vaccines are in an early phase of clinical development with MSP1 being a major antigen candidate. There are limited data on the protective efficacy of antibodies against subunits of MSP1 in the malaria endemic areas of sub-Saharan Africa. This prospective cohort study was nested into a large insecticide-treated mosquito net (ITN) trial during which neonates were individually randomised to ITN protection from birth vs. protection from month six onwards in rural Burkina Faso. A sub sample of 120 children from three villages was followed for 10 months with six measurements of $MSP1_{42}$ antibodies (ELISA based on recombinant 42 kDa fragment) and daily assessment of malaria episodes. Time to the next malaria episode was determined in relation to $MSP1_{42}$ antibody titres. $MSP1_{42}$ antibody titres were dependent on age, season, ITN-group, number of previous malaria episodes and parasitaemia. There were no significant differences in time until the next malaria episode in children with low compared to children with high $MSP1_{42}$ antibody titres at any point in time (101 vs. 97 days in May, $p = 0.6$; 58 vs. 84 days in September, $p = 0.3$; 144 vs. 161 days in March, $p = 0.5$). The findings of this study support the short-lived nature of the humoral immune response in infants of malaria endemic areas. The study provides no evidence for antibodies against a subunit of MSP1 being protective against new malaria episodes in infants.

1. Introduction

Malaria is amongst the leading causes of global morbidity and mortality and remains the most important parasitic disease of humans (Greenwood et al., 2005; Lopez et al., 2006). With the establishment of the Roll Back Malaria (RBM) partnership in 1998 and a number of other major global health initiatives, malaria has received much increased political attention and financial support (Mendis et al., 2009). This is also attributed to the availability of new and highly effective tools, mainly the insecticide-treated mosquito nets (ITNs) and the artemisinin-based combination therapies (ACT). However, it has become clear in the international debate that a breakthrough in controlling and eventually eliminating malaria in the highly endemic areas of sub-Saharan Africa (SSA) will only be possible, if existing health systems are massively strengthened and/or if new and powerful tools such as malaria vaccines are developed and available (Müller, 2011).

Many promising antigens have been identified in recent years and research on malaria vaccines is thriving (Hill, 2011; White, 2009). However, human parasites present a bigger challenge compared to viruses and bacteria, as these are much more complex organisms with multi-stage life cycles in which they express many different antigens of large variability inducing both humoral and cellular immune responses (Hoffman, 2004). There are three principal approaches to a malaria vaccine: (1) development of a pre-erythrocytic stage vaccine, (2) development of a blood-stage vaccine, and (3) development of a transmission-blocking vaccine (Greenwood and Targett, 2011; Hill, 2011). Most progress so far has been achieved in the field of pre-erythrocytic stage vaccine development, with RTS,S currently being the most advanced antigen (Greenwood and Targett, 2011; Hill, 2011). First clinical field studies in different countries of SSA have documented a short-term efficacy of this vaccine of up to 50% against new infections and clinical disease, and a phase III trial on the RTS,S vaccine is currently under way (Greenwood et al., 2005; Abdulla et al., 2008; Bejon et al.,

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2008; Collins and Barnwell, 2008; Hill, 2011). Preliminary results from this trial have just been published. The vaccine showed an efficacy of about 50% against clinical malaria (The RTS,S Clinical Trials Partnership, 2011).

Blood-stage vaccine candidates aim at reducing or eliminating merozoites, and quite a number of candidate antigens have been identified, with MSP1 and AMA1 being the most advanced in clinical studies (Greenwood et al., 2005; Greenwood and Targett, 2011; Hill, 2011; Targett and Greenwood, 2008). The merozoite surface protein (MSP)-1 is a large multiprotein complex at the surface of the merozoites of *Plasmodium falciparum*. During the late stages of parasite maturation, MSP1 is cleaved into the subunits $MSP1_{83}$, $MSP1_{30}$, $MSP1_{38}$, and $MSP1_{42}$ (Woehlbier et al., 2010). The C-terminal 42-kD fragment undergoes a further process which leads to the 19-kD fragment which is important for invading new erythrocytes (Blackman et al., 1990). A potential role of anti-$MSP1_{19\,kD}$ antibodies in the development of protective immunity has been shown in in vitro and in animal model studies (Brown et al., 1982; Chang et al., 1992; Daly and Long, 1995; Kumar et al., 1995). Sero-epidemiological studies have demonstrated an association between anti-$MSP1_{19\,kD}$ antibodies and reduced *P. falciparum* malaria morbidity (Müller et al., 1989; Dodoo et al., 2011), but more prospective cohort studies are needed (Fowkes et al., 2010). Such a temporarily developed protective response appears to be more short-lived in children than in adults (Früh et al., 1991; Tolle et al., 1993). More recently, clinical studies have provided first evidence for the safety and immunogenic character of MSP1 based malaria vaccine candidates (Ellis et al., 2010; Ockenhouse et al., 2006; Withers et al., 2006).

