UNIVERSITAT DE BARCELONA

Improving the management of imported malaria

Leire Balerdi Sarasola

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Improving the management of imported malaria

Doctoral thesis dissertation presented by **Leire Balerdi Sarasola** to apply for the degree of doctor at the University of Barcelona

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Doctoral Program in Medicine and Translational Research
School of Medicine and Health Sciences. University of Barcelona.
September 2024

ACKNOWLEDGMENTS

Lehenik eta behin nere familiari eskerrak eman nahi nizkioke, nire erabakiak babesteagatik eta beti gertu mantentzeagatik.

Siempre estaré en deuda con Dani y Jose, por ser las personas que apostaron por mí desde que era residente, y me han visto crecer a lo largo de mi etapa en Barcelona. Gracias a los dos por la paciencia, por todas las enseñanzas y la motivación contagiosa. Esta tesis no hubiera sido posible sin tu dedicación, Dani. Además, tengo la suerte de poder decir que te has convertido en amigo a la par que director, por lo que me siento totalmente privilegiada.

A Claudio, por saber guiar con precisión, por tus consejos siempre acertados, y por tu apoyo. Es una suerte poder contar con mentes que puedan pensar “outside the box”, por lo que me siento muy afortunada de haberte tenido como director de tesis.

A tot l'equip de Salut Internacional, estaré sempre agraïda per l'acolliment i l'afecte rebut des del primer día. M'heu fet créixer en el professional i en el personal, gràcies per fer el dia a dia més fàcil i pels moments compartits. Com Alba em va dir, a vegades una no imagina, quins ressons arriben a quina cantonada. També podria resumir-ho com “sou els millors del món”.

A Sara, por sabernos acompañar e apoiar no camiño.

FUNDING

ISGlobal acknowledge support from the Spanish Ministry of Science and Innovation and State Research Agency through the “Centro de Excelencia Severo Ochoa 2019–2023” Program (CEX2018-000806-S), CIBER-Consorcio Centro de Investigación Biomédica en Red-(CB 2021), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea and support from the Generalitat de Catalunya through the CERCA Program.

I also acknowledge support from the Hospital Clinic of Barcelona and the Spanish Ministry of Science and Innovation through the program PID2020-116770RJ-I00 and CPP2021-008658.

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I.LIST OF ABBREVIATIONS AND ACRONYMS

AI	Artificial Intelligence
ACT	Artemisinin-based Combination Therapies
AQUAMAT	African Quinine Artesunate Malaria Treatment
ARDS	Acute Respiratory Distress Syndrome
BCE	Before Common Era
CDC	Center for Disease Control and Prevention
CI	Confidence Interval
CM	Cerebral malaria
CRP	C-reactive protein
DTT	Dichloro Dyphenil Trychloroethane
ECDC	European Centre for Disease Prevention and Control
GMEP	Global Malaria Eradication Program
GMP	Good Manufacturing Practice
HRP2	Histidine-rich protein 2
ICU	Intensive Care Unit
IL	interleukin
iRBCs	Infected red blood cells
LFA	Lateral Flow Assay
ML	Machine Learning
NAATs	Nucleic Acid Amplification Tests

OR	Odds Ratio
PADH	Post-artesunate delayed hemolysis
pLDH	*Plasmodium* Pan-malarial Lactate Dehydrogenase
POC	Point-of-care
RBCs	Red blood cells
RDT	Rapid diagnostic test
RR	Risk Ratio
SEQUAMAT	SouthEast Asian Quinine Artesunate Malaria Trial
SMA	Severe malarial anemia
SM	Severe malaria
sTREM-1	Soluble Triggering Receptor expressed on myeloid cells
TNF	Tumor Necrosis Factor
UM	Uncomplicated malaria
USA	United States of America
WHO	World Health Organization

II.LIST OF THE ARTICLES IN THE THESIS

Thesis in compendium of publications format. The thesis consists of 7 objectives and four articles: Article 1 corresponds to the first objective; article 2 corresponds to the second, third, and fourth objectives; article 3 corresponds to the fifth objective and article 4 corresponds to the sixth and seventh objectives.

Article 1

MALrisk: a machine-learning–based tool to predict imported malaria in returned travellers with fever.

Balerdi-Sarasola L, Pedro F, Bottieau E, Genton B, Petrone P, Muñoz J, Camprubí-Ferrer D.

J Travel Med. 2024 Apr 5:taae054. doi: 10.1093/jtm/taae054. PMID: 38578987.

Impact factor 2023: 9.2. Cite Score 20.9. Quartile: Q1 (D1)

Article 2

Not all severe malaria cases are severe: Is it time to redefine severity criteria for malaria in non-endemic regions?

Balerdi-Sarasola L, Muñoz J, Fleitas P, Rodriguez-Valero N, Almuedo-Riera A, Antequera A, Subirà C, Grafia-Perez I, Ortiz-Fernández M, de Alba T, Álvarez-Martínez MJ, Valls ME, Parolo C, Castro P, Camprubí-Ferrer D.

Travel Med Infect Dis. 2024 Jul-Aug;60:102740. doi: 10.1016/j.tmaid.2024.102740.

Impact factor 2023: 6.3. Cite score: 19.4. Quartile: Q1 (D1)

Article 3

Host biomarkers for early identification of severe imported Plasmodium falciparum malaria.

Balerdi-Sarasola L, Parolo C, Fleitas P, Cruz A, Subirà C, Rodríguez-Valero N, Almuedo-Riera A, Letona L, Álvarez-Martínez MJ, Valls ME, Vera I, Mayor A, Muñoz J, Camprubí-Ferrer D.

Travel Med Infect Dis. 2023 Jul-Aug;54:102608. doi: 10.1016/j.tmaid.2023.102608.

Impact factor 2023: 6.3. Cite score: 19.4. Quartile: Q1 (D1)

Article 4

LDH as a prognostic biomarker in imported malaria: Implementing smartphone-based analysis for rapid clinical decision-making

Pedreira J*, **Balerdi-Sarasola L***, Fleitas P, Villanueva G, Petrone P, Muñoz J, Camprubí-Ferrer D, Parolo C.

