Malaria in pregnancy

In search of tools for improved prevention

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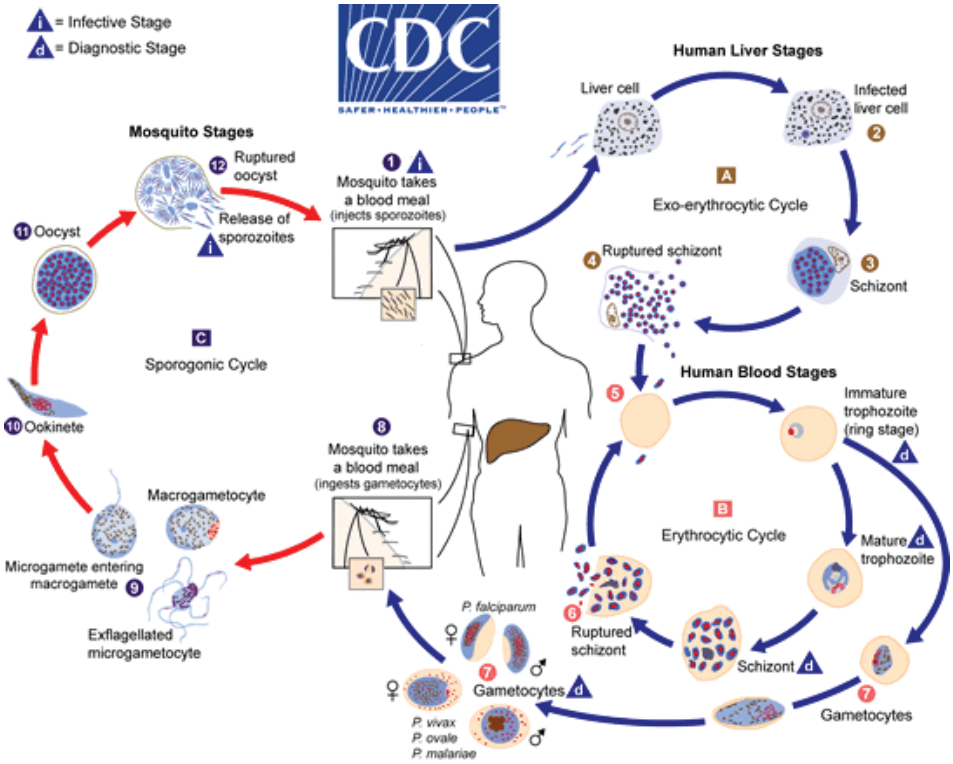
Chapter 1.

General introduction and outline of the thesis

Introduction to malaria

Malaria is a parasitic infectious disease that causes worldwide morbidity and mortality. It was estimated by the World Health Organization that in 2015 a total of 212 million people suffered from malaria of which 429.000 died.[1] 92% of these fatalities occurred in sub-Saharan Africa and about 70% were children under the age of five. There are several *Plasmodium* species causing disease in humans (*P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri and P. knowlesi*), but *P. falciparum* is the most deadliest of species.[1] *P. falciparum* parasites are transmitted to humans by mosquitoes of the *Anopheles* genus. Both the mosquito vector and the (human) host are needed to complete the lifecycle of *P. falciparum*. When a mosquito takes a blood meal, *P. falciparum* sporozoites get injected into the human bloodstream along with the saliva. Sporozoites subsequently migrate to the liver where they infect liver cells and develop into schizonts. After about 7 days the schizonts rupture and release multiple merozoites into the bloodstream. This usually coincides with the first clinical signs, such as fever. Each merozoite invades an erythrocyte, by then known as a trophozoite, and develops into a schizont again. When the schizont ruptures after about 48 hours, merozoites are once again released into the bloodstream. This cycle can be repeated over and over, which can cause rapidly increasing parasite densities. Apart from this asexual cycle, some trophozoites will develop into male or female gametocytes, ready to be ingested by a mosquito taking a blood meal. The gametocytes will reproduce sexually in the mosquito, first forming zygotes after which they transform into an ookinete and subsequently an oocyst. Rupture of the latter stage results in the release of sporozoites that travel to the salivary glands of the mosquito, thereby completing the life cycle (Figure 1).[2]

