Chapter 6

Evaluation of antigen detecting tests, PCR and microscopy for malaria diagnosis during pregnancy


Keywords
Malaria; placental malaria; pregnancy; *Plasmodium falciparum*; diagnostic tests; rapid diagnostic tests; PCR; microscopy; ELISA.

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6.1 Abstract

Rapid diagnostics tests (RDTs) detect malaria specific antigen(s) in the circulation, even when parasites are sequestered in the placenta and not visible by microscopy. However, research on their diagnostic accuracy during pregnancy is limited. Pregnant women (N = 418) were screened for malaria during routine antenatal care (ANC) with two RDTs detecting histidine rich protein 2 (HRP2) or *Plasmodium* lactate dehydrogenase (pLDH) and ELISAs with antibodies detecting dihydrofolate reductase - thymidylate synthase (DHFR-TS) or heme detoxification protein (HDP), and compared to Real-Time PCR (RT-PCR) and microscopy for the evaluation of their diagnostic accuracy. Prevalence of malaria infection was high (53% by PCR). RT-PCR and the HRP2-RDT detected most cases of malaria during pregnancy, whereas microscopy, the pLDH-RDT, and the DHFR-TS and HDP antibodies by ELISA failed to detect several low density infections. HRP2-RDTs could therefore be a useful tool in high transmission areas for the diagnosis of malaria in asymptomatic pregnant women.
6.2 Introduction

Malaria infection is a major public health problem in (sub-)tropical regions throughout the world. Pregnant women, besides children, are at a higher risk of malaria than other adults. Infection with *Plasmodium falciparum* or *Plasmodium vivax* during pregnancy is related to adverse maternal health and poor birth outcomes [3, 4]. During pregnancy, malaria parasites sequester in the placenta, leading to placental changes central to the pathogenesis of placental malaria [33]. In low transmission areas, malaria in pregnancy usually presents as a symptomatic, severe disease that can result in death of the mother and foetus. In high transmission areas, however, malaria infection rarely results in symptomatic disease due to acquired immunity. The main impact in these areas is malaria-related maternal anaemia, low birth weight, and stillbirth [12, 13].

Diagnosis of malaria during pregnancy can be complicated by the absence of parasites in the peripheral blood or by parasite densities below the detection limit of microscopy caused by placental sequestration [5, 33, 57, 92]. Accurate detection of parasite infection in the placenta requires examination of histological sections of fixed placental tissue, which is the gold standard for diagnosing placental malaria. Unfortunately, placental histology and microscopic examination of placental blood can only be performed after delivery. Therefore, it would be beneficial, for both mother and foetus, to diagnose malaria in the peripheral blood earlier, followed by safe and adequate treatment. It is therefore necessary to detect the placental infection with a marker that is present in peripheral blood. Currently available methods for the diagnosis of malaria in peripheral blood are parasite detection by microscopy, DNA- or RNA detection methods such as PCR, and detection of parasite antigens by rapid diagnostic tests (RDTs). RDTs have the advantage of detecting circulating antigens, even when the parasites are sequestered in the deep circulation and not visible by microscopy. Commercially available RDTs for malaria detect one or more of the following antigens: histidine rich protein 2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) or aldolase [62, 63]. RDTs are being widely deployed for the diagnosis of malaria in pregnancy, because of their ease of use and relatively low cost, but their accuracy in this subpopulation has not been extensively evaluated, especially in the case of pLDH-RDTs [5]. Therefore, the aim of the present study was to evaluate the accuracy of an HRP2-based and a pLDH-based RDT for the diagnosis of malaria in pregnancy by using peripheral blood and compare them to PCR and microscopy.

In addition, due to concerns on test stability, accuracy, species detection, antigen persistence, and antigen genetic diversity, there is still a need for improving RDT performances [62, 63, 88–90]. Recently developed antibodies
against the antigens dihydrofolate reductase-thymidylate synthase (DHFR-TS) and heme detoxification protein (HDP) were screened for specificity against \textit{P. falciparum} cultured isolate 3D7 and \textit{P. vivax} samples in ELISA [180]. Two of these antibodies were evaluated on the samples collected for this study for their potential use in malaria diagnosis.