Against this background we have conducted a prospective cohort study on associations between $MSP1_{42}$ antibody titres and *P. falciparum* malaria episodes in infants of rural Burkina Faso, which was embedded into a large ITN trial.

2. Materials and methods

2.1. Study area

The present study took place in the health and demographic surveillance system (HDSS) area of the *Centre de Recherche en Santé de Nouna* (CRSN) in Nouna Health District in north-western Burkina Faso. In 2002, the study area had about 61,000 inhabitants, living in Nouna town and 41 villages. Health services in the research area comprised the District Hospital in Nouna town and at that time six local health centres in the surrounding villages. The predominantly rural and – considering the town of Nouna itself – semi-urban area is a dry orchard savanna, populated mainly by subsistence farmers and cattle keepers. The region has a sub-Sahelian climate with a mean annual rainfall of 796 mm (range 483–1083) over the past five decades. The rainy season usually lasts from June until October. The period November until February is considered the cool dry season whilst the period March until May is called the hot dry season. Most of the malaria morbidity and mortality occurs during and shortly after the rainy season (Becher et al., 2008; Hammer et al., 2006). Malaria was holoendemic in the rural Nouna area at the time of the study (Müller et al., 2001).

2.2. Study population and study design

A prospective cohort of successively recruited newborns from three villages of the Nouna HDSS has been followed up over a ten month period in 2001/2002. This cohort study was embedded into a large randomised controlled community trial on the effectiveness of ITNs. The details of the ITN trial have been published before (Müller et al., 2006). In brief, a birth cohort of 3387 children was enrolled between June 2000 and December 2002 from 41 villages of the rural Nouna HDSS area, with children individually randomised to two interventions: ITN protection from birth onwards (group A) and ITN protection from month six onwards (group B). High compliance of study children with ITN use during the first years of the intervention was demonstrated (Frey et al., 2006). Primary outcomes of the study were all-cause mortality (all children) and malaria incidence (sub-sample of 420 children in six sentinel villages). The cohort which is described here consists of the first 120 children enrolled in three (Bourasso, Sikoro, Koudougou-Mossi) of the six sentinel villages. Data in the ITN trial were collected through active longitudinal surveillance (all-cause mortality, malaria incidence) and through bi-annual cross-sectional surveys (e.g. malaria prevalence, malaria parasitaemia, anaemia).

Children living in the sentinel villages were visited daily by village-based field workers who measured the temperature and in case of fever (axillary temperature $\geq$37.5 °C) also took a blood sample for microscopic malaria diagnosis in the CRSN laboratory. Six points in time were chosen to perform additional regular (every two months) blood sampling through an independent CRSN field team in the sub-group of 120 cohort children (60 from group A, 60 from group B). At every point in time (May 2001, July 2001, September 2001, November 2001, January 2002, and March 2002) a blood sample was taken from the study child to determine malaria antibodies. In addition, a corresponding blood sample was taken from the mothers of respective study children at the time of the May 2001 and the January 2002 survey (data not shown).

2.3. Laboratory work

All blood samples taken were finger-prick samples. Blood samples taken during the trial routine longitudinal surveillance for malaria incidence were processed as described before (Müller et al., 2006). The blood samples taken during the six additional cross-sectional visits in the sub-group of 120 study children were used for establishing malaria blood slides as well as for storing 200 μl of blood in two air-proofed capillaries. After centrifugation in the CRSN laboratory, the serum was transferred into small Eppendorf containers, frozen at −20 °C and stored in the CRSN until transport of frozen samples to Heidelberg in June 2002. The serum samples were then stored in the Department of Infectious Diseases, Heidelberg University at −80 °C until analysis.