Figure 1. Life cycle of malaria



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Pathogenesis of P. falciparum infections

P. falciparum infections are associated with high morbidity and mortality. An important and common manifestation of *P. falciparum* infection is (severe) anaemia.[3,4] Anaemia due to malaria is thought to be caused by destruction of both infected and uninfected red blood cells, as well as inadequate erythropoiesis by the bone marrow.[5] Another important reason why *P. falciparum* infections are associated with high morbidity and mortality is the tendency of the parasites to sequester in the microvasculature of organs, such as brain, heart, liver, lungs and kidneys.[6,7] This sequestration has been associated with severe clinical disease, such as cerebral malaria,[6,7] but importantly it is a way of evading splenic clearance for the parasite.[8] The sequestration in organs is related to the ability of cytoadherence by *P. falciparum* parasites.[9] Cytoadherence is mediated by surface antigens expressed on infected erythrocytes when the parasite is in late trophozoite and schizont stage.[10] There are several families of surface antigens of which the most well-known is the *Pfemp1* family of surface antigens encoded by the *var* genes.[11]

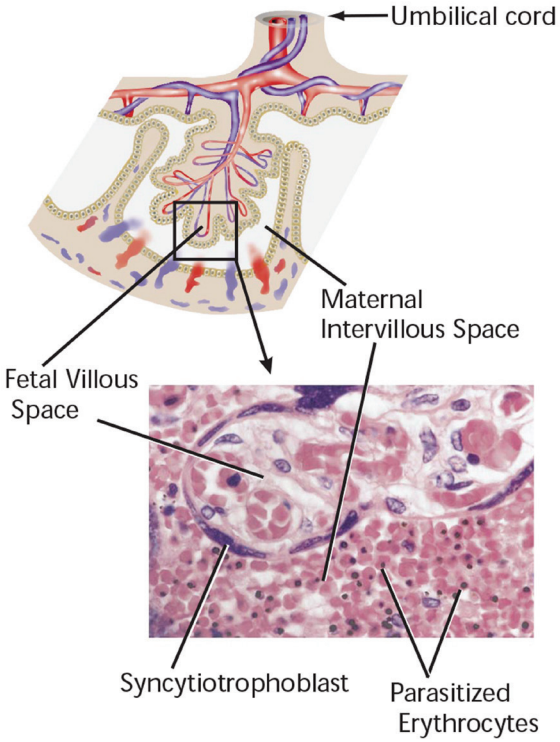
Surface antigens enable binding of *P. falciparum*-infected erythrocytes to a variety of endothelial receptors, such as cluster of differentiation 36 (CD36), intercellular adhesion molecule 1 (ICAM-1) and the more recently discovered endothelial protein C receptor that is associated with severe disease.[10,12–14] Surface antigens are known for their rapid and extensive capability of antigenic variation, which has been shown to be related to *var* gene recombination.[15,16] This antigenic variation also helps the parasite to escape the host immune system, as antibody-mediated immunity is mainly directed against the surface antigens of the malaria parasite.[17,18] An antibody response against one surface antigen is not necessarily effective against another,[19] although there is some cross-reactivity of antibodies against different surface antigens.[20] Frequent exposure to a variety of antigens is therefore needed in order to acquire a broad repertoire of antibodies against the malaria parasite.[19] Infants and young children under the age of five living in malaria endemic areas are therefore susceptible for (severe) clinical disease, but after multiple exposures they eventually acquire an effective antibody response, suppressing clinical symptoms and disease.[21,22] Nevertheless, sterile immunity is not achieved and asymptomatic infections are still frequently observed in adults.[23,24]