Submitted.

III.THESIS SUMMARY

La malaria sigue estando en el pódium de las enfermedades infecciosas más mortales a nivel global. Aunque la malaria importada presenta un panorama diferente al de las zonas endémicas, sigue siendo una fuente considerable de morbi-mortalidad. El diagnóstico precoz es crucial debido a la naturaleza agresiva de la enfermedad, pero el acceso a pruebas microbiológicas puede ser un desafío en algunos entornos médicos. Además, los criterios actuales de malaria grave, basados en los criterios de la OMS para áreas endémicas (y dirigidas principalmente a detectar gravedad en niños), pueden llevar a una clasificación incorrecta en regiones no endémicas, especialmente teniendo en cuenta que la población en estas áreas suele ser adulta y puede no haber estado expuesta previamente al parásito.

La **hipótesis** de esta tesis es que la evaluación de nuevas estrategias para la identificación precoz y estratificación de pacientes optimizaría el manejo de la malaria en regiones no endémicas. Los **objetivos** fueron: 1) Desarrollar una herramienta basada en machine-learning para predecir el riesgo de presentar malaria en viajeros que regresan con fiebre;2) Describir las condiciones potencialmente mortales, incluyendo muertes e intervenciones vitales, así como la prevalencia de coinfecciones en una cohorte de pacientes con malaria atendidos desde 2005 hasta 2023;3)Evaluar una clasificación modificada de malaria grave para regiones no endémicas, para identificar pacientes con mayor riesgo de desarrollar condiciones potencialmente mortales;4) Identificar los factores predictivos asociados con el fallo orgánico y la muerte en pacientes con malaria importada;5) Identificar biomarcadores del huésped asociados con la gravedad y el fallo orgánico en pacientes con malaria importada.6) Identificar biomarcadores del parásito asociados con la gravedad en pacientes con malaria importada.7) Evaluar la capacidad de una prueba de inmunoensayo de flujo lateral convencional HRP2/LDH para identificar pacientes con malaria importada grave.

Los objetivos se respondieron a través de 4 estudios. Para el **artículo 1** se obtuvieron datos de un estudio prospectivo multicéntrico de viajeros con fiebre, para construir un

modelo basado en machine-learning que pudiera predecir casos de malaria entre los mencionados viajeros. Para el modelo se utilizaron características demográficas, clínicas y de laboratorio como variables. Estas variables fueron evaluadas con once modelos de clasificación de machine-learning mediante validación cruzada en un conjunto de entrenamiento. Posteriormente, en el modelo con mejor rendimiento, definido por el área bajo la curva(AUC), se realizó la optimización de parámetros y evaluación en el conjunto de prueba. Finalmente, se elaboró un modelo reducido con aquellas características que más contribuyeron al modelo inicial. El modelo reducido, MALrisk, logró una sensibilidad del 100% y una especificidad del 72% con solo 6 variables (destino de viaje, quimioprofilaxis, erupción cutánea, síntomas respiratorios, recuento de plaquetas y bilirrubina). Por tanto, MALrisk es una herramienta prometedora para identificar malaria en pacientes con fiebre importada, facilitando el tratamiento empírico y el traslado urgente.

En el **artículo 2**, se analizó una cohorte de pacientes (n=506) tratados por malaria en el Hospital Clínic entre 2005 y 2023. Tras el diagnóstico, se aplicó el criterio de la OMS (excepto para la parasitemia, con umbral del 2%). Tras la primera clasificación, se aplicó un criterio modificado de gravedad, que subdividió las malarias graves en malarias muy graves y malarias menos graves. Como resultados, inicialmente, el 35% de los pacientes presentó un episodio de malaria grave según los criterios de la OMS. Tras la aplicar la clasificación modificada, ningún paciente con malaria menos grave desarrolló una condición potencialmente mortal, sugiriendo que las clasificaciones de gravedad de malaria se beneficiarían de una revisión para áreas no endémicas, asumiendo que las malarias menos graves no precisan de vigilancia intensiva.

En el **artículo 3**, se realizó un estudio de casos y controles retrospectivo en el Hospital Clínic (2011-2021), clasificando a pacientes adultos con *P.falciparum* según los criterios de la OMS y comparándolos con controles con fiebres no maláricas. En todos los grupos se midieron biomarcadores como Ang-1, Ang-2, sTREM-1, PCR y plaquetas. Se incluyeron 131 participantes: 52 con malaria grave, 30 con malaria no complicada y 49 con fiebre no malárica. Todos los biomarcadores, excepto sTREM-1, mostraron

diferencias significativas entre los grupos. Usando la clasificación de gravedad modificada de la OMS, Ang-2 y PCR presentaron la mejor AUC; 0.79 (IC 95% 0.64–0.94) y 0.80 (IC 95% 0.67–0.93), respectivamente. Además, un modelo que combina PCR y Ang-2 mostró la mejor AUC, con la mayor sensibilidad y especificidad: 84.6% (IC 95% 58.9–98.1) y 77.4% (IC 95% 65.9–87.7), respectivamente. Por tanto, la combinación de Ang-2 y CRP mostró el mejor rendimiento diagnóstico para casos clínicamente graves.

