Systematic analysis of direct antiglobulin test results in post-artesunate delayed hemolysis

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Abbreviations:

PADH: Post-artesunate delayed hemolysis; DAT: direct antiglobulin test; iv: intravenous; LDH: lactate dehydrogenase; AKI: acute kidney injury; ACT: artemisinin-based combination therapy; RBC: red blood cells AIHA: auto-immune hemolytic anemia; wAIHA: warm AIHA; cAIHA: cold AIHA; Hb: hemoglobin
Abstract

Background: “Post-Artesunate Delayed Hemolysis” (PADH) is common after severe malaria episodes. PADH is related to the “pitting” phenomenon and the synchronous delayed clearance of once-infected erythrocytes, initially spared during treatment. However, direct antiglobulin test (DAT) positivity has been reported in several PADH cases, suggesting a contribution of immune-mediated erythrocyte clearance. The aim of the present study was to compare clinical features of cases presenting a positive or negative DAT.

Method: Articles reporting clinical data of patients diagnosed with PADH, for whom DAT had been performed, were collected from PubMed database. Data retrieved from single patients were extracted and univariate analysis was performed in order to identify features potentially related to DAT results and steroids use.

Results: Twenty-two studies reporting 39 PADH cases were included: median baseline parasitemia was 20.8% (IQR: 11.2-30) and DAT was positive in 17 cases (45.5%). Compared to DAT-negative individuals, DAT-positive patients were older (49.5 vs 31; p=0.01), had a higher baseline parasitemia (27% vs 17%; p=0.03) and were more commonly treated with systemic steroids (11 vs 3 patients, p=0.002). Depth and kinetics of delayed anemia were not associated with DAT positivity.

Conclusions: In this case series, almost half of the patients affected by PADH had a positive DAT. An obvious difference between the clinical courses of patients presenting with a positive or negative DAT was lacking. This observation strongly suggests that DAT result is not indicative of a pathogenic role of anti-erythrocytes antibodies in patients affected by PADH, but is rather a marker of immune activation.
1. Background

Severe malaria is a medical emergency which caused 435,000 globally estimated deaths in 2017 [1]. WHO guidelines [2] recommend the use of intravenous (iv) artesunate to treat severe malaria either in adults and children, considering both efficacy and short-term safety reported in two controlled trials in Asia [3] and Africa [4]. Nevertheless, only limited evidence is available concerning the safety of iv artesunate beyond the initial treatment period because trial designs and lack of infrastructures in endemic countries did not allow for a long-lasting follow-up. Recently, several reports described the occurrence of a late-onset hemolytic syndrome, specifically related to the use of artesunate, defined post-artesunate delayed hemolysis (PADH). It usually occurs more than one week after the iv artesunate course, in approximately 10-40% of hyperparasitemic non-immune travellers [5, 6].

Although not fully elucidated, PADH pathophysiology may be explained by the peculiar pharmacological effect of artemisinin derivatives. The rapid antimalarial action of these drugs is related to “pitting” [7-9], a process whereby artemisinin-exposed parasites are removed from their host erythrocytes, within the spleen microcirculation. After being “pitted”, once-infected erythrocytes re-enter the systemic circulation but with a reduced lifespan [8, 10, 11, 12]. Thus, although “pitting” initially spares parasite-hosting erythrocytes, this positive effect may not be sustained [10]. The delayed synchronous clearance of once-infected erythrocytes would explain major features of PADH, such as the late onset and the occurrence in parasite-free patients.

Despite the evidence supporting “pitting” phenomenon as a leading factor in PADH pathogenesis, an increasing number of cases presenting with positive direct antiglobulin test (DAT) and/or in whom anemia resolved following administration of systemic steroids has been reported [13-20]. This finding suggests a possible role of drug-induced antibody-mediated hemolysis contributing at least to some of the PADH cases [21].

The aim of this review was to collect demographic, clinical, and hematological data of all published PADH cases in which DAT results were reported and to compare the main clinical features of patients
presenting with a positive or negative result. An additional objective was to identify features driving the choice to use or not systemic steroids to manage PADH cases.

2. Methods

All published records of patients affected by PADH with a reported DAT result were included in the analysis. PADH was defined as a drop > 10% of the hemoglobin level occurred after 7 days since the start of artemisinin-based antimalarial therapy, in the context of hemolysis laboratory signs not related to malaria relapse: an increase > 10% of the LDH blood concentration and/or LDH plasma level > 390 UI/l, or a plasma haptoglobin level < 0.1 g/l [23].

