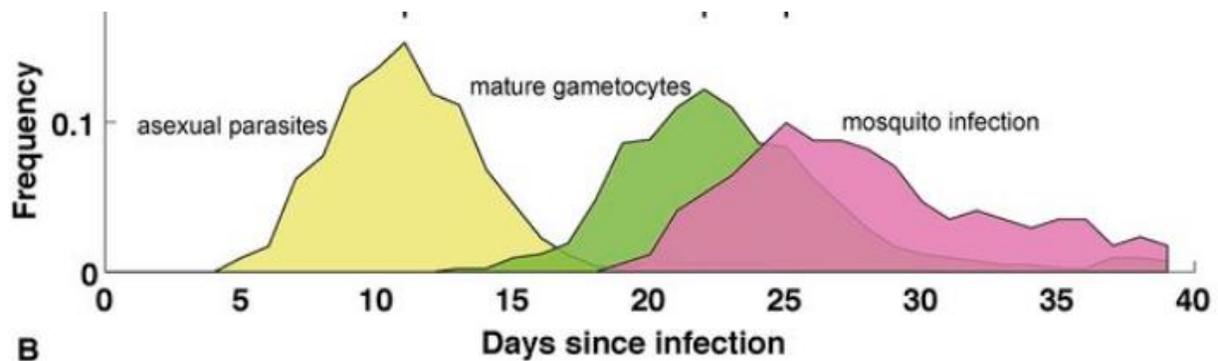


# Artemisia afra blocks transmission of gametocytes

## Abstract.

Gametocytes are loaded with hemozoin resulting from hemoglobin consumption. Their voracious need of this food may explain why they sequester in the bone marrow where they find a large supply of young red blood cells generated by erythropoiesis. Malaria leads to hemolysis and anemia, often severe, and this triggers and amplifies erythropoiesis. Over the years it became evident that not only intravenous artesunate often causes hemolysis, but also ACT therapy. In a recent large-scale clinical trial in Maniema, RDC, it was found that *Artemisia afra* void of artemisinin causes less hemolysis than *Artemisia annua* rich in artemisinin. This may explain why *Artemisia afra* rapidly and completely eliminates gametocytes and consequently blocks transmission to mosquitoes.



## Introduction

Gametocytes of *Plasmodium falciparum* go through five (I-V) stages for their development, which under the microscope have different shapes and motilities, those of stage V with their sickle shape having the highest motility. During stage II to V gametocytes hide in the bone marrow for their development. This may explain why they do not generate any signs of pathology.

*Plasmodium falciparum* immature gametocytes are not observed in peripheral blood. The results of a Spanish study highlight the high prevalence and abundance of early sexual stages in bone marrow. Selective accumulation of immature gametocytes in bone marrow during the 8 to 12 days they need for maturation may provide them with a better niche for survival than the lumen of small vessels. It cannot be ruled out that cytoadhesion of erythrocytes infected by sexually committed trophozoites to specific receptors in the bone marrow may contribute to the enrichment of early sexual stages in this organ.

Ruth Aguilar, Ariel Magallon-Tejada, Ariel H. Achtman. Molecular evidence for the localization of *Plasmodium falciparum* immature gametocytes in bone marrow. *Blood*. 2014;123(7):959-966.

Additionally, the invasion of erythroid precursor cells in the bone marrow allows the gametocytes to develop in young erythrocytes with a median life expectancy much longer than that of circulating erythrocytes that become increasingly rigid with age and thus allow these long-lived gametocytes to

circulate for the maximum time possible. Developing gametocytes undergo remarkable shifts in their erythrocyte membrane elasticity, which may allow them to be retained within the bone marrow until maturation. Once mature, and when their membrane elasticity returns to that approximating an uninfected erythrocyte, they then can egress the bone marrow and return to the peripheral circulation to be ingested by the mosquito during a blood meal.