Malaria in pregnancy

Besides young children, pregnant women represent another group vulnerable to malaria infections. Given the natural course of acquiring immunity to malaria as explained above, one would expect adult women living in high endemic settings to have acquired a certain level of immunity that is sufficient to protect them from malaria disease. While this is true, the situation is different when a woman becomes pregnant. During pregnancy the malaria parasite favorably expresses a distinct *Pfemp1* type surface antigen that is not, or only rarely, expressed in non-pregnant individuals.[25] With this antigen, the variant surface antigen 2-chondroitin sulphate A (VAR2CSA), *P. falciparum* parasites can adhere to chondroitin sulphate A (CSA) that, attached to a core protein, forms a receptor abundantly present in the intervillous space and on syncytiotrophoblasts lining the chorionic villi of the placenta.[26–28] Due to this adherent property, the parasites can sequester within the placenta resulting in placental malaria (PM) (Figure 2). The unique expression of VAR2CSA during pregnancy means that even though women living in high endemic areas have pre-existing malaria immunity, a specific antibody response against VAR2CSA is lacking. This is particularly apparent in primigravidae, who are pregnant for the first time.[29–33] Over multiple pregnancies women will however also gain antibodies against VAR2CSA antigens.[27,29,34–36] High antibody levels against VAR2CSA or multiple domains of VAR2CSA during pregnancy have been associated with reduced risk of PM.[35,37] Multigravid women who have gained VAR2CSA antibodies over multiple pregnancies are therefore less prone to malaria

in pregnancy (MiP) and its negative effects; they experience less placental malaria and have fewer low birth weight babies.[32,38–40]

Figure 2. Sequestration of *P. falciparum* parasites in the placenta



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Placental malaria and clinical consequences

A chronic malaria infection in the placenta can result in attraction of mononuclear cells and deposition of haemozoin pigment, a waste product of the digestion of haemoglobin by the parasite, in phagocytic cells or in fibrin in the placenta.[42,43] There is also evidence for villous morphological changes of the placenta during PM.[44] Furthermore, a concurrent disturbance of the physiological cytokine balance in the malaria-infected placenta has been observed, with increased placental concentrations of T-helper 1 (Th1) type inflammatory cytokines such as tumour necrosis factor α (TNF- α) and interferon γ (IFN- γ), but also of the anti-inflammatory cytokine interleukin 10 (IL-10).[45,46] PM has been associated with preterm birth, fetal loss and more commonly with impaired growth of the fetus, resulting in low birth weight babies (<2500 grams) and, indirectly, in approximately 100.000 infant deaths each year.[47–51] There is conflicting evidence as to the timing of malaria in-

fection in pregnancy (early versus late) and the risk of low birth weight.[52–54] Besides the negative effects on the unborn child, the woman may suffer from (severe) anaemia.[55–57] After birth, there have been indications that infants born to mothers with PM, in particular multigravid mothers, have an increased risk of malaria infections in early life, even after adjusting for risk of exposure.[58,59] Immune tolerance of the child due to prenatal exposure to the parasite is one of the theories to explain this phenomenon.[60] Although there are detrimental effects of MiP, the pre-existing anti-malarial immunity in women living in high endemic settings is apparently sufficient to suppress overt clinical symptoms. Characteristic symptoms of a malaria infection, like fever in non-immune individuals, are therefore often lacking and as a result the infection can remain unnoticed.[55–57] The sub-clinical presentation of MiP is one of the important issues impairing identification of pregnant women with malaria infections.

Diagnosis of malaria in pregnancy

In pregnant women, there are two compartments where the parasites can remain: in the peripheral circulation or sequestered in the placenta. A lack of evidence for a peripheral malaria infection does not necessarily mean there is no placental infection and vice versa.[61] This is problematic, as during pregnancy there are no methods to analyze the placental compartment, so diagnosis is based on peripheral blood tests only.

In most African settings, diagnosis of *P. falciparum* infections is based on rapid diagnostic tests (RDT) or microscopy of peripheral blood slides. RDT is an easy to perform point-of-care test based on the detection of circulating *P. falciparum* antigens, without the need for specialized equipment or highly trained staff. The most used RDT for diagnosing *P. falciparum* infections is based on detection of the histidine rich protein 2 (HRP2). Other RDTs are based on the detection of *Plasmodium* lactate dehydrogenase (pLDH) or *Plasmodium* aldolase, but these are more in use in regions where other *Plasmodium* species are common and they are generally less sensitive for detecting *P. falciparum* than HRP2-based RDTs.[62,63] However, HRP2-based RDTs are known to occasionally give false positive results in areas with frequent malaria exposure due to persistent antigen circulation after clearance of infection.[64] Microscopy of peripheral blood slides can provide both species identification and parasite density and does not suffer from prolonged positivity after clearance of infection. However, well-trained staff and more specialized equipment are needed. Despite these differences, both RDT and microscopy have a detection limit of about 100 – 200 parasites/µL, depending on the type and brand of RDT and the skills of the microscopist.[63,65] This is usually sufficient for diagnosis of clinical malaria disease in young children. However, parasite densities in pregnant women are much lower, due to pre-existing immunity and to the

