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SEED HAEMATOLOGY

Malaria – the global burden

Introduction

Malaria has plagued humankind since ancient times and is still a significant threat to around half of the world's population. Although malaria infections are primarily a problem in tropical regions, they occur ever more frequently also in non-endemic areas due to tourism and globalisation.

In 2012, 5,161 confirmed cases of malaria were reported by 26 European countries. 85 % of these cases were reported by five countries alone: France, United Kingdom, Germany, Spain and Belgium. 99 % of those malaria cases were reported as imported [1].

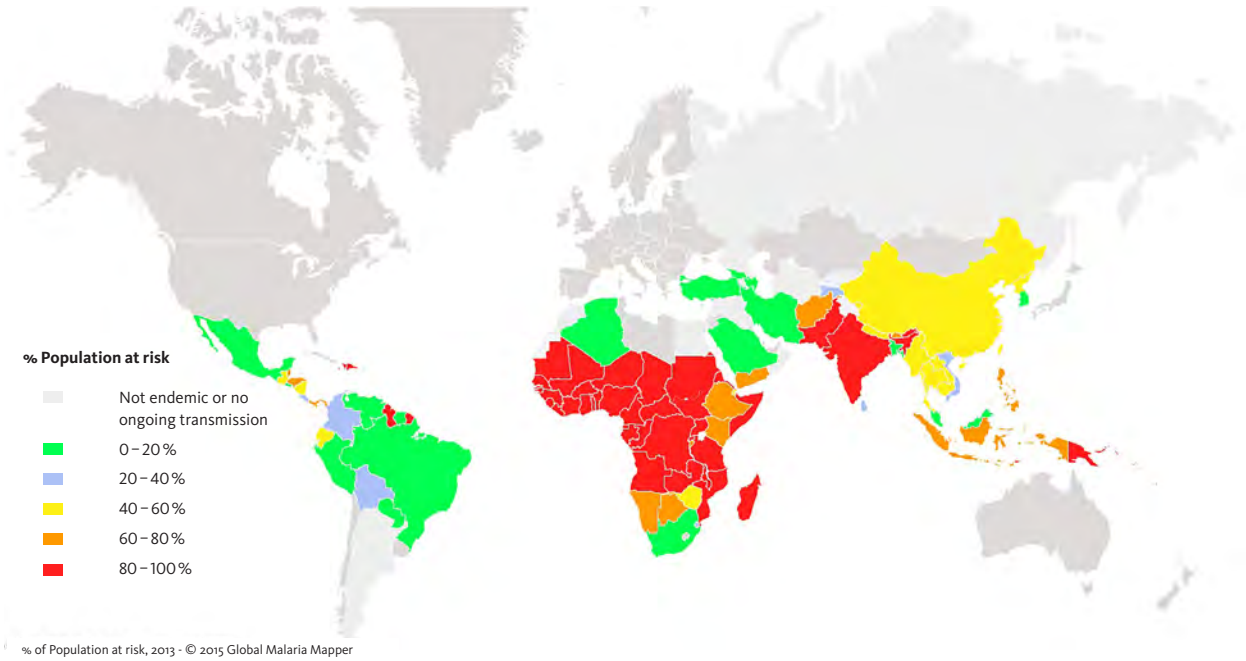

Fig. 1 *Population at risk of contracting malaria according to data from 2013 [3]*

According to the World Health Organization (WHO), globally an estimated 3.3 billion people in 97 countries and territories are at risk of being infected with malaria and developing disease (Figure 1), and 1.2 billion are at high risk (>1 in 1,000 chance of getting malaria within a year). According to the latest estimates, 198 million cases of malaria occurred globally in 2013 (uncertainty range 124 – 283 million) and the disease led to 584,000 deaths (uncertainty range 367,000 – 755,000), representing a decrease in malaria case incidence and mortality rates of 30 % and 47 % since 2000, respectively. The burden is heaviest in the WHO African Region, where an estimated 90 % of all malaria deaths occur with children under the age of five accounting for 78 % of all deaths [2].