The analysis of antibodies against $MSP1_{42}$ of the serum samples was performed with an established $MSP1_{42}$ ELISA. The recombinant antigen used is a 42 kDa fragment of the major surface antigen (MSP1) of *P. falciparum* expressed in *Escherichia coli* (Epp et al., 2003).

The ELISA technique followed standard laboratory procedures (Wakilzadeh, 2009). Ninety-six-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 μl of 100 nM recombinant $MSP1_{42}$ protein per well. Microtitre plates were kept overnight at room temperature in a humid chamber. The recombinant antigen was diluted in a 0.2 M carbonate-bicarbonate buffer, pH 10.6, The plates were washed two times with buffer (TBST; 10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0) and incubated with 100 μl blocking buffer (1% skim milk in TBST) per well for 1 h at room temperature, the blocking buffer was then discarded, followed by two washing steps. A volume of 100 μl serum samples at a 1:200 dilution were added to each well, each sample was assayed in duplicate and further serial dilutions were performed in the microtitre plates and allowed to react for 1 h at room temperature. After two washing steps, phosphatase labelled anti-human IgG antibody (Promega, Madison, WI, USA) was diluted 1:1000 in blocking buffer, added and incubated for 1 h at room temperature. After repetition of the washing steps, 100 μl of 1 mg/ml *p*-nitrophenyl phosphate (Sigma Aldrich, Taufkirchen, Germany) in substrate buffer (1 mM $MgCl_2$,

0.96% diethanolamine, pH 9.6) were added and incubated for 1 h at room temperature in the dark. Reactions were stopped by the addition of 100 μl 2 N NaOH. The optical density was determined photometrically at 405 nm with 620 nm as reference filter. Only mean optical density (OD) values in the linear range (0.1–1.0 OD) were used, 7.1% of the results exceeded OD of 1.0 and were excluded from further analysis (Wakilzadeh, 2009). Antibody titres were calculated by multiplication of the OD by the serum dilution and are expressed as reciprocal endpoint titre.

Study questions

1. What is the rate of acquisition of $MSP1_{42}$ antibodies during the first year of life in a malaria holoendemic area of Burkina Faso?
2. Which factors are associated with the humoral immune response against $MSP1_{42}$?
3. What is the association between $MSP1_{42}$ antibody titres and new episodes of *P. falciparum* malaria in young children?

2.4. Statistical methods

2.4.1. Malaria episodes

For every child in this sub-cohort, specific and daily information on fever and malaria episodes was available through the routine longitudinal follow-up procedures. In addition, $MSP1_{42}$ antibody titres were available every two months. A malaria episode was defined as fever (axillary temperature $\geq 37.5\,^{\circ}C$) accompanied by *P. falciparum* parasites/μl > 0, in the presence or absence of *Plasmodium malariae* and/or *Plasmodium ovale* parasites and with no other obvious cause for the fever.

To measure the influence of $MSP1_{42}$ antibody titres on subsequent malaria episodes the study children were divided into two groups at every point in time: in the first group were children with high $MSP1_{42}$ antibody titres (above median) and the second group with low $MSP1_{42}$ antibody titres (below median). Then the duration in time until the first malaria episode occurred was calculated for each group. In addition, the mean number of *P. falciparum* parasites during these malaria episodes was compared with $MSP1_{42}$ antibody titres in each group.

2.4.2. Regression analysis

The influence of the covariables age (grouped in 3–5 months, 6–8 months, 9–11 months, . . ., 21–22 months), sex (male, female), village (Bourasso, Sikoro, Koudougou), ethnic group (Bwaba, Mossi, Dafin/Peulh/Samo), season (rainy and dry), and intervention arm (A and B) on the $MSP1_{42}$ antibody response were compared over the 6 points in time.