Finalmente, en el **artículo 4**, se realizó un estudio de casos y controles (enero de 2011-enero de 2021), de viajeros que regresaron con enfermedades febriles agudas al Hospital Clínic tras un viaje internacional. Se aplicó la misma metodología de clasificación de casos que en el artículo 3. Tras ello, en las muestras obtenidas se realizó la medición de pLDH y HRP2, biomarcadores del parásito, utilizando un inmunoensayo multiplex de alto rendimiento basado en la plataforma Luminex. Posteriormente, se realizó una lectura a simple vista de una prueba de inmunoensayo de flujo lateral convencional y finalmente se realizó la cuantificación de las bandas de dicho test a partir de las imágenes de un smartphone analizados con el software ImageJ. En total, 121 participantes fueron incluídos en el estudio: 75 pacientes con malaria (de los cuales 50 eran malarias graves) y 46 participantes con fiebres no maláricas. En Luminex, la sensibilidad de pLDH como marcador pronóstico fue de 84.0%(95%IC:73.8-94.2), con una especificidad de 88.0% (95%CI: 75.3-100). Además, la combinación de pLDH con HRP2 no aumentó significativamente la habilidad pronóstica. Por último, el AUC de pLDH determinada por el test comercial tras el análisis de smartphone fue de 0.85 (95% IC: 0.76-0.93), permitiendo estimar una sensibilidad de 73.9% (95%IC: 61.2-86.6) y una especificidad de 88.0% (95%IC: 75.2-100). Con todo ello, la pLDH puede ser una firme candidata como herramienta pronóstica. Para finalizar, el uso de pruebas rápidas y análisis con smartphone podría proporcionar un método accesible y eficiente para diagnosticar y predecir la gravedad de la malaria.

IV.INTRODUCTION

Historical view of malaria

Malaria has afflicted humanity since almost its inception; neolithic dwellers suffered it before they could even imagine that the life cycle of the disease involved a mosquito. Many civilizations throughout history have documented the havoc caused by intermittent fevers, starting in 2700 BCE with the NeiChing (the Chinese Canon of Medicine), where malaria symptoms were discussed and the relationship between fevers and enlarged spleens was proposed[1]. In the 6th century BCE, the cuneiform script from Mesopotamia also documented it. Such was the impact of malaria that Indian writings of the Vedic period (1500 to 800 BCE) called malaria the "king of diseases"[1]. More directly, malaria antigen was detected in Egyptian excavations from 3200 and 1304 BCE[2]. The early Greeks and Roman Empire, since their beginnings, also suffered from this disease that came down from the Nile.

The term malaria appears documented in Italy for the first time around the mid-18th century. It derives from the contraction of the Italian words "mal" and "aria" meaning "bad or unhealthy air". In the Middle Ages, malaria was thought to be transmitted by humid and stale air, a situation often found in swamps[3]. In the same way, the word "paludisme" comes from the French, where "palus" is the Latin word for swamp, once again emphasizing the idea that staying close to swamps and marshes caused the disease[4]. In Spain, the word is registered in 1861[5]. Shortly thereafter, in 1880, the *Plasmodium* parasite was discovered by Charles Louis Alphonse Laveran, and thanks to the studies of Ronald Ross in 1897 and several Italian scientists (to note Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava) between 1898 and 1900, mosquitoes were described as obligatory vectors in malaria cycle[6]. Since then, it has been understood that malaria is a life-threatening disease caused by *Plasmodium* spp. parasites, transmitted through the bites of previously infected *Anopheles* mosquitoes. Although several zoonotic *Plasmodium* spp.

infections have been documented in humans (mostly six), *Plasmodium falciparum* is the most widespread and aggressive one, being responsible for the majority of deaths from malaria[7].

Global trends on malaria and impact of malaria control strategies

At the beginning of the 20th century, the course of each continent has been influenced, in one manner or another by malaria, coinciding this fact with the establishment of national and international programs for the control and eventual eradication of malaria, such as the Center for Disease Control (CDC) and Prevention in the USA[8]. In Europe, England and Italy were pioneers in carrying out campaigns to drain swamps, vector-control, and using quinine systematically to tackle malaria, being the first countries in Europe to achieve disease control[3]. Spain eliminated malaria in 1964, being one of the last countries in Western Europe to be declared free of malaria by the World Health Organization (WHO)[9]. Many other countries achieved this status right after Second World War[10](**Figure 1**). Behind these achievements, there was also the involvement of WHO that, since 1955, implemented the Global Malaria Eradication Program (GMEP).

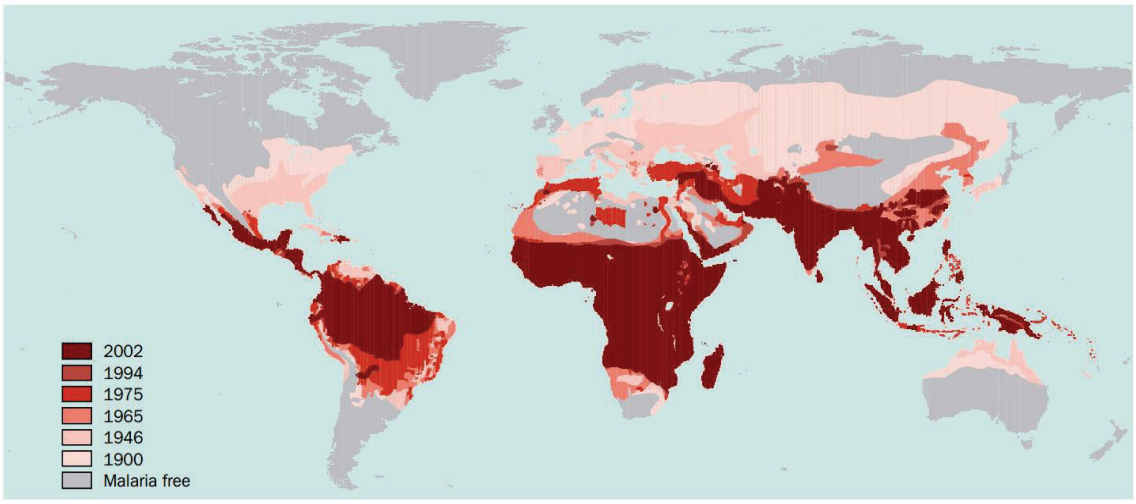


Figure 1. The global distribution of malaria since 1900 to 2002. [11]