Diagnostic challenges

Whereas diagnostics and treatment are commonplace in those countries where the disease is endemic, this is unfortunately not the case in Europe. Frequently, the treating physicians have difficulties giving advice on prophylactic measures or coming up with a fast and clear diagnosis in the event of an infection. Unfamiliar with the clinical picture, they might misinterpret primary clinical symptoms such as fever and chills, headaches, muscle pains, enlarged liver or pancreas, nausea and vomiting, abdominal pain and/or diarrhoea as an influenzal infection or some similar illness. Whilst the absence of a travel history to a malaria endemic region is unusual, it definitely does not preclude a diagnosis of malaria. The problem in such instances however is that malaria would typically not be considered in the differential diagnosis by the majority of clinicians with little or no prior exposure to malaria patients. Valuable time will elapse before the proper diagnosis is made and treatment can begin. Depending on the type of pathogen, such a delay may be fatal for the patient.

Therefore, for the patient as well as for the treating physician and the laboratory, it is of utmost importance to use any available information in a way that enables fast diagnosis and treatment. This is particularly true for Europe, where no rapid malaria test forms part of the standard programme for an initial investigation of a patient with flu-like symptoms. Such a test will only be specifically requested if malaria infection is suspected. In addition to the clinical symptoms and information about the patient's travels, deviations in the blood count can also be used under certain conditions to trigger the request for a malaria test or blood films ('thick smear' and 'thin smear'). The entire range of malaria tests requires skilled examiners with a good morphological knowledge and familiarity with the possibilities and limitations of haematology analysers and the patterns to be expected in the blood count, especially in the early stage of infection. In this respect, also the sensitivity of the 'thick smear' reference test is to be taken into account. Even in expert hands, 'thick smears' lack sensitivity giving rise to false negative results [4].

Since blood counts are frequently performed in febrile patients, a careful study of the results provided by automated haematology analysers in malaria patients has shown that several changes, mainly related to abnormal white blood cell (WBC) scattergrams, were peculiar enough to suggest malaria infection in these patients, without knowledge of their clinical status [5].

Malaria

Malaria is caused by five species of the parasite belonging to the genus *Plasmodium*. Four of these – *P. falciparum, P. vivax, P. malariae* and *P. ovale* – are human malaria species, which are spread from one person to another by female mosquitoes of the genus *Anopheles*. In the following the two major types of malaria pathogens, *P. falciparum* and *P. vivax*, will be described in more detail as they are the most frequently occurring and have very characteristic differences, not only in the course of the disease but also in the blood smear and results of the haematology analyser.

1. Plasmodium falciparum

This pathogen is most prevalent on the African continent, and is responsible for most deaths from malaria [2]. After a person is bitten by an infected female *Anopheles* mosquito – the intermediate host for all malaria pathogens – the pathogen first infests the hepatocytes. In the hepatocyte, organisms mature into tissue schizonts after an incubation time of 8 to 12 days. These schizonts release thousands of merozoites into the bloodstream, which infect red blood cells (RBC), forming ring trophozoites which are named after the signet ring appearance of the chromatin dot and blue circle of parasite cytoplasm (see Figures 2 and 3). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites, and these again infect other red blood cells. Some trophozoites differentiate into gametocytes. The gametocytes do not show any further growth in the human host.

During its intraerythrocytic asexual development, the trophozoite feeds on haemoglobin by ingesting small amounts of red cell cytoplasm. The globin component is further digested into amino acids for the parasite's metabolic needs. However, haeme is toxic to the parasite and is therefore aggregated to the insoluble dark-brown crystal called haemozoin, which can accumulate in the parasitised red blood cell. Haemozoin is released by the rupture of parasitised red cells, reaches high concentrations in the circulation, and is actively phagocytized by circulating monocytes and neutrophils as well as by tissue macrophages of the liver and spleen. Haemozoin activates the release of various proinflammatory mediators by monocytes/macrophages, including TNF-α, IL-1β and the chemokines MIP-1α and MIP-1β, but also plays an inhibitory role in the maturation and function of immature dendritic cells and may be responsible for certain features of the immune suppression that is characteristic of malarial infection [6]. Haemozoin is also produced by all other *Plasmodium* species. Further propagation and development of the pathogens will result in the red blood cells' bursting and – due to the released parasites and their metabolic products – this will finally result in the clinically apparent symptoms, such as fever and chills. The usual incubation period is around 12 days for an infection with *P. falciparum*. Unique to the clinical picture is the change in the surface characteristics of the infected red blood cells; they virtually 'stick' to the endothelial cells that line the lumen of the vessels. This adhesive property of the red blood cells bears the greatest risk as vascular occlusions – especially of capillaries, e. g. in the brain (the so-called 'cerebral malaria') or in the kidneys – can result in hypoxia and serious organ damage, the main cause for the high fatality rate with this type of pathogen.