To analyse which variables have a significant influence on the $MSP1_{42}$ antibody response a multivariate regression model was built. The six measurements per child were assumed to be independent and the log of antibody and parasite density was used to achieve normal distributed values, so we got the following model:

$$\log(\text{MSP1 antibody}) = \beta_0 + \beta_1 \log(\text{parasite}) + \beta_2 \text{fever} + \beta_3 \text{age} + \beta_4 \text{sex} + \beta_5 \text{season} + \beta_6 \text{ethnic group} + \beta_7 \text{village} + \beta_8 \text{ITN-group} \quad (1)$$

Data entry and all analysis were conducted using Excel 7.0, Microsoft Access 97, and SAS 9.1 software (SAS Institute Inc., 2003).

3. Results

Table 1 shows the means of the $MSP1_{42}$ antibody titres in dependence on age and the month of examination. Besides the relatively high antibody titres in May (end of dry season) in the very first age group (3–5 months), which is most likely attributed to residual maternal antibodies, the following dynamic can be seen: in May there were overall the lowest antibody titres observed, in July (rainy season) the titres became higher, in September (peak of the rainy season) all age groups had the highest values, which stayed relatively constant high in November (start of the dry season), and then for January and March (mid of dry season) the antibody titres clearly decreased.

Table 1
Means of $MSP1_{42}$ antibody titres in dependence of age group and season in young children of rural Burkina Faso.

Age in months \ Season	May (end of hot dry season)	July (beginning of rainy season)	September (peak of rainy season)	November (beginning of cold dry season)	January (peak of cold dry season)	March (beginning of hot dry season)
3-5	1:362 [a] (31) [b]	1:565 (6)				
6-8	1:83 (44)	1:3167 (32)	1:7120 (14)			
9-11	1:170 (35)	1:3905 (33)	1:9967 (42)	1:3329 (29)	1:358 (4)	
12-14		1:2056 (17)	1:5705 (36)	1:4119 (37)	1:1802 (31)	1:1400 (23)
15-17			1:3770 (8)	1:4692 (40)	1:2833 (45)	1:2248 (36)
18-20				1:390 (1)	1:2233 (23)	1:1680 (34)
21-22						1:1455 (2)
Total number of children	110	88	100	107	103	95

[a] Mean of $MSP1_{42}$ antibody titres.
[b] Number of children (children without $MSP1_{42}$ antibody titres mean values were not included).

The relation of mean $MSP1_{42}$ antibody titres and the number of previous malaria episodes (last two months) has been analysed by season (Fig. 1). Having experienced at least one malaria episode in the two months before the survey was clearly associated with an earlier and higher antibody response when compared with the none-episode group, but this reached statistical significance only in July ($p < 0.0001$).

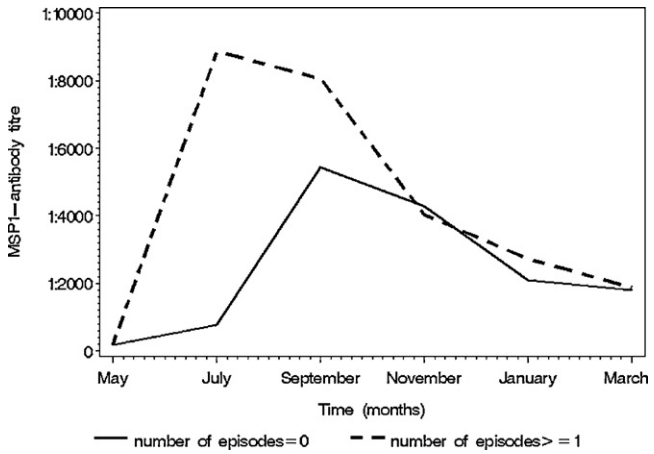

Fig. 1. Means of $MSP1_{42}$ antibody titres by number of *P. falciparum* malaria episodes (last two months) in dependence on season in young children of rural Burkina Faso.