PubMed database was investigated using a string of MeSH terms ["hemolysis" OR "anemia, hemolytic") AND ("artemisinins" OR "artemisinine" OR "artesunate" OR "artemether" OR "artemether, lumefantrine drug combination" OR "dihydroartemisinin") and all the papers published before October 31, 2019 were reviewed. All articles in English, French, Portuguese or Spanish language were included in the analysis. The reference list of all selected studies was investigated to identify other relevant articles. Titles and abstracts were independently screened by two authors. Data collected from every single patient were extracted from the selected studies by the same two authors and the final database was finally approved by the whole authors’ consensus. One clinical case included in the analysis is part of the severe malaria Italian multicenter study and has not yet been published before (Figure 1).

Collected information included demographic data, travel history, clinical presentation, hematological, biochemical and parasitological tests, clinical management and follow-up.

Statistical analysis was performed using STATA software. Comparisons between the groups of patients presenting with positive and negative DAT were performed using Mann-Whitney U-test,
while Fisher’s exact test was used to analyze categorical variables. Furthermore, univariate analysis was performed to assess variables eventually related to clinician’s choice to use systemic steroids.

3. Results

Figure 1 shows the algorithm used to select the studies to be included in the review. Twenty-two articles reporting clinical features of 38 patients affected by PADH, who had been tested with DAT, were identified. Another unpublished case, managed in our center and included in the Italian multicenter malaria cohort, was also included in the analysis. Epidemiological, clinical, and therapeutic characteristics of the cohort patients are summarized in table 1. Cumulative data are presented in table 2 as aggregate case series and stratified according to positive (17/39 patients, 43.6%) or negative (22/39 patients, 56.4%) DAT result. DAT results were reported in detail in 13 out of the 17 positive patients (76.5%): five of them presented an IgG pattern (38.5%), six showed a C3d positivity (46.1%), while the remaining two cases were characterized by a mixed condition (15.4%).

Thirteen of the patients were females (33.3%) and the overall median age was 44 years (IQR 24.5-51.5): 49.5 years (IQR 43.2-54.7) and 31.0 years (IQR 20.5-48.0) in positive and negative DAT patients, respectively (p=0.01). Data concerning place of birth and residency were available for only 26 and 28 patients, respectively. Out of 26 patients, 4 (15.4%) were born in endemic areas, whereas 7 out of 28 (25%) were living in malaria endemic countries. Overall, 35 malaria infections were sustained by *Plasmodium falciparum* (89.7%) and one by *P. vivax* (2.6%), while the remaining three patients were affected by a *P. falciparum/P. vivax* mixed infection (7.7%). The median reported baseline parasitemia was 20.8% (IQR 11.2-30); parasite count was higher in DAT positive comparing to DAT negative PADH patients [27% (IQR 21-34) versus 17% (IQR 9.0-22.0) respectively (p=0.03)]. An overall median of three WHO severe malaria criteria (IQR 2-3) was reported. Patients with positive DAT had a not statistically significant higher prevalence of previous acute kidney injury (AKI) (p=0.09). Thirty-four patients (87.2%) had been treated with artesunate (either intravenous or
intrarectal), usually combined with a second antimalarial drug and/or followed by oral artemisinin-based combination therapy (ACT). Five patients (12.8%) had received oral ACT, only.

The median time between antimalarial treatment start and PADH onset was 12 days (IQR 9-14). At baseline, a median hemoglobin level of 12.4 g/dl (IQR 10.7-13.8) was registered, while the median hemoglobin lowest detected level (nadir) was 6.0 gr/dl (IQR 5.2-6.8). Median time occurred between the start of antimalarial treatment and hemoglobin nadir was 14 days (IQR 11-15). Blood transfusions were prescribed to 29 patients (74.4%) using a median number of two red blood cells (RBC) units considering the whole PADH cohort. No significant differences in clinical features and outcomes of PADH were observed between patients presenting with a positive or negative DAT. Median hemoglobin level at nadir was 6.5 g/dl in DAT-positive (IQR 5.4-6.9) and 5.9 g/dl in DAT-negative patients (IQR 4.8-6.4) respectively (p=0.2), while median time to nadir was 14.5 days (IQR 12.2-16.7) in DAT-positive and 13 (IQR 11.2-15) in DAT-negative patients respectively (p=0.3). Furthermore, the median amount of packed erythrocytes needed for transfusion support was similar between the two groups: two (IQR 0-4) and 3.5 (IQR 0-4) transfused RBC units in DAT-positive and DAT-negative patients, respectively (p=0.5). The lack of an obvious difference between the two groups strongly suggests that DAT positivity is not an operator of anemia in this context but rather just a marker of autoimmunity.