*Christopher L. Peatey, Jolanta A. Watson, Enhanced gametocyte formation in erythrocyte progenitor cells: a site-specific adaptation by Plasmodium falciparum. The Journal of Infectious Diseases. 2013-208 DOI:10.1093/infdis/jit309*

*Valeria Messina, Mauro Valtieri, Gametocytes of the Malaria Parasite Plasmodium falciparum Interact With and Stimulate Bone Marrow Mesenchymal Cells to Secrete Angiogenetic Factors. Front Cell Infect Microbiol. 2018; 8: 50.*

The goal to prevent *Plasmodium falciparum* transmission from humans to mosquitoes requires the identification of targetable metabolic processes in the mature (stage V) gametocytes, the sexual stages circulating in the bloodstream. This task is complicated by the apparently low metabolism of these cells, which renders them refractory to most antimalarial inhibitors and constrains the development of specific and sensitive cell-based assays. Gametocytes remain in a state of arrested cell development until ingested by a feeding mosquito. Dark pigmented *hemozoin* crystals are present in multiple *gametocyte* developmental stages and are generated from the biocrystallization of hemozoin, a toxic intermediate derived from the parasite digestion of hemoglobin.

*Giulia Siciliano, T. R. Santha Kumar, Roberta Bona, A high susceptibility to redox imbalance of the transmissible stages of Plasmodium falciparum revealed with a luciferase based mature gametocyte assay, Mol Microbiol 2011; 79, 306-18*

## **Gametocytes and transmission**

An essential element for continuing transmission of *Plasmodium falciparum* is the availability of mature gametocytes in human peripheral circulation for uptake by mosquitoes. Natural immune responses to circulating gametocytes may play a role in reducing transmission from humans to mosquitoes.

*Joice, R. et al. Plasmodium falciparum transmission stages accumulate in the human bone marrow. Sci. Transl. Med. 6, 244re5 (2014).*

*Farfour, E., Charlotte, F., Settegrana, P. The extravascular compartment of the bone marrow: a niche for Plasmodium falciparum gametocyte maturation? Malar. J. 11, 285 (2012).*

Recent evidence suggests that the antibodies which intervene during the sporozoite invasion, the merozoite multiplication and the development of immature stage I-IV gametocytes are not the same as those active for stage V mature gametocytes. Antibody recognition was evaluated in a cohort of Ghanaian school children by flow cytometry. The findings support the existence of antigens on the surface of a sub-population of mature gametocyte-infected erythrocytes which induce specific antibody responses. Children with asymptomatic malaria carry antibodies that recognize antigens on the surface of in vitro-

cultured erythrocytes infected with mature *Plasmodium falciparum* gametocytes. These antibodies did not recognize immature gametocyte-infected erythrocytes. The level of recognition varied among individuals. But gametocytes of East African origin can be recognized by antibodies from West African individuals.

*Dinko B, King E, Targett GA, Sutherland CJ. Antibody responses to surface antigens of Plasmodium falciparum gametocyte-infected erythrocytes and their relation to gametocytaemia Parasite Immunol. 2016 Jun;38(6):352-64. doi: 10.1111/pim.12323*

## **Hemozoin in gametocytes**

Severe infections are a major source of stress on hematopoiesis, where consequences for hematopoietic stem cells have only recently started to emerge. It was found that during *Plasmodium* infection the hematopoietic compartment turns over significantly faster than in steady state.

*Myriam L.R. Haltali, Samuel Watcha, Malaria-induced remodelling of the bone marrow microenvironment mediates loss of haematopoietic stem cell function. <https://doi.org/10.1101/477190>*

Trophozoites have variable amounts of pigment, depending on the species of *Plasmodium* and the stage of infection. In *Plasmodium falciparum* infection, blood smear preparations fall into two categories that are distinguishable at all levels of parasitemia; one type of preparation contains only pigment-deficient trophozoites, and the other type contains only pigment-rich trophozoites. This finding dates back to 1983.

*Jamjoom GA. Dark-field microscopy for detection of malaria in unstained blood films. J Clin Microbiol. 1983 May;17(5):717-21.*

The presence of clinical peripheral blood samples of *P. falciparum* with high parasitemia containing only hemozoin-deficient (non-pigmented) asexual forms has been repeatedly confirmed. Such samples stand in contrast with other samples that contain mostly pigmented circulating trophozoites and gametocytes, indicating that pigment accumulation is a prominent feature of gametocytogenesis.