At the same time, this adhesive property also has the effect that only specific forms of affected red blood cells are detectable in peripheral blood – mainly the aforementioned ring form.

Aside from the fact that the ring form is the primarily detectable one in the red blood cells, there are other special features in the blood smear to be observed:

1. The infected red blood cells detectable in the blood count are not enlarged.
2. Infected red blood cells contain detectable parasitic nucleic acid, as opposed to the non-infected red blood cells. In this case, ring forms with two chromatin dots can occur (see centre cell in Figure 2).
3. Also possible are several ring forms inside a single red blood cell (see Figure 3).
4. Gametocytes only occur after approximately four weeks in peripheral blood. However, they are rarely visible. This sexual form will be ingested by the intermediate host and there develops further until being transmitted to yet another human.
5. *P. falciparum* invades red blood cells of all ages, reaching parasitaemias of over 50 % causing severe anaemia. This is especially true in view of the fact that the number of pathogens increases greatly while the number of red blood cells will simultaneously decrease.
6. An additional haematological characteristic is a worsening anaemia.
7. Aside from that, thrombocytopenia and leukocytopenia are also typical for the clinical picture, as well as the occurrence of monocytosis. In a very late stage, the number of leukocytes may then increase again.

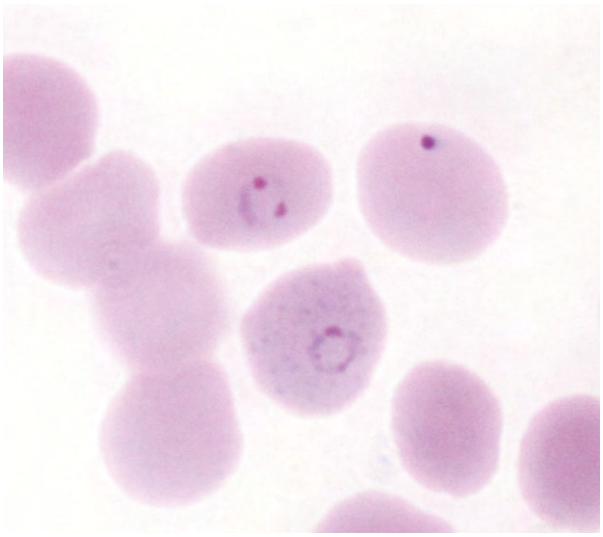

Fig. 2 *Intraerythrocytic trophozoites, or ring forms, with a* Plasmodium falciparum *infection*

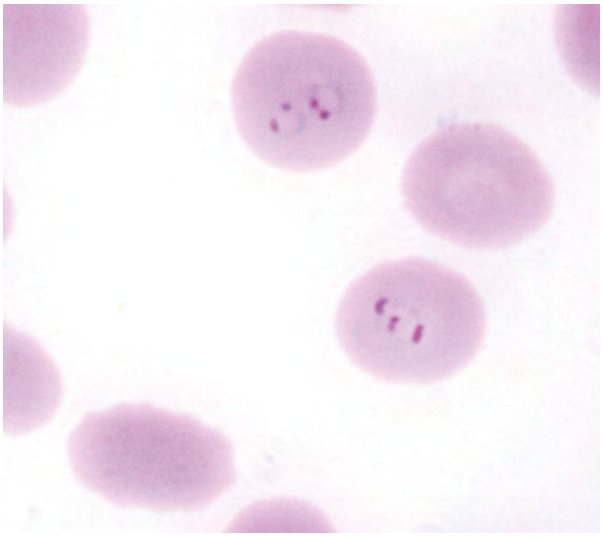

Fig. 3 *Two ring forms of the* Plasmodium falciparum *parasite in a single red blood cell*

Fortunately, an infection caused by *P. falciparum* which was overcome does not bear the risk of the fever recurring months or years after the initial infection since the parasite does not remain in the liver after the first development cycle, as opposed to *P. vivax* and *P. ovale*.