After WHO's first malaria initiative in 1955, the organization shifted its approach in 2000 with the launch of the Global Malaria Program (GMP). The focus moved from global

malaria eradication to creating country-specific roadmaps, setting control goals for all countries and elimination targets for some. The interventions focus on vector control with dichloro dyphenil trychloroethane (DTT), the distribution of insecticide-treated bed nets, as well as to secure access to diagnostic tests and treatment. Briefly, from 2000 to 2015, malaria cases and mortality decreased significantly[12,13]: Globally, the number of malaria cases fell from an estimated 262 million in 2000 (range 205–316 million) to 214 million in 2015 (range 149–303 million). The same occurred with mortality, as the number of malaria deaths fell from an estimated 839.000 in 2000 (range 653.000 to 1.1 million), to 438.000 in 2015 (range 236.000–635.000)[13]. The percentage of total malaria deaths in children aged under 5 years decreased from 87% in 2000 to 76% in 2015[14]. Although there was a pause in the trend from 2015 onward, the COVID-19 pandemic definitively altered the global downward trajectory of cases; due to disruptions in GMP implementation (among other factors), there was a global increase in cases and deaths[15]. The World Malaria Report, published annually by WHO, summarizes the global epidemiological situation, interventions undertaken, and future challenges. By the end of 2022, there were an estimated 249 million malaria cases (an increase of 5 million cases compared with 2021) and 608.000 deaths (fewer cases than the previous year but still more cases than in 2019[14]. On the other hand, the year 2022 left with Azerbaijan, Belize, and Tajikistan achieving the status of malaria-free countries, being 85 the total account for malaria-endemic countries and territories. In January of 2024 WHO certified Cabo Verde as a malaria-free country too. **Figure 2** from World Malaria Report 2023 shows global trends in malaria incidence and mortality.

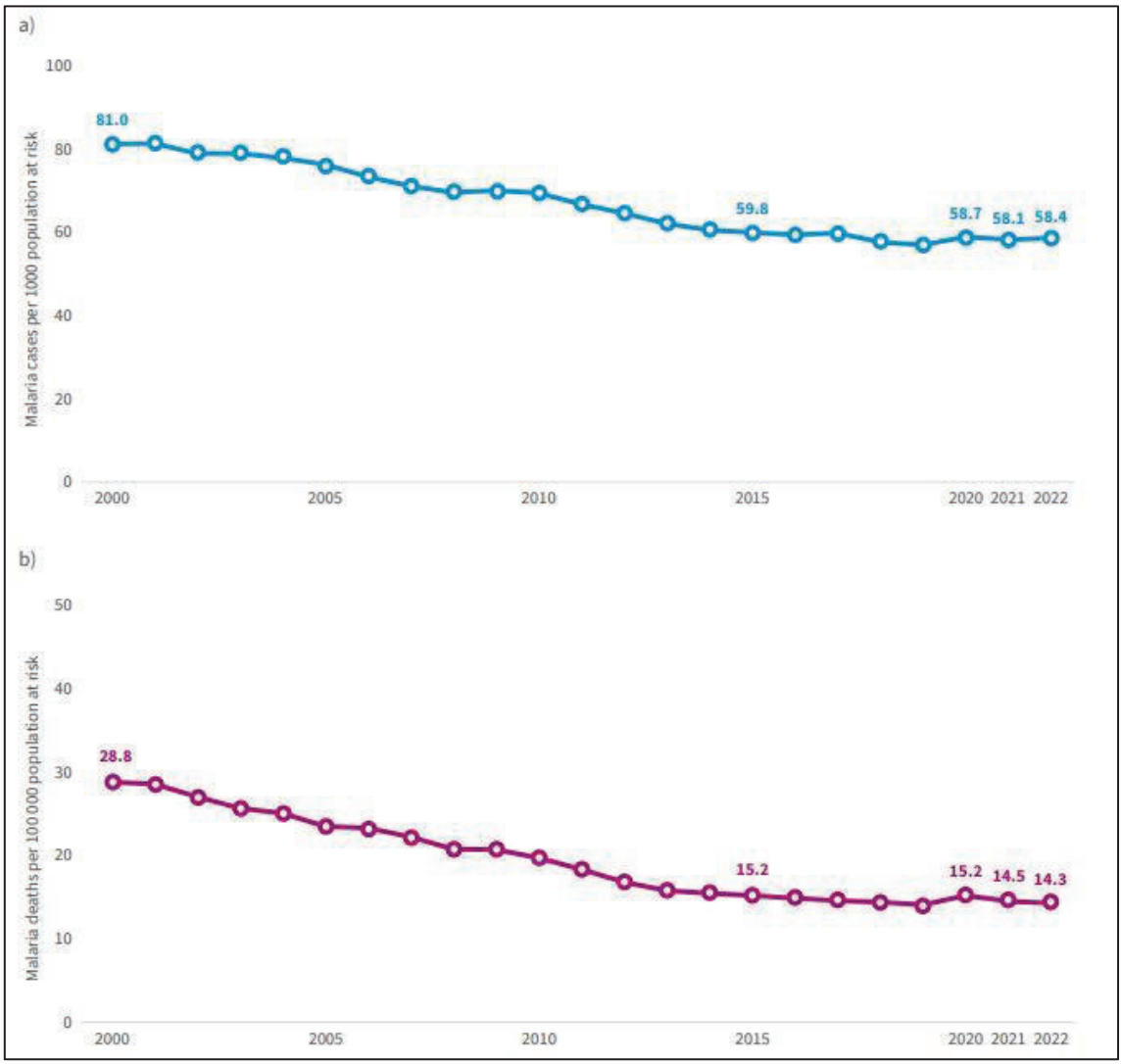


Figure 2. Global trends in a) malaria case incidence (cases per 1000 population at risk) and b) mortality rate (deaths per 100 000 population at risk), 2000–2022.[14]

On the other hand, in contrast to these historical trajectories, Africa has consistently had the heaviest burden of malaria cases and associated impact over the centuries. Nowadays, about 93.6% of malaria cases globally occur in Sub-Saharan Africa, and specifically, 4 countries (Nigeria, Democratic Republic of Congo, Uganda and Mozambique) host 50% of global malaria cases[14].