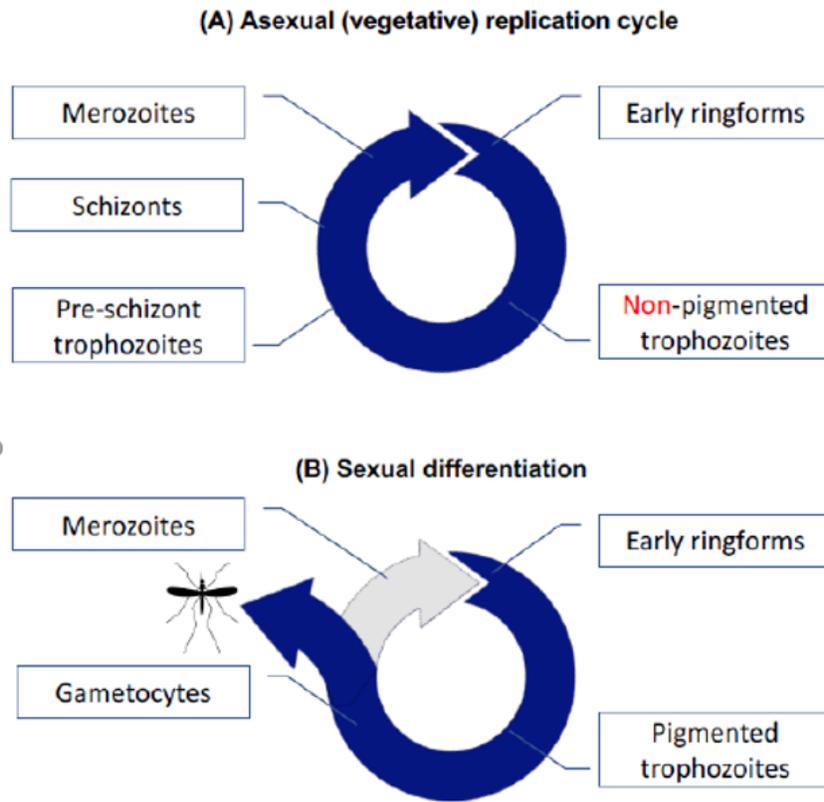


Fig.1. Asexual multiplication circle (A) and sexual differentiation in *Plasmodium falciparum* (B)

. Ghazi A. Jamjoom. Evidence for a role of hemozoin in metabolism and gametocytogenesis. *MalariaWorld Journal*, 2017, 8:10. ISSN 2214-4374

If a drug or a plant inhibits the biocrystallization of hemozoin it may inhibit or reduce the generation of gametocytes. This is the case for example for *Artemisia* herbal tea. *Artemisia afra* appears to be more potent in this respect than *Artemisia annua*, based on the results obtained by Mutaz Akkawi at the University of Al Quds.

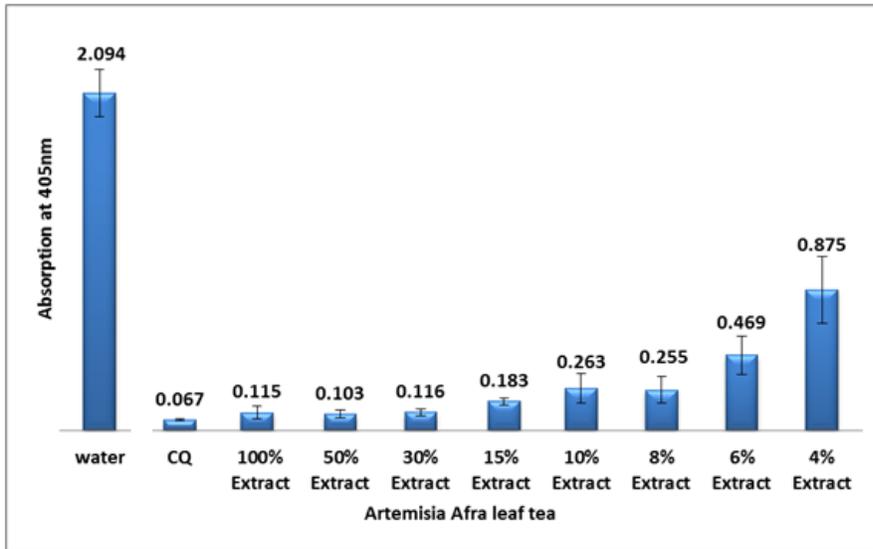


Figure 2. Column diagram representing the potential anti-malarial activity of *Artemisia afra* leaf, water extract using method B, compared to the negative and positive control: CQ-chloroquine 0.1mg/ml showing the absorption values of dissolved  $\beta$ -hematin (alkaline hematin) at 405 nm using ELISA reader, according to E. Deharo semi-quantitative method. The absorption is inversely proportional to drugs efficiency, the lower the absorption is, the drug is considered to be more efficient.