2. Plasmodium vivax

P. vivax is more geographically widespread (mostly in Asia, Latin America and some parts of Africa) than *P. falciparum*. Because of the population densities especially in Asia it is probably the most prevalent human malaria parasite. Basically, the life cycle of *P. vivax* is very similar to that of *P. falciparum*. However, from its clinical picture, *P. vivax* malaria is not nearly as serious as malaria caused by *P. falciparum*.

Preferentially younger red blood cells are infected by *P. vivax* which considerably reduces the total number of infected red blood cells and significantly reduces the pathogenicity of the pathogen. The infected red blood cells do not adhere to the endothelial cells since they lack the 'sticking' properties. Therefore, the serious complications as seen with *P. falciparum* infections are not present. Bouts of fever occur frequently, however not always at very regular intervals of approx. 48 hours, with nearly fever-free phases in between.

The blood count also shows some differences to one of an infection by *P. falciparum*:

1. The haemolysis of young red blood cells stimulates the production of reticulocytes, with their percentage slightly increasing in the course of the infection. In turn, these young cells are a target for free merozoites in the blood.
2. Since red blood cells do not stick to the endothelial cells of the blood vessels, all maturation forms are found in the red blood cells in peripheral blood, i. e. aside from trophozoites (ring forms) also schizonts and gametocytes (see Figure 4).
3. Also *P. vivax* breaks down haemoglobin to haemozoin, a dark-brown haeme crystal, morphologically visible as a crystalline structure in the infected red blood cell.
4. Red blood cells infected by *P. vivax* are enlarged. With the growth and increasing maturity of the pathogens inside the cells their size increases before they burst. Adding to this is the fact that the young RBC, which are usually infected, are inherently larger than more mature ones.

In *P. vivax* a dormant stage (hypnozoite) can remain in the liver and cause malaria relapses by invading the bloodstream weeks or even years later.

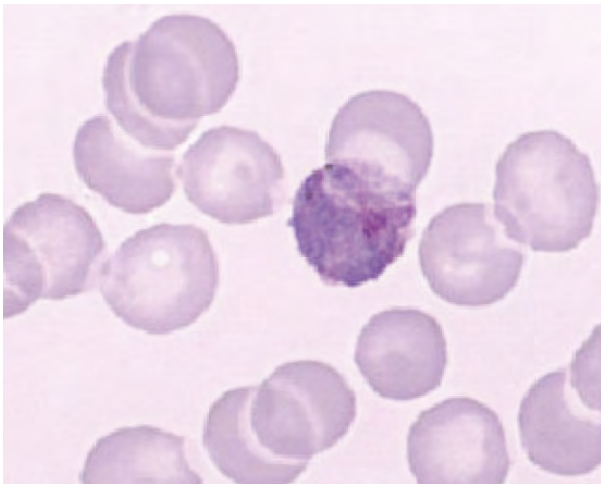

Fig. 4 *Gametocyte in a red blood cell*

Sysmex's fluorescence-based technology – where are malaria pathogens found in the scattergrams?

Nearly all of the haematological characteristics described under the two malaria pathogens can be detected in the blood smear as well as by haematology analysers. There are primarily two measurement channels in Sysmex instruments in which the blood parasites can be detected, provided the infections are sufficiently strong: the reticulocyte channel (RET channel) and the WBC differential channel (hereinafter called WDF channel). For both channels, very specific fluorescence markers that label nucleic acids are used. The higher the content of intracellular nucleic acids (DNA and RNA) is, the higher the resulting fluorescence signal will be. In the following part, both channels are described in more detail and information is provided as to where and why red blood cells infected by Plasmodia are found in the scattergrams.