Data concerning PADH pharmacological management were available for 37 patients; of them, 14 patients (37.8%) received either oral or intravenous steroids (table 3). Systemic steroids were prescribed to 11/16 DAT-positive patients (68.7%) and to 3/21 DAT-negative patients (14.3%), respectively (p=0.002) (table 2). All the cases described resulted in a complete clinical recovery and no deaths were reported. However, the outcomes description and the follow-up duration were quite heterogeneous between the articles included.
4. Discussion

PADH is a non-recurring highly expected event in non-immune hyperparasitemic patients after artesunate administration [22]. In 2011, 6 out of 25 patients (24%) included in a severe malaria European cohort, developed PADH with a median onset of 15.5 days (IQR 15-32) after the start of intravenous artesunate. Five of them presented with severe anemia requiring blood transfusions (83.3%), all resulting in a full recovery [23]. Furthermore, other authors registered a similar PADH occurrence rate. Kurth F. et al. [24] reported a 27.1% incidence of PADH in a cohort of 70 patients with severe malaria managed in European countries. Three patients (15.8%) with PADH received blood transfusions, and two (10.5%) needed to be re-hospitalized (for 3 and 5 days, respectively). Jauréguiberry S. et al. [6] described a French cohort of 123 imported severe malaria cases, in which 27% of patients experienced PADH after a successful antimalarial treatment. The 85% of the PADH recorded were mild and only one patient required blood transfusion. Lastly, few PADH cases after oral artemisinin-based combination therapy (ACT) have been already reported [5, 16, 25].

Several factors can contribute to anemia in patients affected by malaria, including acute intravascular hemolysis related to mechanical rupture of both infected and uninfected RBC and extravascular retention [26] and/or phagocytosis of erythrocytes during splenic circulation. This latter might be induced by mechanical splenic retention of RBC that lost their deformability or by opsonisation/phagocytosis related to the effect of immunoglobulins, complement cascade, low levels of CD55, membrane expression of ring-surface protein 2 (RSP-2) and Rhoptry associated protein 2 (RAP-2). Dyserythropoiesis, bone marrow failure and drug-related side effects are other relevant factors contributing to malarial anemia. It might be difficult to distinguish PADH when superimposed on that pathophysiological background [27-42].

PADH has attracted attention because it often occurs well after resolution of malaria-related symptoms and after complete parasite clearance [23, 24] and is neither directly related to active infection nor to the presence of parasites. Currently, weekly follow-up visits up to one month
following artemisinin derivatives treatment are strongly recommended to detect and manage this condition, mainly, in case of both high baseline parasitemia and intravenous artesunate treatment [11]. In confirmed PADH cases, clinical tests performed to exclude common hemolytic disorders, immunologic diseases or drug-induced hemolysis, such as glucose-6-phosphate dehydrogenase deficiency, usually give inconclusive results [23, 24].