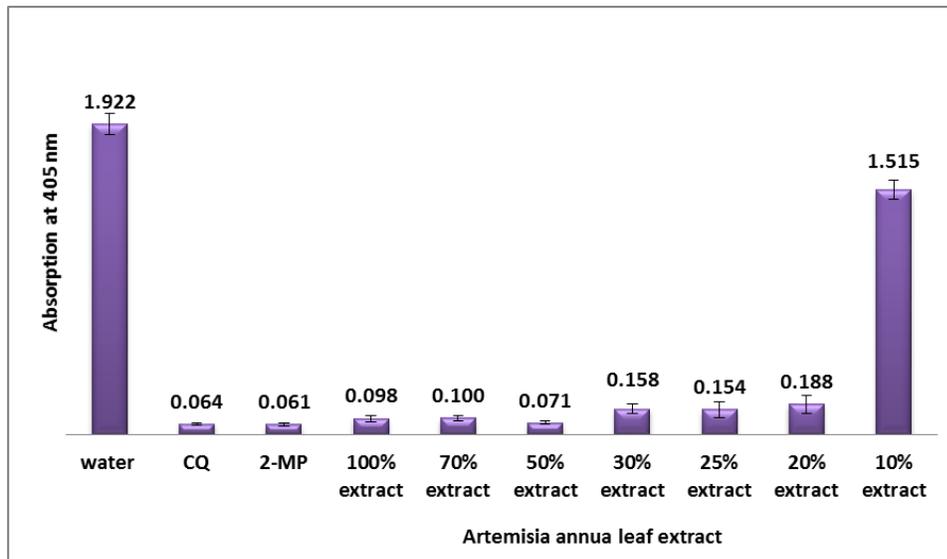


Figure 3 : Column diagram representing the potential anti-malarial activity of *Artemisia annua* leaf, water extract using the same method method.

This stronger inhibition by *Artemisia afra* may explain the apparently stronger reduction in the gametocyte carriage for *Artemisia afra* than for *Artemisia annua*, as noticed in the clinical trials in Maniema, RDCongo

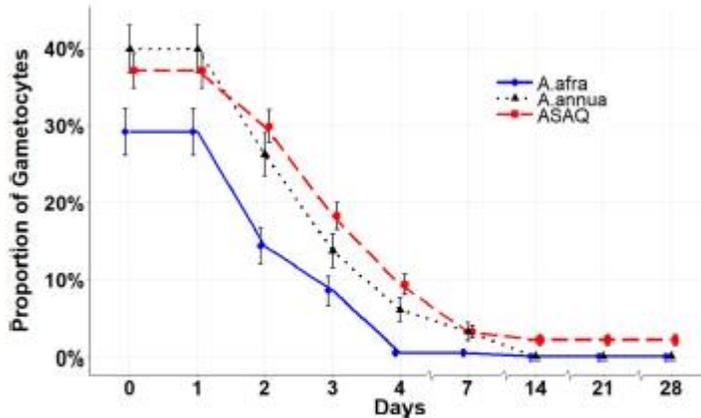


Fig. 4. Microscopically determined proportion of patients with gametocytes (carriers) throughout the trial period.

*Artemisia annua* and *Artemisia afra* tea infusions vs. artesunate-amodiaquine (ASAQ) in treating *Plasmodium falciparum* malaria in a large scale, double blind, randomized clinical trial  
 Jerome Munyangi, Lucile Cornet-Vernet, Michel Idumbo, Chen Lu, Pierre Lutgen, et al., *Phytomedicine* 57 (2019) 49–56

## Gametocytes, artemisinin and hemolysis

There are thousands of studies and papers on the therapeutic effects of artemisinin, its derivatives and artemisinin combined therapy (ACT) on the asexual, trophozoite stage. But those on the effect of these drugs on the sexual, gametocyte stage are scarce.

The effects of the therapeutic response to ACTs have been studied 20 years ago. Delay in the time taken to clear the initial parasitemia increased the risk of subsequent gametocyte carriage significantly. Patients whose infections recrudesced were also more likely to become gametocytemic during follow up. Once formed, the sexual stages often persist for up to three weeks and show a persistent ability to infect mosquitoes.