sequestration of parasites in the placenta, compromising sensitivity of RDTs and microscopy.[66–69] This is problematic as low density infections have also been associated to malaria-related morbidity.[70] Molecular detection of *P. falciparum*, such as (real-time) polymerase chain reaction (PCR) is a useful alternative in terms of sensitivity as PCR-based methods can detect very low parasite densities, down to ~20 parasites/μL,[71] but even a limit of detection below 1 parasite/μL has been reported.[71] Unfortunately, the need for highly specialized, fairly expensive equipment and educated and trained staff as well as the fact that these tests usually are not available near the bedside in most places, are currently major bottlenecks for implementation of molecular methods for routine diagnosis in most rural settings of sub-Saharan Africa.

At delivery, there is the possibility to diagnose malaria in the placental compartment. Naturally, this usually only serves research purposes as this diagnosis is too late for any treatment interventions. PM can be diagnosed by microscopy or real-time PCR of placental blood, but the gold standard is histology of a placental biopsy. Histological analysis of the placenta does not only provide information about the current status of infection, but it also informs on previous infections in the placenta. This is summarized in a histological classification scheme for PM consisting of four categories: I. No infected erythrocytes and no haemozoin deposition (pigment) means there is no evidence of PM; II. Infected erythrocytes without signs of pigment deposition is considered an acute infection; III. The presence of both infected erythrocytes and pigment deposition suggests an active-chronic infection; IV. The presence of pigment only without evidence of infected erythrocytes is considered a past infection.[72]

Preventive strategies for malaria in pregnancy

Pregnant women with a confirmed malaria infection can be treated with quinine plus clindamycin in first trimester, or artemisinin-based combination therapy (ACT) in second and third trimester.[73] However, as described above the lack of symptoms and reliable diagnostics often precludes case detection as an adequate measure to prevent morbidity and mortality due to MiP in high endemic settings. Instead of passive case detection, most sub-Saharan African countries have implemented or promoted several preventive strategies. Distribution of insecticide-treated bed nets at antenatal care visits (ANC) and indoor residual spraying are among efficient preventive measures.[48,74] Another preventive strategy widely implemented in sub-Saharan Africa is intermittent preventive treatment during pregnancy with sulfadoxine-pyrimethamine (IPTp-SP). IPTp-SP consists of offering prophylactic SP treatment to pregnant women at ANC visits. As the name implies, it is mainly meant as a preventive strategy. Due to the extended period of detectable plasma concentrations of the two components of SP, providing an estimated protective

window of several weeks, SP does not only contribute by curing active infections, it is also considered to prevent new infections.[75] IPTp-SP has been shown to effectively reduce malaria-related low birth weight prevalence and neonatal mortality.[76] While in earlier years two doses of IPTp-SP were given for this purpose, it has been shown that increasing the dosing frequency improves the protective efficacy.[77,78] It is therefore currently recommended by the World Health Organization to offer IPTp-SP at each ANC visit from second trimester onwards, taking a one month interval between visits into account.[79] Unfortunately, although IPTp-SP coverage has increased over the past years, still less than one third of pregnant women living in moderate to high endemic areas receive three or more doses of IPTp-SP.[1] This is because there are several barriers impairing the uptake of IPTp-SP by pregnant women. First of all, women need to (regularly) visit the ANC in order to receive SP. However, many pregnant women book their first visit to the ANC late in second trimester, leaving them unprotected in the earlier stages of pregnancy. Moreover, ANC attendance might be low in general, in particular by young adolescent women, due to cultural constraints or other domestic duties.[80,81] Also, pregnant women may refuse taking SP because they are not always well educated about its purpose or are afraid of side-effects.[82] Apart from these barriers, an increase in resistance against SP by *P. falciparum* has been observed throughout sub-Saharan Africa (described below). Therefore other treatment regimens are considered for IPTp, such as the drug combination dihydroartemisinin-piperaquine.[83] Furthermore, in recent years more attention has been given to the possibility of replacing IPTp-SP by intermittent screening and treatment (IST). During IST, pregnant women are screened for malaria with a RDT and given anti-malarial treatment in case of a positive test result. Because of inferior results to IPTp-SP and reduced cost-effectiveness,[84] IST as replacement for IPTp-SP is currently not recommended by the WHO.[85]