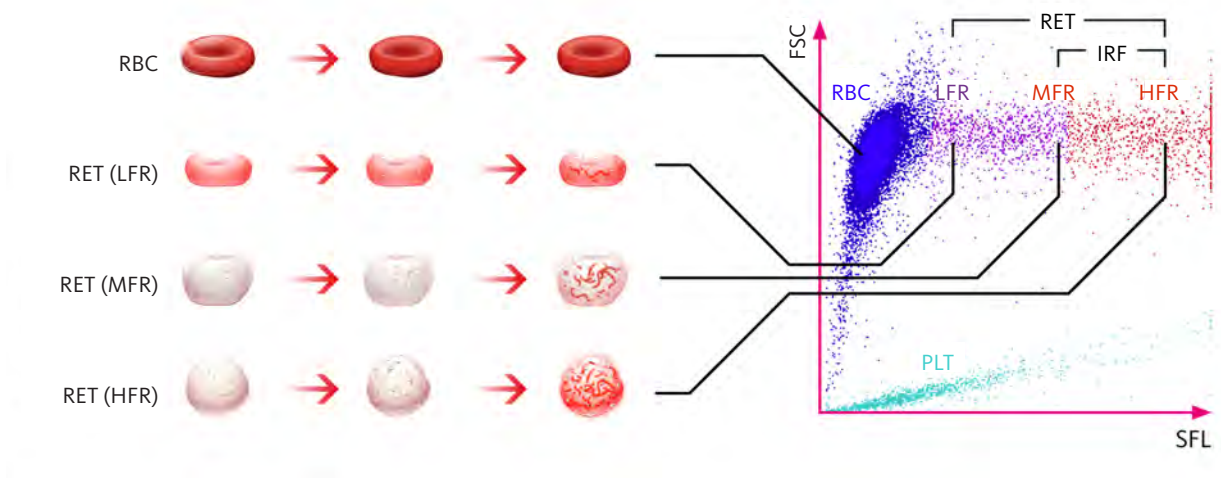

Fig. 5 *RET channel of Sysmex haematology analysers: on the X-axis, the fluorescence intensity (SFL) based on the cells' amount of nucleic acids is plotted ascendingly whereas on the Y-axis the size of the cells is plotted ascendingly.*

RET channel

The amount of RNA in reticulocytes presents the single morphological differentiation criterion between mature red blood cells and reticulocytes, and this also applies for automatic counting (Figure 5). This amount of nucleic acid is specifically labelled by means of a patented fluorescence marker in the RET channel. The higher the reticulocytes' amount of nucleic acid is, the younger the cells are and also the greater their fluorescence intensity signal measured in the RET channel will be. This signal is proportionate to the amount of RNA inside the reticulocytes; i. e. mature red blood cells have only a very low or no fluorescence signal and are accordingly found very far left on the X-axis. White blood cells, which are not lysed in this channel, have a cell nucleus containing significantly more nucleic acids than reticulocytes and therefore lie outside the RET scattergram. Malaria pathogens also contain nucleic acids. These too are labelled and detected in the RET channel.

In view of the different haematological characteristics of *P. falciparum* and *P. vivax*, the appearance of the two species is very different in the RET scattergram. Due to the low total number of infected red blood cells and the initially mentioned low sensitivity for the detection of these blood parasites, infestation with *P. vivax* pathogens cannot be detected, at least not in the RET channel, by the Sysmex haematology analysers.

The situation is quite different for a malaria infection with *P. falciparum*. The percentage of parasitised red blood cells is relatively high at times while, at the same time, several ring forms and those with several chromatin dots can occur. Both will result in a significant increase in the percentage of red blood cells with a quite considerably increased concentration of nucleic acids.

Figure 6 shows where the affected red blood cells are found in the RET scattergram (⬭). The red blood cells infected with *P. falciparum* have a higher fluorescence signal than uninfected ones due to the parasitic nucleic acids. At the same time, however, the infected cells are not enlarged and, therefore, the forward scatter light signal is not increased.

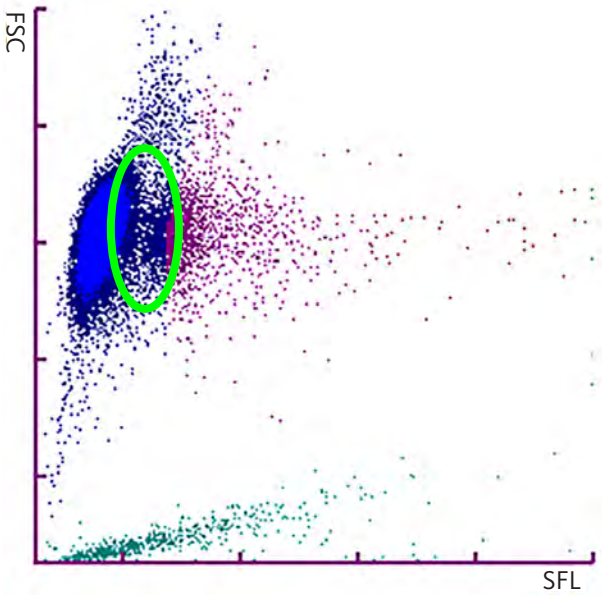

Fig. 6 *RET scattergram of a patient infected with* P. falciparum. *The ellipse marks the position where RBC infected with* P. falciparum *interfere with the normal distribution of cells.*