*Rik Price, François Nosten; Julie Simpson, Christine Luxemburger. Risk factors for gametocyte carriage in uncomplicated falciparum malaria Am. J. Trop. Med. Hyg., 60(6), 1999, pp. 1019–1023*

Artemisinin-based combination treatments (ACTs) or intravenous artesunate are used in over 100 countries for uncomplicated or severe falciparum malaria. Although intravenous artesunate may cause delayed haemolytic anaemia, there is little evaluation of the temporal changes in haematocrit following ACTs. In a study in Nigeria clinical and parasitological parameters were measured before and following treatment of uncomplicated falciparum malaria in children with artesunate-amodiaquine or artemether-lumefantrine over 6-weeks.

*Akintunde Sowunmi, Adejumoke I. Ayede, Temporal changes in haematocrit following artemisinin-based combination treatments of uncomplicated falciparum malaria in children BMC Infect Dis. 2015; 15: 454.*

Episodes of delayed hemolysis 2–6 weeks after treatment for severe malaria with intravenous artesunate have been observed in non–malaria-immune patients in Europe. This phenomenon, recently referred to as post-artemisinin-delayed-hemolysis (PADH), has been confirmed in other nonimmune patients and in children in Africa. Approximately 20%–30% of nonimmune patients given intravenous artesunate show signs of PADH that vary in intensity and duration.

The pathophysiology of hemolysis after artemisinin therapy is not fully understood. Once-infected erythrocytes, that have been cleared of parasites in the spleen, have a shorter life span and play a role. Patients with higher concentrations of once-infected erythrocytes after artemisinin treatment are at higher risk for PADH.

In a study in Mali, standard oral doses of artesunate were administered for 7 days and patients were followed up for 28 days. The proportion of gametocyte carriers was unchanged at the end of treatment and did not significantly decline until day 21 of follow-up. Artesunate did not clear mature gametocytes during treatment and did not prevent the appearance of new stage V gametocytes as assessed by light microscopy. In this geographical area in Mali, baseline gametocyte carriage was significantly higher 6 years after the deployment of artemisinin-based combination therapies in this setting

*Abdoulaye A. Djimde, Amelia W. Maiga, Gametocyte clearance dynamics following oral artesunate treatment of uncomplicated falciparum malaria in Malian children. Parasite 2016, 23, 3*

Delayed hemolysis occurs not only after intravenous treatment for severe malaria but also in a substantial number of patients given oral ACTs for uncomplicated malaria. Because delayed hemolysis has not been captured by safety studies on ACTs, we assume that delayed hemolysis after oral ACTs is less pronounced and occurs to a subclinical degree.

A prospective observational study was conducted at the University Hospital of Charité–Universitätsmedizin, Berlin (Berlin, Germany). All patients who sought treatment at the hospital and were found to have microscopically confirmed uncomplicated *P. falciparum* malaria were included in the study after written informed consent was obtained. This analysis evaluated data for all patients given oral ACTs during the first 12 months of the study. The primary objective was to assess the proportion of patients with posttreatment hemolysis. All malaria infections had been acquired in Africa.

The criteria for posttreatment hemolysis on day 14 were met by 8 (40%) of 20 patients. Data from this prospective study confirm the hypothesis that delayed posttreatment hemolysis also occurs after oral artemisinin treatment and provide insight into its frequency and clinical course. The role of this observation for clinical practice in malaria-endemic and non–malaria-endemic settings remains to be defined but should prompt increased vigilance for hemolytic events, particularly for patients with preexisting anemia. This delayed hemolysis by ACTs has also been found in other studies in Mali.

*Florian Kurth, Tilman Lingscheid, Hemolysis after Oral Artemisinin Combination Therapy for Uncomplicated Plasmodium falciparum Malaria. Emerging Infectious Diseases, 2016 Vol. 22, No. 8,*

*Issaka Sagara, Renaud Piarroux, Abdoulaye Djimde, Delayed anemia assessment in patients treated with oral artemisinin derivatives (ACT) for uncomplicated malaria: a pooled analysis of clinical trials data from Mali. Malaria Journal volume 13, Article number: 358 (2014)*

Looking for an explanation we also found in the clinical trials from Maniema RDCongo that the ACT ASAQ and *Artemisia annua* reduced hemoglobin, but *Artemisia afra* did not. The latter does not contain artemisinin.