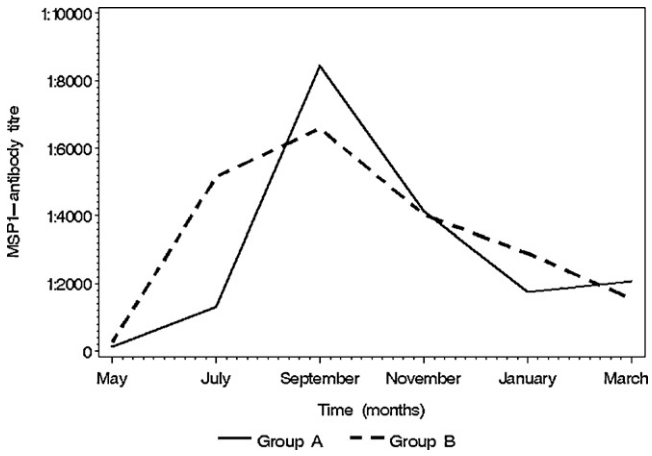

Fig. 2. Means of $MSP1_{42}$ antibody titres by both ITN-group (A = ITN protection from birth onwards; B = ITN protection from month six onwards) in dependence on season in young children of rural Burkina Faso.

In Fig. 2 the relation between mean $MSP1_{42}$ antibody response by ITN study group and season is illustrated. Group B children (not protected with ITN during the first six months of life) developed $MSP1_{42}$ antibody titres earlier compared to children of group A, but this reached statistical significance only in July ($p = 0.04$).

Table 2 shows the results of the multivariate regression model. The final model did not include the covariables fever, sex, ethnic group and village because these factors had no significant effect on the MSP1-antibody titres and did not change the remaining parameters appreciably. The covariables parasites, age of study children and season had a highly significant effect on the $MSP1_{42}$ antibody titres ($p < 0.0001$ each). The covariable ITN-protection was significant at the level of $p = 0.02$.

The duration (in days) until the first *P. falciparum* malaria episode occurs has been investigated for the three baseline points in time May, September, and March, depending on $MSP1_{42}$ antibody titres at these points in time (Table 3). Mean duration varied from 99 days in May, over 68 days in September, to 152 days in March, without significant differences between children with high and children with low $MSP1_{42}$ antibody titres (p-values were $p = 0.6$, $p = 0.3$ and $p = 0.5$ for May, September and March respectively), tested by Student's t-test. The results of the non-parametric two sample Wilcoxon test did not differ from the results of Student's t-test.

Table 2
Multivariate regression model of $MSP1_{42}$ antibody titres in dependence of parasites, age, season, and ITN-group in young children of rural Burkina Faso.

Risk factor	β[a]	95% CI lower	upper	p value
Log (parasites)	0.182	0.137	0.226	<0.0001
Age	0.003	0.001	0.005	<0.0001
Season	−1.476	−1.866	−1.087	<0.0001
ITN-group	−0.425	−0.777	−0.073	0.0177

[a] β = Estimate of the coefficients of the multivariate regression model.

For the same three baseline points in time May, September, and March, the mean and median *P. falciparum* parasite densities during the next *P. falciparum* malaria episode has been analysed by $MSP1_{42}$ antibody group (Table 4). No significant differences between children with high and children with low $MSP1_{42}$ antibody titres were found. The resulting p-values were $p = 0.3$, $p = 0.3$ and $p = 0.4$ for May, September and March respectively, likewise tested by Student's t-test.

4. Discussion

The first asexual blood stage vaccine candidates including MSP1 based vaccines have reached phase I and II clinical trials, but progress is slow (Moorthy et al., 2004). Such vaccine candidates are considered to carry the potential to provide protection against disease but not against infection (Targett and Greenwood, 2008). Semi-immunity acquired under real life conditions in endemic

Table 3
Mean duration between survey points in time and the following *P. falciparum* malaria episode in young children of rural Burkina Faso.

	Number of children	Minimum [days]	Maximum [days]	Median [days]	Mean[b] [days]	p value[c]
May 2001						
≤1:52[a]	53	24	231	95.0	101.4 ± 41.4	0.6
>1:52	50	25	174	95.5	97.2 ± 37.8	
September 2001						
≤1:4789	49	1	337	23.0	57.8 ± 84.4	0.3
>1:4789	48	2	721	29.5	83.9 ± 155.7	
March 2002						
≤1:802	46	68	502	157.0	144.1 ± 93.9	0.5
>1:802	45	41	576	140.0	161.1 ± 159.5	

[a] $MSP1_{42}$ antibody titre median at survey time point.
[b] Plus–minus values are means ± SD (standard deviation).
[c] Student's t-test.