Impact of artesunate in mortality

From the arrival of quinine (found in the bark of the cinchona tree) in Europe from Peru in the 17th century, brought by the Jesuits[1], to the discovery of artemisinin by the Nobel Prize Professor Youyou Tu in 1972[16,17], the history of malaria is also a narrative of the quest for a cure for the so-called 'intermittent fevers'.

Until the early 2000s, quinine was the first-line, core treatment for severe malaria (SM)[18]. By that time SM mortality in endemic areas ranged between 15 to above 30% (when presenting with cerebral malaria or with multiple vital organ dysfunction)[18,19]. With this global health challenge, two large trials were conducted to prove the superiority of artesunate as a first-line treatment against malaria. SEQUAMAT (SouthEast Asian Quinine Artesunate Malaria Trial) was the first trial, published in 2005[20], that consisted of an open-label controlled trial that randomized 1461 patients admitted to hospital with severe *P. falciparum* malaria in Bangladesh, India, Indonesia, and Myanmar. Being the primary endpoint in-hospital mortality, death in artesunate recipients was 15% compared with 22% in quinine recipients, with an absolute reduction of 35% (95% CI 18.5–47.6%; $p<0.001$)[20]. These findings started the path for a paradigm shift in SM management, that was ratified by the AQUAMAT (African Quinine Artesunate Malaria Treatment) trial. AQUAMAT results were published in 2010[21]; 5425 children were enrolled in this open-label, randomized trial undertaken in 11 centers in nine African countries. In this trial, mortality in the artesunate group was 8% while in the quinine group was 11%[21] . Data from these two big trials along with smaller ones (8 in total) were grouped in a meta-analysis[22] that showed a 39% (95% CI 25% to 50%) reduction of the risk of death in adults, and 24% in children (95% CI 10% to 35%)[22](**Figure 3).** Apart from that, artesunate was shown to be safer than quinine with fewer adverse events[22]. Although no trial included in the meta-analysis reported discontinuation of medication due to severe adverse events, mild adverse events including tinnitus, hearing impairment, nausea, and vomiting were less commonly described in the artesunate group. Besides, artesunate was associated with a better safety profile, with statistically significant reduction in episodes of hypoglycemia [Risk Ratio (RR) 0.55, 95% 0.41 to 0.74][22].

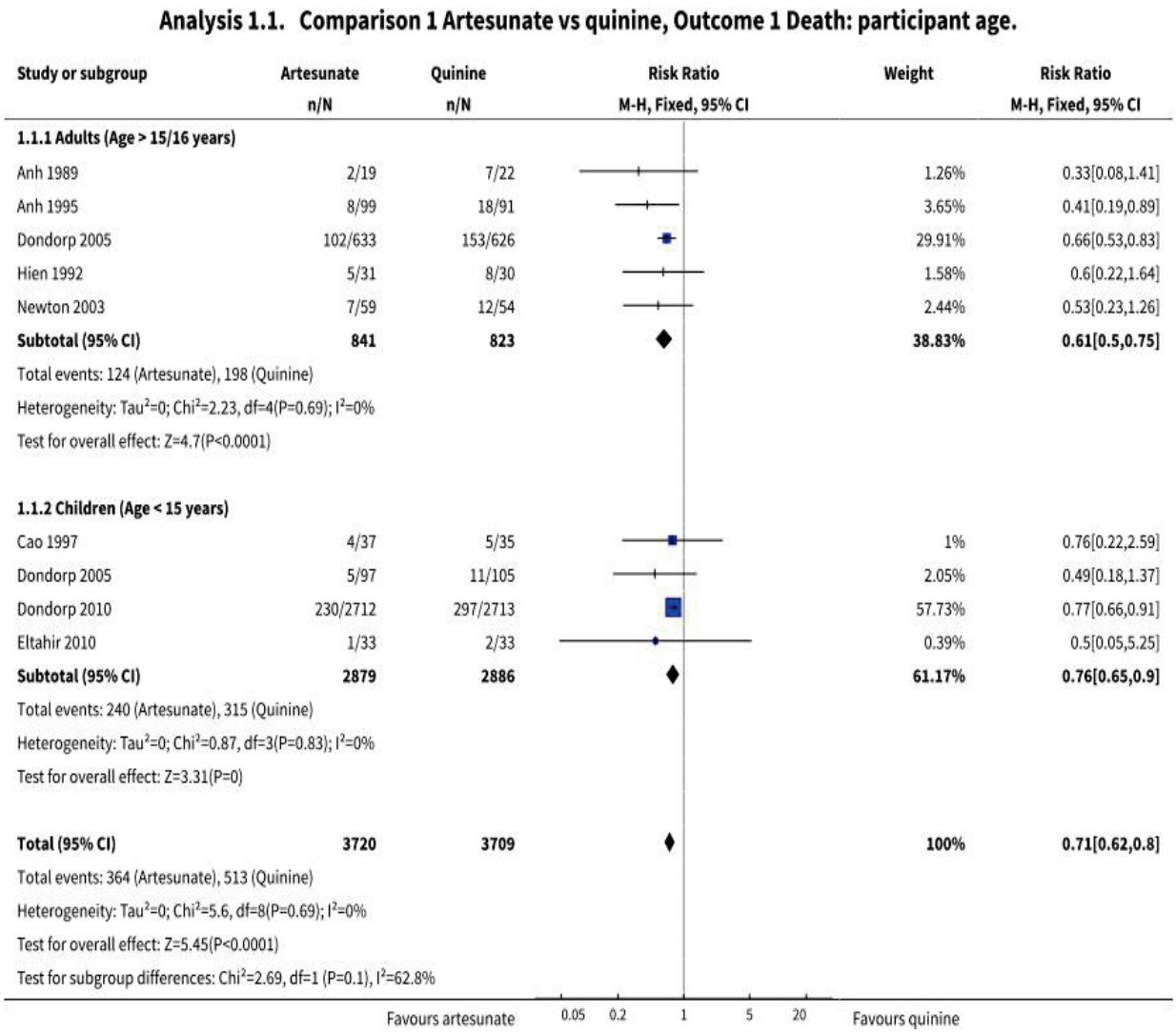


Figure 3. Forest plot comparing mortality of *P. falciparum* malaria treated with artesunate and quinine[22].