Resistance against sulfadoxine-pyrimethamine (SP)

SP was previously used as first-line treatment for malaria in non-pregnant individuals. Due to increasing resistance of *P. falciparum* against SP and the fact that artemisinin-based combination treatments became available, its use as first-line treatment has long been abandoned. Because of its prophylactic effects and sufficient safety profile it is still in use however as IPTp-SP, or as intermittent preventive treatment of infants (IPTi) or seasonal chemoprevention. The reduced efficacy of SP in clearing malaria infections in the non-pregnant population initially did not translate in a reduced efficacy of IPTp-SP in preventing low birth weight babies.[86,87] However, it seems that resistance against SP is further increasing, which has been demonstrated by the increase in prevalence of mutations over the last decade in

two genes of *P. falciparum* associated with SP resistance: dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*).[88–91] Combinations of several point mutations in these genes (*dhfr* codons N51, C59, S108 and *dhps* codons S436, A437, K540) have been associated with reduced efficacy of SP.[92,93] The quintuple mutant (triple *dhfr* N51, C59 and S108 mutation and double *dhps* A437, K540 mutation), but more importantly the newly emerging sextuple mutant (quintuple mutation plus *dhps* A581 mutation) in East Africa, have been associated with failure of IPTp-SP in preventing MiP or MiP-related morbidity.[94–96] In West Africa, thus far quintuple and sextuple mutants are rarely described.[97]

Aims of this thesis

Malaria infections in pregnant women may, amongst other sequelae, result in maternal anaemia, low birth weight babies and increased malaria risk for infants during early life. Currently, there are several issues that are hampering optimal prevention of malaria-related morbidity. First of all, due to the general lack of symptoms, passive case detection is not useful for identifying women with malaria. Therefore, intermittent preventive treatment strategies with SP have been implemented to protect pregnant women from malaria infection. However, the uptake of IPTp-SP is currently not sufficient to protect women throughout their pregnancy. Also, resistance of the malaria parasite against SP still seems to be increasing in many parts of sub-Saharan Africa. As a solution to these issues, we developed a new addition to the preventive strategy, based on an existing strategy to prevent malaria morbidity in children, named community case management of malaria (CCMm). In CCMm, malaria care is brought closer to home by the use of community health workers (CHW). CHWs are lay health workers specifically trained for diagnosis and treatment of malaria. Children who are suspected of malaria can thus visit the CHW in their own village.[98] Having CCMm as an example, an intervention trial was designed in which CHWs were now deployed for providing malaria care to pregnant women. From second trimester onward, women were visited monthly by the CHW and were screened for malaria with a RDT (irrespective of clinical symptoms). In case of a positive RDT result the CHW would provide a course of artemether-lumefantrine. This intervention was in addition to the regular care at the ANC, where women would receive IPTp-SP according to national guidelines as usual. By this intervention we aimed to capture and treat any woman not fully protected by monthly IPTp-SP doses, either due to insufficient attendance to the ANC, resistance against SP, or other reasons.

An important consideration in this strategy is whether the sensitivity of RDTs is sufficient for screening malaria in pregnancy. RDT performance when executed by CHWs for the screening of MiP is unknown. Two aims of this thesis were therefore to evaluate RDT performance when used by CHWs and to evaluate whether CHWs

are confident in making treatment decisions for malaria in pregnant women, in order to provide insights into the feasibility of such a community-based intervention as well as to interpret results of the intervention trial.

Another aim of this thesis was to gain insights into the occurrence of SP resistance in parasites in these pregnant women. If there is evidence of high resistance against SP by *P. falciparum*, the screen and treat intervention may be more effective, as women will be less protected by IPTp-SP. Furthermore, as it has been suggested that IPTp-SP itself may also select for SP resistant parasites, monthly screening may result in treatment of these parasites with artemether-lumefantrine which could actually prevent selection of SP resistant parasites.