Table 4
Means of *P. falciparum* parasites in dependence of high and lower $MSP1_{42}$ antibody titre groups at three survey time points in young children of rural Burkina Faso.

	Number of children	Minimum [days]	Maximum [days]	Median [days]	Mean[b] [days]	p value[c]
May 2001						
≤1:52[a]	53	25	110,000	4850	16,086 ± 26,165	0.3
>1:52	50	66	248,125	7700	23,743 ± 42,526	
September 2001						
≤1:4789	49	25	151,600	13,800	25,683 ± 33,815	0.3
>1:4789	48	25	224,000	11,850	19,824 ± 23,490	
March 2002						
≤1:802	46	25	301,125	7696	27,099 ± 51,568	0.4
>1:802	45	25	224,000	5400	19,012 ± 37,304	

[a] $MSP1_{42}$ antibody titre median at survey time point.
[b] Plus–minus values are means ± SD (standard deviation).
[c] Student's t-test.

areas depends on the development of variant-specific responses to the large array of *P. falciparum* strains and does usually take years to develop (Ouattara et al., 2010; Rogier and Trape, 1993; White, 2009).

The findings presented in this study largely support the long-standing knowledge on the dynamic of the malaria antibody response in young children of malaria endemic areas, including published examples from other countries in West Africa (Dodoo et al., 2011; Riley et al., 1992; Sarr et al., 2007). The data support the short half-life of the maternal MSP1 antibodies in young infants and a positive correlation between the age of the children and the MSP1 antibody response depending on the number of subsequent *P. falciparum* malaria episodes in malaria endemic areas (Al-Yaman et al., 1996; Apio et al., 2000; Hogh et al., 1995; Müller et al., 1989; Sehgal et al., 1989). The observed rapid decline of the MSP1 antibody titres after the rainy season supports the ephemeral nature of the immune response during the first year of life (Branch et al., 1998; Früh et al., 1991; Tolle et al., 1993). Infants' ability to develop a long-term humoral response against MSP1 and other malaria antigens may be physiologically restricted (Clerici et al., 1993). The findings from this study also confirm the relationship between malaria antibody titres and the number of previous malaria episodes, as seen in many other studies in endemic areas (Al-Yaman et al., 1996; Cavanagh et al., 2004; Müller et al., 1989; Shi et al., 1996). Such an association is furthermore supported by the findings from this study that children protected with ITNs since birth developed a delayed $MSP1_{42}$ antibody response, due to a lower number of *P. falciparum* infections.

The mean duration between survey points in time and the following *P. falciparum* malaria episode varied by season, with the shortest time observed at the height of the malaria transmission time in September and the longest time in the middle of the dry season, which is likely explained by the likelihood to be exposed to infective mosquito bites. The finding of this interval not being significantly associated with $MSP1_{42}$ antibody titres supports the assumption that antibody responses towards individual asexual blood stage antigens may have no effect on malaria incidence (Targett and Greenwood, 2008). However, as there was also no clear association between previous $MSP1_{42}$ antibody titres and the mean parasite density, the assumption that a vaccine targeting individual blood stage antigens may decrease disease severity (Hill, 2011) is not supported by this study. Previous approaches to developing blood stage vaccines against *P. falciparum* malaria were mainly based on single antigens which did not show protection in field trials (Greenwood and Targett, 2011; Hill, 2011). However, allele-specific responses may well be protective against subsequent allele-specific infections, as recently shown in a study in Mali (Thera et al., 2011). The use of more complex mixtures and/or the combination of a number of key parasite antigens may thus become a more promising approach in the future (Hill, 2011).

This study has some strength and some limitations. Strength is the fact that it is a prospective study which was conducted under real life conditions in a malaria holoendemic area of rural SSA. Moreover, the availability of daily quality information on the morbidity of children including malaria parasite data in such a newborn cohort is an important prerequisite for responding to the research questions. Limitations are that the cohort of newborns was small and had been followed up only for ten months. As a consequence the age and also the number of children were different at every point in time and during seasons.

In conclusion, this study supports the short-lived nature of the humoral immune response in infants of malaria endemic areas but provides no evidence for antibodies against a subunit of MSP1 being protective against new malaria episodes.

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