6.3 Methods

Study area and population

The study was conducted in Nanoro, Boulkiemdé Province, Burkina Faso between November 2010 and August 2011, where \textit{P. falciparum} is the dominant malaria species. Malaria transmission in the study region has a peak during the rainy season (June to December). The study population comprised pregnant women >15 year old and at a gestational age $\geq$ 15 weeks, visiting the health centre for their routine antenatal care (ANC). As the women were screened during their antenatal care visit, they were mostly asymptomatic and not febrile. This study was performed in conjunction with an ongoing study assessing the safety and efficacy of three ACTs (Artesunate-Amodiaquine, Arthemeter-Lumefantrine or Artesunate-Mefloquine) for the treatment of malaria in pregnant women (clinicaltrials.gov: NCT00852423). Ethical approval to conduct this study in conjunction with the PREGACT-trial was obtained from the Ethical Committee of the University Hospital in Antwerp (registration number ITG 10 30 2 732), and from the Institutional Ethics committee of Centre Muraz, Burkina Faso (registration number 019-2010/CE-CM).

Study design and sample collection

Pregnant women attending regular antenatal care (ANC) were screened at each visit with an HRP2-based RDT, SD-Bioline Malaria Antigen P.f. (Standard Diagnostics Inc.), for the purpose of patient recruitment for the PREGACT study. The women were recruited in the PREGACT study if positive in the RDT and confirmed with microscopy and subsequently allocated to an ACT treatment group, and then followed actively after treatment. When informed consent was obtained, finger prick blood (250-500 $\mu$L; from one or two finger pricks) was collected in an EDTA tube (microvette, Sarstedt or capiject, Terumo). The blood sample was transferred to the laboratory where it was used to prepare thick and thin blood smears, blood was spotted on filter paper for Real-Time PCR (RT-PCR) and the sample was stored at 4$^\circ$C. A second RDT, Advantage Malcard Pf and PAN (J. Mitra & Co), detecting pLDH, was performed immediately or several weeks later depending on the test availability. The presence of
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the DHFR-TS and HDP antigens was tested by ELISA when all samples were collected. Women that were screened for the purpose of this study and were found positive in the SD-Bioline RDT received free anti-malarial treatment, either according to national treatment guidelines of Burkina Faso (chloroquine), or according to the PREGACT trial protocol (artesunate-amodiaquine, artemether-lumefantrine or artesunate-mefloquine), if the women were included in the PREGACT trial.

**Laboratory procedures**

Finger prick blood was applied to the RDTs, SD Bioline Malaria Antigen P.f. (Standard Diagnostics Inc.) and Advantage Malcard (J. Mitra & Co) according to the procedures described by the manufacturer. The RDTs were selected based on the WHO evaluation; the SD-Bioline RDT was one of the top-10 performing HRP2-RDTs, and the Advantage Malcard RDT was the best performing pLDH-tests detecting both PAN and Pf [62, 63]. Manufacturer's storage temperature specifications (4-30°C) were maintained during transportation (on ice) and at storage (in a monitored cold room at 18°C). Microscopy was performed according to international and GCP guidelines by local expert microscopists [189, 190]. Briefly, thick and thin blood smears were Giemsa-stained and parasites were counted against 200 leukocytes, with parasite negative results based on screening of 100 microscopic fields at 1000× magnification. In case of lower parasitemia (<10 parasites/200 leukocytes) parasites were counted against 500 leukocytes. A leukocyte count of 8000/µL was assumed to calculate the parasite density per microliter [191]. Slides were examined by two readers and in case of discordant results by a third reader. Discordant results were defined as a difference between the two readers in 1) positive and negative, 2) with parasitemia >400/µL if the higher count divided by the lower count was >2 or 3) with parasitemia ≤400/µL if the higher reading density was more than one log10 higher than the lowest reading. The final result was recorded as the geometric mean of the readings. Blood was spotted on Whatman 903 protein saver cards, air dried and stored at room temperature in sealed bags with desiccant until transport and further processing in the Netherlands. DNA was isolated from the protein saver cards according to the Boom method and as described before and kept at -20°C until use [186, 190]. A *P. falciparum* specific 18s rDNA RT-PCR was performed on a CFX96™ real-time PCR detection system (BioRad) with a FAM-labelled Taqman probe as described before [83, 187, 190]. If there were discordant results in the duplicates (i.e. one positive result and one negative result), the RT-PCR reaction of that sample was repeated. Samples were not considered positive until they gave a signal in at least two of the duplicates. For the ELISA, 37.5 µL of EDTA
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**Figure 6.1 Percentage of women positive in the different tests**