This evidence, regardless of the age of the patient, clearly supported the superiority of parenteral artesunate over quinine, prompting revisions and changes in global treatment guidelines. Nowadays, artesunate and artemisinin-based combination therapies (ACT) are indisputably the first-line treatment for severe and uncomplicated malaria worldwide. [23]

Malaria in non-endemic areas

The current situation of malaria in Europe presents a completely different scenario compared to Africa. After centuries of shared suffering due to the morbidity and mortality associated with malaria, the WHO European Region was at first declared malaria-free in 1975. However, by the 1990s, local malaria transmission was re-established in the Caucasus and the Central Asian republics. In response to this situation, the affected countries committed through the Tashkent Declaration in 2005 to eliminate malaria in the WHO Europe Region by 2015. This objective was successfully achieved, and WHO certified again WHO Europe Region malaria-free in 2016[24,25]. Focusing on Spain, historically, malaria epidemics occurred in the 18th century and after the Civil war[10]. During the 1950s and until the last cases were declared in 1961, Western Andalusia with its wetlands represented the last important focus of endemic malaria in Spain[26]. Finally, Spain was certified as malaria-free in 1964[9].

Spain and Europe thus became non-endemic regions, and since that time the number of imported cases reported over the years has been constant. According to 2023 European Centre for Disease Prevention and Control (ECDC) epidemiological report, a total of 4.856 malaria cases were reported in Europe in 2021[27]. Among 4.257 cases with known importation status, 99.7% were travel-related[27]. Although declaration is not obligatory in France, they reported the highest number of cases (2.322), followed by Germany (605), Italy (443), Spain (430), and Belgium (365). However, this numbers are lower compared to those of pre-pandemic years; Spain reported 851 cases and France 2.839 in 2018[28].

Going into more detail regarding malaria cases, a recent meta-analysis highlighted a pooled prevalence of SM of 12.5% (95% CI 10.3%–14.6%)[29] among imported malaria cases. Besides, the ECDC annual report described a case fatality ratio of 1.1% among 1.944 malaria cases with a known outcome in Europe[27], and the systematic review highlighted a pooled prevalence of deaths attributable to severe imported malaria of 5.1% (95% CI 4.0%–6.2%). In terms of temporary trends, the systematic review described a pooled prevalence for death of 8 % between 2000 and 2009, descending to 4.4 %

between 2010 and 2021[29] . However, ECDC only receive data from passive surveillance and information about clinical outcomes are missing in almost 50% of cases. In the same way, large cohorts specifically describing imported SM cases in non-endemic regions are scarce. A small cohort of 20 patients attended in an intensive care unit in the Hospital Clinic of Barcelona between 1991 to 2007, described a mortality as high as 25%[30] in the pre-artesunate era. Besides, one of the most representative studies addressing SM is a French cohort with 400 patients, that collected data from 2000–2006. In the mentioned cohort, the reported mortality of severe cases was 10.5%[31], showing that referral centers concentrate the most severe cases and higher mortality rates.

Fortunately, the introduction of artesunate shifted the paradigm in the management of malaria. After WHO recommended using artesunate as a first-line treatment for SM, non-endemic countries also started to change their guidelines, even though controlled trials were not conducted in non-endemic settings due to the strong evidence already collected in endemic regions. European guidelines for the management of malaria were drafted in 2012[32], and incorporated artesunate as first-line therapy. Since then, the introduction of intravenous artesunate into treatment practice has been slow because a formulation meeting standards of Good Manufacturing Practice (GMP) as well as prospective clinical safety data required for regulatory approval were not available. To address this issue, a first retrospective multicentric study was conducted, monitoring the treatment practices and outcomes of 185 patients with SM across 12 European countries between 2006 and 2014[33]. The study added evidence in favor of artesunate demonstrating that artesunate reduced the duration of Intensive Care Unit (ICU) and hospital stay, with faster parasite clearance time (median, 36 vs 48 hours; $p = 0.02$, $n = 100$). Subsequently, a propensity-score analysis obtained similar results in terms of reduction of ICU stay[34]. However, the latter study, which included patients treated for SM in France between 2011 to 2017 ($n = 1544$), did not find any differences in terms of 28-day mortality when comparing quinine and artesunate groups. On top of that, in Spain, the availability of artesunate has been related with a decrease in mortality[35].

In parallel, a safety concern about the development of hemolytic anemia after artesunate treatment came up. In this line, a review which summarized 13 studies that included a total of 574 patients showed that post-artesunate delayed hemolysis (PADH) occurred in 15% of patients, but no death or sequelae were reported. In PADH patients presenting anemia, overall blood transfusion was administered in 50% of travelers[36]. Afterward, a French multicentric prospective study enrolling 1391 patients between 2011–2017, recorded clinical and epidemiological features of artesunate-treated patients[37]. In this article, the incidence of PADH was 42.8% when specifically assessed in a 98-patient subgroup (of which hematological parameters were available during the 28-day follow-up period) but was not associated with fatal outcomes or sequelae. Interestingly, PADH was twice as frequent in patients of European origin compared with patients of African origin. As other adverse events, cardiac events were recorded in 24 patients, being more frequently reported in patients with European origin. This data showed that indeed Artesunate is highly efficacious, however follow-up is necessary to detect the mentioned side effects; a sort of toll to pay for a life-saving treatment.

Severe malaria

After providing a general overview of malaria from a chronological perspective, as well as its evolution in different regions, it is important to delve into two key aspects of the term "severity" in this context. The first is to understand severity as the consequence generated by the interaction of a pathogen with the host. The second is to recognize that defining a disease as severe is a necessary classificatory label to standardize clinical decisions and establish a common language. These two aspects coexist in the term "severe malaria". Understanding both aspects is essential to fully appreciate the spectrum of disease caused by malaria and the corresponding medical response.