Warning messages generated by the RET channel

All haematology analysers currently available on the market are designed to determine anomalies in the blood count, but not necessarily to detect specific components inside blood cells. The sensitivity for reliably diagnosing a malaria infection – for example in the form of a possible warning message, such as 'Malaria?' – by the haematology analyser is too low. To do so, the 'thick smear' test still remains indispensable. This also applies to the RET channel, which detects very specifically the intracellular nucleic acid content of blood cells but is unable to distinguish between parasitic nucleic acids, cell-specific DNA/RNA or RNA of reticulocytes. For malaria infections by *P. falciparum* that have a visible impact on the RET scattergram, Sysmex analysers usually show the following result abnormalities including flags:

- 'RET Abn Scattergram' is the indicated warning message due to the fact that no clear separation between the RBC and LFR population can be made.
- The analyser counts all red blood cells containing RNA as reticulocytes resulting in a pseudo-reticulocytosis.
- Especially the more mature reticulocytes detected as 'Low Fluorescence Ratio' (LFR) within the reticulocyte population can be significantly increased. The number of very young reticulocytes – Immature Reticulocyte Fraction (IRF) – is low or normal while at the same time the reticulocyte count is apparently greatly increased. Physiologically, such a reticulocytosis is not possible with normal cell production. However, the abnormal constellation of these parameters may be caused by an infection with *P. falciparum*.
- In particular the number of reticulocytes and the percentage value of LFR could be used to examine whether the therapy is successful in case of a malaria infection. The number of 'reticulocytes' as detected by the analyser should decrease significantly if the parasite infestation in the red blood cells is declining.

WDF channel

The WDF channel differentiates leukocytes according to their nucleic acid content and their internal structure, not according to their size (Figure 7). This ensures an exact count of lymphocytes, monocytes, eosinophils, neutrophils and immature granulocytes.

Even if the red blood cells infected with malaria parasites have a significantly increased amount of nucleic acid versus non-infested red blood cells, the resulting fluorescence signal will still be much smaller than that from any nucleated cell, which contains DNA that will always result in a stronger fluorescence signal. Accordingly, red blood cells or reticulocytes, whether infected or not, will be found in the WDF scattergram in the 'ghost' area (bottom dark blue area below the lymphocytes in Figure 7).