Left: Percentages of positive cases with the SD-Bioline RDT (HRP2), Microscopy and PCR per month. Results from June are not available, because women could not be included in the study during that month. The numbers of participants screened for each month is presented in brackets. In November, however, for RT-PCR, N = 80 and for microscopy, N = 43. Right: Percentages positive for all tests during the whole study period. For HRP2-RDT N = 418, for RT-PCR N = 417, for microscopy N = 380, for the pLDH-RDT N = 412, and ELISAs N = 347.

blood was lysed with 75 µL of cold distilled water, then mixed with an equal amount of 50 mM sodium carbonate pH 9.6 and coated in duplicate on two ELISA plates (medium binding, Greiner) for 1h at room temperature. The next day wells were washed three times with PBS, 0.1% Tween-20 (PBST). Subsequently, the ELISA was performed with α-HDP H16 (5 µg/mL) or α-DHFR-TS D20 (5 µg/mL) as described before [190].

**Data collection and statistical analysis**

The sample size was calculated to be able to determine with 95% confidence if the sensitivity and specificity was 90% ± 5%, resulting in at least 150 positive women and 150 negative women to be recruited [192]. With an expected parasite prevalence of 30% and expected study-participant dropout rate of 5-10%, recruitment was continued until at least 165 (150 + 10%) women had been found positive in the HRP2-RDT. Test outcome was collected on case record forms and double entered in an Access database (Microsoft 2003). Calculations, including those for sensitivity, specificity and prevalence, were performed in STATA (Special edition 11.2, StataCorp LP). The Shapiro-Wilk test was used to test for normality (W > 0.9) and not-normal distributed variables such as parasite densities were log transformed for the analyses when required. The difference in mean parasite density as determined by microscopy and by RT-PCR was determined with a paired t-test. The difference in
mean parasite density (as determined by Real Time PCR) between microscopy positive- and negative cases was tested with a two-sample t-test. p-values below 0.05 were considered as statistically significant. Kappa-values were calculated to measure the level of agreement between the diagnostic tests. Confidence intervals (95%) of calculated values are indicated with CI between square parentheses, when applicable.

6.4 Results

Between November 2010 and August 2011, 418 pregnant women were recruited and all women were routinely tested with the SD-Bioline RDT (HRP2). With this RDT, 194/418 (46% [CI 42-51%]) women had a positive test result for *P. falciparum*. Overall, the HRP2-RDT and RT-PCR detected more cases than microscopy (Figure 6.1). Because some samples were too coagulated or there was little blood left, not all women could be tested with the pLDH-RDT (N = 6) and ELISA (N = 71). The filter paper for PCR was missing for one participant and in the first period of the study (November) no microscopy slides were prepared (N = 38). When restricting the analysis to the available microscopy results, malaria prevalence in pregnant women that were included in the study was 30% [CI 25-34%] (112/380) by microscopy, while it was 47% [CI 42-52%] (178/380) by HRP2-RDT and 53% [CI 48-58%] (201/380) by RT-PCR. Prevalence by RT-PCR was slightly higher, 54% [CI 49-59%] (224/417), when all recruited women are included. The Advantage Malcard RDT (pLDH) and both ELISAs detected fewer cases than RT-PCR and the HRP2-RDT (Figure 6.1). The two RDTs had a good agreement with each other and with microscopy (Table 6.1). All infections detected by microscopy, RT-PCR and HRP2-RDT were due to *P. falciparum*. The pLDH-RDT, however, was positive in only the PAN-line and not the *P. falciparum*-line in three cases, but they were all detected by the *P. falciparum* specific RT-PCR and had low parasite densities, i.e. 26, 28 and <4 parasites/µL.