Another important issue to solve, which could improve prevention of malaria-related morbidity, is the lack of reliable diagnostics for MiP. An innovative approach for new diagnostic methods is to target the host response rather than detection of parasites as an indication of infection. As described above, PM is characterized by a disturbance in (anti-) inflammatory cytokines. In this thesis, we aimed to identify host biomarkers that can support diagnosis of MiP. Furthermore, the immune response against *P. falciparum* is unique in pregnant women in that it is mainly directed against VAR2CSA surface antigens. As high levels of VAR2CSA antibodies during pregnancy have been shown to be associated with a reduced risk of PM, another aim of this thesis was therefore to evaluate whether we could identify pregnant women at increased risk of malaria-related morbidity based on their levels of VAR2CSA antibodies early in pregnancy. Identifying such a risk group would enable more targeted preventive strategies.

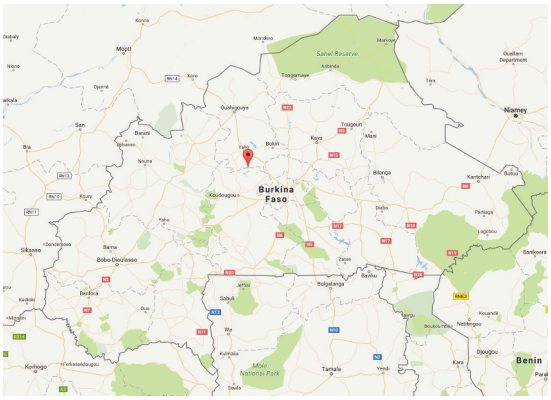
Study area

The research described in this thesis was conducted in the Nanoro health centre catchment area, a rural area about 85 km northwest of Ouagadougou, the capital of Burkina Faso (Figure 3). It is a holoendemic area for malaria characterized by perennial transmission, with a peak during and directly after the rainy season that lasts from June to October. In this area, mainly *P. falciparum* and sporadically *P. malariae* or *P. ovale spp.* are found in both pregnant and non-pregnant individuals.[99,100] This thesis covers only *P. falciparum* infections during pregnancy.

Thesis outline

This thesis is divided in two parts. In Part I, the new intervention strategy is explained and evaluated, and local SP resistance in *P. falciparum* is investigated, while in Part II, the potential use of host biomarkers to support diagnosis of MiP was evaluated, and it was studied whether antibodies against VAR2CSA surface antigens can identify women at risk of malaria-related morbidity.

Figure 3. Nanoro, Burkina Faso



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The successes and barriers of CCMm related to quality of care and sustainable implementation are reviewed in **Chapter two** of this thesis, so that lessons could be learned for the community-based intervention in pregnant women. In **Chapter three**, the design of the intervention for pregnant women is described in detail. The performance of CHWs using RDTs for malaria screening in pregnant women and the adherence to test results is evaluated in **Chapter four**. The current local level of SP resistance in *P. falciparum* was examined in **Chapter five** by studying the prevalence of mutations associated with SP resistance.

A systematic literature review on potential diagnostic or predictive markers of (placental) malaria infections is described in **Chapter six**. A selection of cytokines was subsequently studied in a case-control population of women with and without malaria in **Chapter seven**, to evaluate their potential use in supporting malaria diagnosis. In **Chapter eight** the potential of VAR2CSA antibody levels as a marker for the risk of (placental) malaria infections and low birth weight babies was examined. Finally, the findings of this thesis are discussed in **Chapter nine.**

Abbreviations

CCMm: community case-management of malaria; CHW: community health worker; dhfr: dihydrofolate reductase; dhps: dihydropteroate synthase; HRP2: histidine rich protein 2; IPTp-SP: intermittent preventive treatment during pregnancy with sulfadoxine-pyrimethamine; IST: intermittent screening and treatment; MiP: malaria in pregnancy; PCR: polymerase chain reaction; PM: placental malaria; RDT: rapid diagnostic test; SP: sulfadoxine-pyrimethamine; VAR2CSA: variant surface antigen 2-chondroitin sulphate A

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