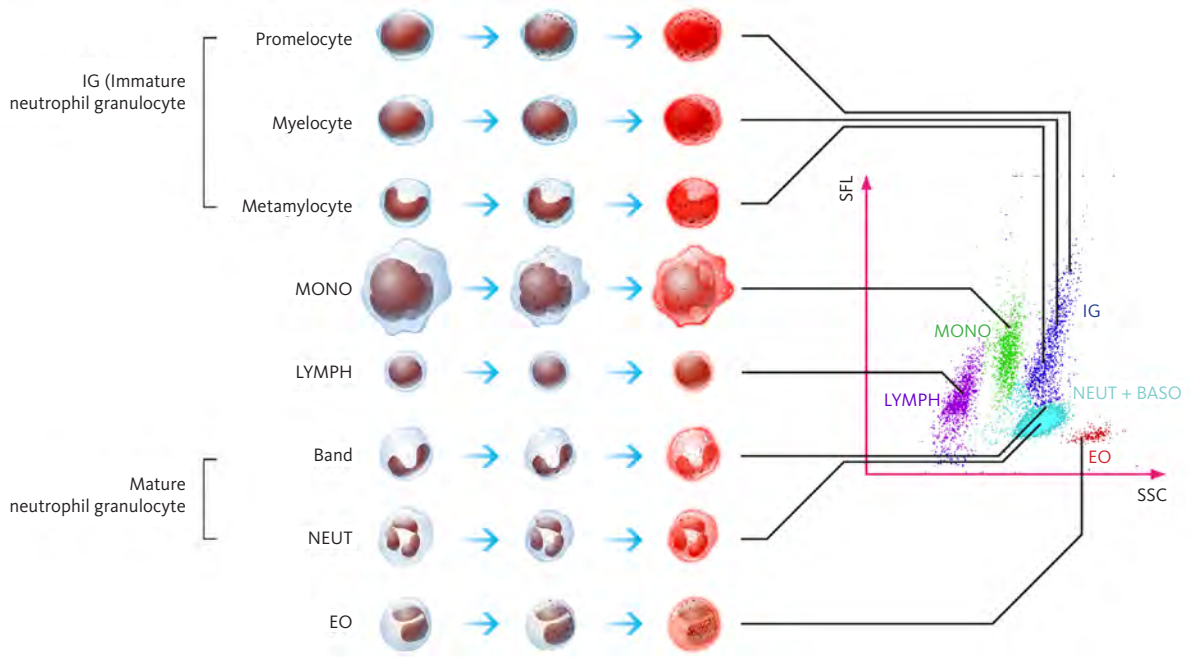

Fig. 7 *WDF channel: on the Y-axis, the fluorescence intensity (SFL) based on the cells' amount of nucleic acids is plotted ascendingly whereas the X-axis displays the side scattered light (SSC) ascendingly, reflecting the increasing complexity of the cells.*

Especially with *P. vivax* infections, increased side scatter (SSC) and side fluorescence light signals (SFL) can be observed in the area of the neutrophils and/or eosinophils in the WDF scattergram when those free pathogen forms of schizonts and gametocytes, which remain undestroyed by lysis, are present (Figure 8). Even the released haemozoin might contribute to these increased side scatter and fluorescence signals. In contrast to this, infections with *P. falciparum* are less distinctly revealed in the WDF scattergram.

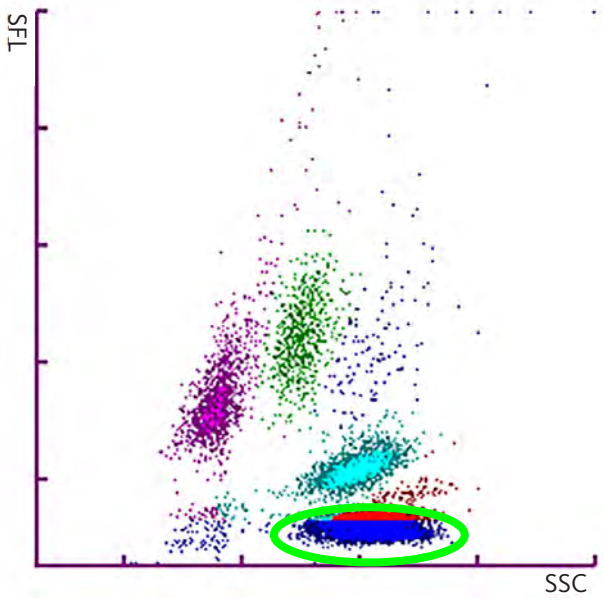

Fig. 8 *Schizonts and gametocytes cause a clearly increased side scatter light signal, especially in the eosinophils' area ( ).*

Warning messages generated by the DIFF channel

With malaria infections by *P. vivax* that have a visible impact on the WDF scattergram, Sysmex analysers usually show the following results including flags generated from the WDF scattergram:

- Since the WDF scattergram in Figure 8 ( ) shows no clear separation between the 'ghost' and the eosinophils' area, the flag 'WBC Abn Scattergram' will be generated. The lower area is cut in two parts by a threshold: the upper red one is counted by the analyser as eosinophils while the lower blue one is neglected by the analyser because it lies beneath the threshold.
- The flag 'Eosinophilia' is a customisable flag and can be helpful in these cases to detect the pseudo-eosinophilia.
- Frequently, the flag 'Atypical Lympho?' is also found, caused by reactive lymphocytes, which might occur during the infection.

Conclusions

- Infections with *P. falciparum* and *P. vivax* generate different patterns in scattergrams:
  - *P. falciparum* causes a change of the normal pattern in the RET scattergram.
  - *P. vivax* causes a change of the normal pattern in the WDF scattergram.
- In the past several authors using the Sysmex XE-series analysers have already described pseudo-eosinophilia or abnormal WBC scattergrams as a result of haemozoin-containing neutrophils. [5, 7, 8] Although the XE's overall sensitivity is limited compared with the sensitivities of conventional diagnostic methods, such as Giemsa-stained thick blood films, its specificity is high.

References

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