Pathophysiology of severe malaria

A thorough understanding of the intricate life cycle of *P. falciparum*, comprising multiple developmental stages, is essential for assessing the clinical impact of malaria on patients. It begins with the bite of an infected female *Anopheles* mosquito, while clinical symptoms start when merozoites are released from the liver and invade red blood cells (RBCs). Within the RBCs, they replicate asexually, leading to the rupture of RBCs and release of more merozoites, which can infect other RBCs.[38] Once the blood stage is reached, *P. falciparum* shows a unique characteristic: cytoadherence. Thanks to the ligands expressed by the parasite in the surface of infected RBCs, they have the ability to produce the clumping of uninfected to infected RBC ("rossetting"), together with the clumping between infected RBC (denominated "auto-agglutination"). More importantly, cytoadherence permits infected RBC to stick in post-capillary distal venules' endothelium, causing sequestration and further contributing to the impairment of microcirculatory flow, causing tissue hypoxia and organ damage[39]. Therefore, peripheric parasite count does not entirely reflect the *P. falciparum* pathogenic mechanisms, and cytoadherence is a cornerstone for the most severe clinical presentations of malaria, as directly damages the microvasculature and endothelium, having as a final result a diffuse tissue ischemia and organ failure. On the other hand, cytoadherence triggers the host reaction, based on a potent inflammatory and immune response. Pro-inflammatory cytokines [e.g., interleukin (IL)-1β and tumor necrosis factor (TNF)], neutrophils and neutrophil-produced proteins are key actors in the onset of severe malaria pathology [40] , but both immune and inflammatory response converge on the endothelium activation. This change in the endothelium's state makes it permeable and pro-inflammatory, allowing it to accommodate pathogen sequestration and elimination while simultaneously working to repair vascular damage[41]. If the aggression continues, endothelial activation results in endothelial dysfunction, leading to organ damage too. This interaction shows that there is a fine balance between activating a response that is strong enough to limit parasite replication and avoiding a response that damages the host[39,42]. **Figure 4** shows the most remarkable aspects of host responses to the parasite.

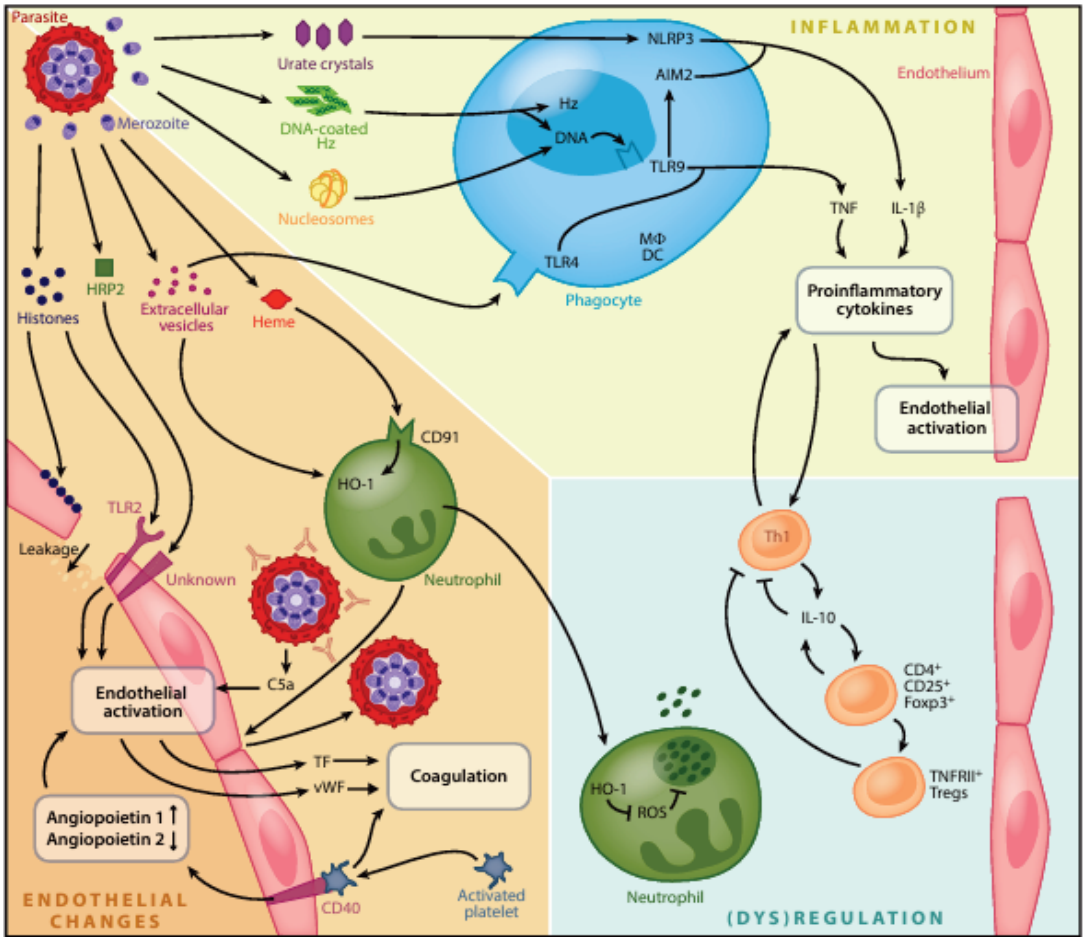


Figure 4: Image showing host-parasite interaction during *Plasmodium* spp. infection.[39]

Identification of severe cases through WHO definitions

By shifting the focus from the pathophysiology of severe malaria to the medical response to malaria, the initial step of adopting guidelines was taken. This was done in an attempt to standardize the clinical approach to malaria patients and to identify individuals at higher risk of developing severe disease. In 1985 a WHO committee set a severity classification, based on experts' opinion and evidence gathered in South-East Asia[43]. This event became an important accomplishment in the management of malaria because it provided a concrete guide to define malaria's clinical presentations and to decide on initial antimalarial treatment. Since 1985, severity criteria have undergone several modifications until nowadays (**Table 1)** [19]. (Note: The severity criteria and

classification of malaria apply to *P*. *falciparum*. If not specified otherwise in the text, reference is always made to *P*. *falciparum*.).