Accuracy with microscopy as reference test

When microscopy was used as a reference test, the RT-PCR had the highest sensitivity, followed by the HRP2-RDT (Table 6.2). The pLDH-RDT had the highest specificity (90% [CI 86-93%]). The other tests have significantly lower specificities (Table 6.2). The sensitivity for both ELISAs is very low while the specificity is higher. This specificity, however, was not sufficient to justify the use of the ELISAs as diagnostic test (Table 6.2).
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Tabel 6.1 Percentage of agreement and Kappa-value ± standard error to measure the level of agreement between the diagnostic tests. p < 0.05 for all comparisons

A kappa value <0.20 is considered a poor agreement; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 good; 0.81-1.00 very good.

<table>
<thead>
<tr>
<th>Test</th>
<th>Microscopy</th>
<th>HRP2-RDT</th>
<th>pLDH-RDT</th>
<th>RT-PCR</th>
<th>DHFR-TS ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP2-RDT</td>
<td>80.5%</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(0.60±0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDH-RDT</td>
<td>85.7%</td>
<td>83.0%</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(0.66±0.05)</td>
<td>(0.65±0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>75.0%</td>
<td>87.0%</td>
<td>74.9%</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(0.51±0.05)</td>
<td>(0.74±0.05)</td>
<td>(0.51±0.04)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHFR-TS ELISA</td>
<td>66.1%</td>
<td>62.0%</td>
<td>66.2%</td>
<td>58.7%</td>
<td>x</td>
</tr>
<tr>
<td>(0.19±0.06)</td>
<td>(0.22±0.05)</td>
<td>(0.19±0.05)</td>
<td>(0.20±0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDP ELISA</td>
<td>67.7%</td>
<td>62.0%</td>
<td>66.2%</td>
<td>57.8%</td>
<td>74.6%</td>
</tr>
<tr>
<td>(0.24±0.06)</td>
<td>(0.22±0.05)</td>
<td>(0.21±0.05)</td>
<td>(0.18±0.05)</td>
<td></td>
<td>(0.40±0.05)</td>
</tr>
</tbody>
</table>

Tabel 6.2 Diagnostic test accuracy with microscopy as reference test

CI: confidence interval.

<table>
<thead>
<tr>
<th>Test</th>
<th>N</th>
<th>Sensitivity 95% CI</th>
<th>Specificity 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>380</td>
<td>97.3% (109/112)</td>
<td>92 - 99% 65.7% (176/268) 60 - 71%</td>
</tr>
<tr>
<td>HRP2-RDT</td>
<td>380</td>
<td>96.4% (108/112)</td>
<td>91 - 99% 73.9% (198/268) 68 - 79%</td>
</tr>
<tr>
<td>pLDH-RDT</td>
<td>378</td>
<td>75.9% (85/112)</td>
<td>67 - 84% 89.8% (239/266) 86 - 93%</td>
</tr>
<tr>
<td>DHFR-TS ELISA</td>
<td>316</td>
<td>41.2% (40/97)</td>
<td>31 - 52% 77.2% (169/219) 71 - 83%</td>
</tr>
<tr>
<td>HDP ELISA</td>
<td>316</td>
<td>47.4% (46/97)</td>
<td>37 - 58% 76.7% (168/219) 71 - 82%</td>
</tr>
</tbody>
</table>

Accuracy with PCR as reference test

When PCR was considered as the reference test, the highest sensitivity was found with the HRP2-RDT, followed by the pLDH-RDT and microscopy, and then the ELISAs, whose sensitivity was <50% (Table 6.3). Conversely, microscopy had the highest specificity, closely followed first by pLDH-RDT, and then HRP2-RDT. The ELISAs had the lowest specificity, around 78% (Table 6.3).