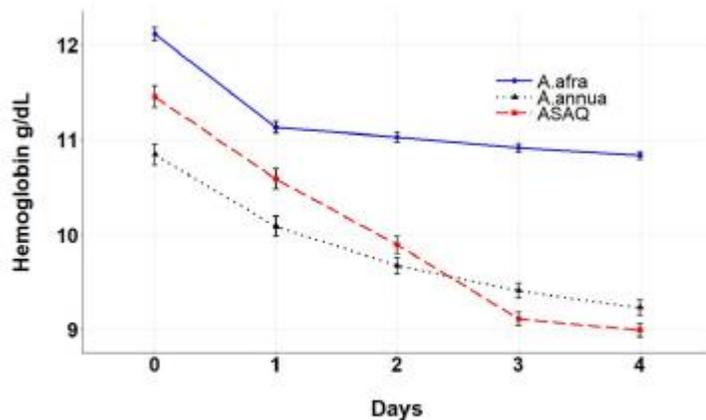


Fig. 5. Hemoglobin levels during the first four days of treatment.

*Artemisia annua* and *Artemisia afra* tea infusions vs. artesunate-amodiaquine (ASAQ) in treating *Plasmodium falciparum* malaria in a large scale, double blind, randomized clinical trial. Jerome Munyangi, Lucile Cornet-Vernet, Michel Idumbo, Chen Lu, Pierre Lutgen, et al., *Phytomedicine* 57 (2019) 49–56

The French Artesunate Working Group relates delayed hemolysis to pitting. When invaded and occupied by *Plasmodium falciparum*, normally pliable red blood cells become rigid and inelastic, properties that contribute to the occlusion of capillaries and the symptoms of malaria. Pitting is a phenomenon that occurs in the spleen, in which the bulk of an infected erythrocyte has to enter a 2- $\mu$ m tube, leaving the parasite jammed at the entrance; the membrane ruptures, leaving the parasite behind, and the parasite-free red cell emerges out the other end. In the spleen, such “pitted” erythrocytes can then be returned to circulation.

As PADH is related to artesunate, but not to quinine, its mechanism may be linked to a specific effect of the artemisinin class of drugs. The rapid parasite clearing action of artesunate is based on the pitting process. The erythrocytes then reseal rather than lyse. As a mechanism of microbial clearance, pitting is unique because it initially spares the host cell. 20 After being pitted, once-infected erythrocytes reenter the circulation, now parasite free but with a reduced lifespan. Thus, although pitting initially spares ring-hosting erythrocytes, this positive effect is not sustained. The delayed clearance of once-infected erythrocytes would explain major features of PADH, specifically the syndrome’s delayed occurrence in parasitologically cured patients and the observed hemolytic profile.

*Stéphane Jauréguiberry, Papa A. Ndour. Postartesunate delayed hemolysis is a predictable event related to the lifesaving effect of artemisinins. Blood* 2014 124, 167-175

The effect of RBC hemolysates on erythropoiesis was investigated by a research team in Mexico, already in 1963. The results furnish strong evidence that the products of erythrocytic destruction have an enhancing effect on the recovery of anemia.

*Luis Sanchez-Medal, Juan Labardini, Hemolysis and Erythropoiesis. Blood (1963) 21 (5): 586–593.*

And in 1968 the Colorado School of Medicine studied the hemoglobin production in response to marked blood loss. Bone marrow erythroblasts increased in the same extent as hemoglobin production, up to 3.5-4.6 times normal.

*RD Hamstra, MH Block, Erythropoiesis in response to blood loss in man. J Appl Physiol. 1969, 27, 503-507.*

In a French INSERM study hemoglobin concentrations were negatively correlated with peak gametocyte counts and gametocyte carriage durations. Severe malaria cases were more likely to have gametocytes than mild malaria cases.

*Nacher M, Singhasivanon P, Silachamroon U, Decreased hemoglobin concentrations, hyperparasitemia, and severe malaria are associated with increased Plasmodium falciparum gametocyte carriage. J Parasitol. 2002 Feb;88(1):97-101.*

## **Conclusion**

**Hemolysates increase erythropoiesis in the bone marrow and this provides the large supply of hemoglobin needed by young gametocytes. An additional hypothesis is that the hemolytic effect of the epoxide artemisinin ruptures the young erythrocytes in the bone marrow, and that the voracious gametocytes take advantage of the easy and increased supply of reticulocytes (young red blood cells) and hemoglobin. And create a numerous population of strong stage V gametocytes. It would be dramatic if ACTs recommended by WHO since 20 years, had increased malaria transmission.**

**A process of this complexity is of course difficult to study and reproduce *in vitro*.**