In our review, we identified 39 PADH case reports in which clinical and immune hematological characteristics of patients, including DAT, were described. Seventeen (43.6%) of the included patients presented a positive DAT result, either with an IgG, C3d or mixed pattern. DAT is a method used to demonstrate the presence of antibodies or complement fractions bound to RBC membranes. The test is performed using anti-human globulins that cause, in case of a positive result, a visible agglutination reaction [12]. Different reagents can be used to elicit RBC agglutination: a polyclonal one recognizing both IgG and C3d and two monospecific agents recognizing only one of the two molecules. DAT positivity in the context of hemolysis usually defines “auto-immune hemolytic anemia” (AIHA) [43]. Different subtypes of AIHA can generally be classified according to DAT result: warm AIHA (wAIHA) is usually the result of polyclonal IgG binding on RBC surface, while cold AIHA (cAIHA) is related to the effect of clonal or oligoclonal IgM with further activation of the complement cascade, this latter recognized from C3d fraction bound to RBC [44]. A mixed form, characterized by the recognition of both IgG and C3d on RBCs membrane, has also been described. Noteworthy, a positive DAT alone does not allow for AIHA diagnosis. The test indeed, can be positive in 0.1% of healthy donors and positivity prevalence can rise up to 1-15% in hospitalized patients affected by acute illnesses, even in absence of hemolysis [43, 44]. Moreover, malaria and other systemic infections can be associated with a positive DAT, but the true role of immune mechanisms in contributing to malarial anemia is not easy to determine [45, 46]. Finally, AIHA can be idiopathic or secondary to infections, drugs exposure, autoimmune or lymphoproliferative disorders.
The observation of a positive DAT in almost half of the PADH patients reported in the available literature, may suggest the possibility of different mechanisms underlying late-onset hemolysis in malaria patients. This finding may indicate the need for an active diagnostic attitude aimed at excluding a possible AIHA, in every case meeting PADH diagnostic criteria. This approach would help to early identify patients who could benefit from AIHA-specific treatment strategies, such as systemic steroid administration.

On the other hand, no significant clinical differences were observed between the groups of patients presenting with a positive or negative DAT. Most of the patients included in the review showed a similar clinical course, with a median time to PADH onset occurring 12 days after artemisinin-derivatives administration start, a low Hb nadir (6 g/dl) reached at day 14, a high need for RBC transfusion (74% of the patients) and a positive outcome. This observation suggests that, despite different DAT results, pathophysiologic mechanisms underlying the hemolytic process may be similar for all included patients and not related to DAT positivity. According to the latter hypothesis, the finding of a positive DAT may be only a nonspecific marker of systemic immune activation [47], triggered by the recent malarial episode, instead of a real determinant of the hemolytic anemia.

Our results show that use of systemic steroids in patients affected by PADH is currently driven by clinical decision, mainly supported by a positive DAT result, despite limited clinical evidence. Data concerning long-term follow-up, time to recovery and outcomes are partially lacking in most of the included articles. This additional information could be useful to collect in order to uncover possible differences in clinical course of patients presenting with a positive or a negative DAT. Considering the relatively low number of severe malaria cases in non-endemic countries, the issue should be further evaluated in a multicenter prospective observational study. This methodology would help to define the real benefit of implementing a specific strategy for early diagnostic approach and best therapeutic options in patients with severe PADH in the context of a positive DAT.
5. Conclusions

The present review points out that DAT positivity is a common finding among patients affected by PADH. Our data suggest that a positive DAT is probably not related to a significant auto-immune hemolytic process, but it is rather a nonspecific expression of malaria-related systemic immune activation. Nevertheless, data included in the review are heterogeneously collected and information concerning PADH time to recovery and follow-up is lacking. Therefore, a prospective observational study is needed in order to understand if DAT positivity may identify a subpopulation of patients in which antibody-mediated hemolysis contributes to PADH features, eventually leading to a worse outcome and possibly requiring a targeted therapeutic approach.

Declarations

Ethics approval and consent to participate

The present research is based on data reported in previously published works. The patient enrolled at INMI “L. Spallanzani” IRCCS, whose data have never been analyzed and published before, has signed an informed consent to participate to observational research protocols.

Consent for publication

The present research is based on data reported in previously published works. The patient enrolled at INMI “L. Spallanzani” IRCCS, whose data have never been analyzed and published before, has signed an informed consent for data publication.

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The research team of Pierre Buffet received support from Guilin Laboratories in 2017-2020 for the surveillance of artesunate efficacy and toxicity in France. The author provided expertise for FastTrack Drugs & Biologics LLC and Sigma-Tau Pharmaceuticals from 2013 to 2016, and provided expertise to Sanofi Aventis Research & Development from 2013 to 2015.

All other authors report no potential conflicts.

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**Authors' contributions**

**TA: conceptualization, investigation, writing – original draft**

**LL: investigation, writing – original draft**

**AD: methodology, writing – review draft**

**GA: formal analysis**

**AC: conceptualization**

**LS: writing – review draft**

**LG: writing – review draft**

**NB: methodology**

**CP: investigation**
AM: writing – review draft
GI: Supervision
PB: Conceptualization, writing – review draft
EN: Project Administration, conceptualization, writing – review draft

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None

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