Tabel 6.3 Diagnostic test accuracy with RT-PCR as reference test

CI: confidence interval.

<table>
<thead>
<tr>
<th>Test</th>
<th>N</th>
<th>Sensitivity 95% CI</th>
<th>Specificity 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>380</td>
<td>54.2% (109/201)</td>
<td>47 - 61% 98.3% (176/179) 95 - 100%</td>
</tr>
<tr>
<td>HRP2-RDT</td>
<td>417</td>
<td>81.3% (182/224)</td>
<td>76 - 86% 93.8% (181/193) 89 - 97%</td>
</tr>
<tr>
<td>pLDH-RDT</td>
<td>411</td>
<td>54.8% (120/224)</td>
<td>48 - 62% 97.9% (188/192) 95 - 99%</td>
</tr>
<tr>
<td>DHFR-TS ELISA</td>
<td>346</td>
<td>37.5% (69/184)</td>
<td>31 - 45% 82.7% (134/162) 76 - 88%</td>
</tr>
<tr>
<td>HDP ELISA</td>
<td>346</td>
<td>40.2% (74/184)</td>
<td>33 - 48% 77.8% (126/162) 71 - 84%</td>
</tr>
</tbody>
</table>
6.4. RESULTS

Figure 6.2 Sensitivity vs. parasite density
The sensitivities (y-axis) of the different diagnostic tests compared to RT-PCR as a reference test stratified by parasite density in parasite/µl (x-axis) that was determined by RT-PCR.

Effect of parasite density on diagnostic test accuracy

The parasite densities that were detected by microscopy and RT-PCR were statistically different (p <0.0000). Parasite density ranged from 30 to 64,471 parasites/µL by microscopy and from ≤4 to 4066 parasites/µL by RT-PCR. Among women positive for RT-PCR 75% (169/224) had a parasite density by RT-PCR below 100 parasites per µL, with a low mean density (GMPD 15.7 [CI 11.5-21.3]). The sensitivity of pLDH-RDT and microscopy depended on parasite density (Figure 6.2). Mean parasite density of women with a negative blood slide but a positive RT-PCR (sub-microscopic infections, N = 92) was significantly lower (2.9 p/µL [2.2-3.9]) than those having both tests positive (62.1 p/µL [42.0-91.9]) (p < 0.0000).
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6.5 Discussion

RDTs are increasingly implemented for the diagnosis of malaria in pregnancy, but research on their diagnostic accuracy in this sub-population is limited [5]. In the present study, six diagnostic tests, comprising microscopy, two RDTs (SD-Bioline RDT and Advantage Malcard RDT), RT-PCR and two ELISAs (detecting HDP or DHFR-TS), were evaluated for routine testing of malaria infections among pregnant women attending antenatal clinics. Both the Advantage Malcard RDT (pLDH) and microscopy were unable to detect a large proportion of the low-density infections that were detected by RT-PCR and the SD-Bioline RDT in asymptomatic pregnant women. As expected, the sensitivity of all tests varied with parasite density, with a higher sensitivity at high parasite density. This effect was greatest with microscopy and the pLDH-RDT.

The specificity of PCR and both RDTs were low when microscopy was taken as the reference test, probably because microscopy cannot detect placental sequestered parasites. PCR and RDT detect circulating antigen or nucleic acids, which may be adequate for identifying women with placenta malaria [5]. Three microscopy-positive cases with parasite densities of 93, 277 and 484 parasites/µL were negative by RT-PCR. Possible explanations for this discrepancy could be degradation of target DNA or the presence of an amplification-inhibiting factor.

The difference in RDT performance is most likely caused by the type of antigen that is detected by the RDT, and does not mean that tests from one of the manufacturers of the tests in this study is superior to the other. This is reflected by the HRP2 detecting test from J. Mitra & Co (Advantage P.f. Malaria Card) that performs similarly to the SD-Bioline Malaria Antigen P.f. in the WHO-evaluation of RDTs [62, 63]. This difference in test performance between pLDH and HRP2 based tests was indicated in a previously conducted meta-analysis as well [5].

The retrieved accuracy for RT-PCR and the RDTs as compared to microscopy was very close to the summary values reported in a previous meta-analysis, although the specificity in this study was slightly lower [5]. The sensitivity of the pLDH-RDTs in the meta-analysis was lower than the Advantage Malcard RDT in this study. In the WHO-evaluation of RDTs, the performance characteristics of the Advantage Malcard RDT were shown to be superior to other pLDH-RDTs as well [5,62,63]. The pLDH-RDT and the ELISAs were performed on stored blood samples, which might have negatively influenced the accuracy of these tests due to degradation of antigen. However, no significant difference in accuracy of the pLDH-RDT performed on stored blood (N = 246) versus fresh blood samples (N = 172) was found.

Both ELISAs, compared to PCR and to microscopy, did not have sufficient
accuracy for the diagnosis of malaria during pregnancy. These antibodies were selected on the basis of antibody affinity, detection limit and sufficient difference in signal between positive (with *P. falciparum* culture 3D7) and negative whole blood [180]. Other antibodies are available and if more sensitive tests detecting DHFR-TS and HDP could be developed, they might be good options for malaria diagnosis, but not in the form of the assays used in this study.

This study has shown that a remarkably high proportion of pregnant women in the study area had an asymptomatic malaria infection, detectable by HRP2-based RDT and PCR, despite the implementation of intermittent preventive treatment (IPTp) in the country and study area. It is remarkable that in July and August all women in the studied population were parasite-positive by RT-PCR and the HRP2-RDT. Women could not be recruited between June 1st and July 10th 2011, because of unavailability of research staff, and therefore the percentage of infected pregnant women in that period could not be determined. Although it has been demonstrated that HRP2 persists considerably after parasite clearance [190], most women were RT-PCR positive as well, indicating they had active infections, or had cleared their infection very recently. Unfortunately information on prior malaria episodes and treatment history (including last dose of IPTp-SP) was unavailable. If these positive results indeed are an active infection and are left untreated, these malaria infections may cause severe maternal anaemia, a risk factor for maternal mortality and for intrauterine growth retardation and low birth weight [12].

There were many sub-microscopic infections (detected by RT-PCR) and a large proportion of these cases (64%; 59/92) were detected by the HRP2-RDT as well. In the absence of a gold standard for malaria infection during pregnancy, it was difficult to make conclusions on the accuracy of these tests and to conclude whether the HRP2-RDT and RT-PCR were really detecting more cases or whether they are detecting false positive cases. Sub-microscopic infections detected by PCR, however, have been associated with maternal anaemia, low birth weight, and premature delivery [84]. This indicates that although malaria control measures such as IPTp are implemented, routinely screening pregnant women for malaria during their antenatal care visits might be necessary to further eliminate infection and its related adverse effects. In areas with low malaria transmission or high sulfadoxine-pyrimethamine resistance, screening and treatment of women during antenatal care is already being done, but this strategy requires an easy-to-use test with sufficient accuracy [56, 193]. However, the complexity of RT-PCR does not make it suitable for large scale implementation. Therefore, the RDT based on HPR2 would be the most practical and economic alternative.

In conclusion, microscopic examination of peripheral blood of pregnant
women as well as the Advantage Malcard RDT (pLDH) failed to detect a large proportion of low density infections that were detected by RT-PCR and the HRP2-RDT, which is similar to earlier reports [5]. However, more research is needed to determine if PCR and HRP2-RDTs are really detecting low density infections rather than recently cleared infections. For that purpose, results from histological examination of the placenta should be compared to those obtained by RDTs and PCR conducted at delivery.

### 6.6 Acknowledgements

We acknowledge the work of Anneke Taal and Yao Mnimou for the collection of the blood samples and for the performance of the RDTs, preparation of slides and PCR spots and Janneke Zoeten for performing RT-PCR. We thank the clinical and laboratory staff at the Clinical Research Unit of Nanoro (CRUN) for their contribution to the work and all patients for participating in the study.

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