The main target of these criteria was children (under the age of 5 years) from malaria-endemic areas, the most vulnerable population for SM that needed enhanced management. After gathering experience from studies in African children, in 1995 a new meeting was held by WHO where a more inclusive and pragmatic definition of SM in children included severity criterion such as prostration (defined as the inability to sit upright in a child normally able to do so or to drink in the case of children too young to sit) and respiratory distress (acidotic breathing) as severity criteria[44]. The new inclusive definition ensured high sensitivity, but low specificity in identifying potentially fatal infections[43]. However, in the same meeting, the contrasting needs of more specific criteria were highlighted[43]. In addition, the parasite density threshold to consider hyperparasitemia was sub-divided according to transmission intensity: 4% was recommended for low transmission settings, and 20% in high transmission settings. Subsequently, a meeting was held in 2013, marking the latest engagement by the WHO on this issue. During the meeting, certain aspects of SM were reformulated, including a change to the hyperparasitaemia criterion, which was set back to a 10% cut-off value. Interestingly, severe *Plasmodium vivax* and *Plasmodium knowlesi* definitions were also proposed[45]. For all patients fulfilling the severity criteria, admission in the highest monitoring unit available at hospital was also advised. This 2013 meeting incorporated substantial changes in the therapeutical aspect's recommendations, including parenteral artesunate as a first-line treatment for SM. The introduction of artesunate also enabled the development of recommendations for clinical situations that while not strictly classified as severe, could still result in worse patient outcomes.[38,45, 46]

Currently, clinical criteria for use of intravenous artesunate are:

- Unable to take oral medications due to repeated vomiting.
- Uncomplicated hyperparasitaemia.

Uncomplicated hyperparasitemia refers to patients exhibiting parasitemia between 4-10%, without other severity criteria, thereby constituting a subgroup where parenteral treatment could be prioritized without any other specific recommendations. This

threshold was proposed to be even lower (≥2%) in low-transmission regions and in non-immune travelers[23,47].

CRITERION	ACTUAL DEFINITION	1985's DEFINITION
Impaired consciousness	Glasgow Coma Score <11 in adults and Blantyre Coma scale <3 in children *Post-critic period needed after convulsion: 30 minutes	A Glasgow Coma Score <11 *Post-critic period needed after convulsion: 6h
Pulmonary oedema	Radiologically confirmed or oxygen saturation <92% on room air with a respiratory rate >30 per min, often with chest indrawing and crepitations on auscultation	Not included initially
Substantial bleeding	Including recurrent or prolonged bleeding from the nose, gums, or venipuncture sites; hematemesis or melena	Not included initially
Shock	Systolic blood pressure <70 mm Hg in children, <80 mm Hg in adults, with evidence of impaired perfusion	Not included initially, making part of: Fluid, electrolyte or acid–base disturbances requiring intravenous therapy
Acidosis	A base deficit of >8 mEq/L, or plasma bicarbonate <15 mmol/L, or venous plasma lactate ≥5 mmol/L.	Not included initially, making part of: Fluid, electrolyte or acid–base disturbances requiring intravenous therapy
Hypoglycaemia	Blood or plasma glucose 2.2 mmol/L (<40 mg/dL)	Blood or plasma glucose 2.2 mmol/L (<40 mg/dL)
Severe anaemia	Haemoglobin ≤5 g/dL / haematocrit ≤15% in children aged <12 years. Haemoglobin concentration <7 g/dL / haematocrit <20% in individuals aged ≥12 years with a parasite count >10000 per µL	Haematocrit < 20%
Severe acute kidney injury	Plasma or serum creatinine >265 µmol/L (3 mg/dL) or blood urea >20 mmol/L in individuals aged >12 years. (not defined by WHO in children aged <12 years)	Urine output of less than 400mL in 24h and serum creatinine >3mg/dl failing to improve after rehydration
Jaundice	Plasma or serum bilirubin >50 µmol/L (3 mg/dL) with a parasite count >100000 per µL	Total bilirubin > 50 µmol/L alone was a criterion
Hyperparasitaemia	*Plasmodium falciparum* parasitaemia >10% of infected erythrocytes in stable high endemicity area	> 5% parasitaemia
Prostration	The person is unable to sit, stand, or walk without assistance	Not formulated initially

Multiple convulsions	More than two episodes within 24 h	Not formulated initially
Body temperature	Removed	> 39 °C (Hyperpyrexia) was a criterion
Hemoglobinuria	Removed	Its presence was a criterion

Table 1. Severity criteria changes according to initial definitions. Adapted. [38, 19]

Challenges of malaria management in non-endemic areas

Regardless of the perspective from which the severity of malaria is viewed, it can be an aggressive disease, with imported cases presenting unique challenges that complicate their definition. Early diagnosis is the first step to implementing the subsequent measures, and the WHO recommends the prompt initiation of artesunate and management at the highest monitoring level available at hospital for severe malaria. Although expert ICU management is essential for the most severe cases (we must not forget that the mortality of imported cases remains up to 5% [29,37]), the pandemic has evidenced that a high level of healthcare is a precious resource not universally accessible. Additionally, it must be considered that the population attended in non-endemic areas consists mainly of adults without previous exposure to the infection (or with a distant past exposure), which is why the response to the infection may diverge. With all this in mind, malaria in non-endemic regions faces some particular challenges.

First challenge for malaria in non-endemic settings: Identification of malaria cases

Unraveling the etiology of febrile illnesses after international travel can be complicated: practitioners in non-endemic areas should consider cosmopolitan pathogens and microorganisms of imported origin, which are less familiar to them [48,49]. Besides, there is frequently a clinical overlap between life-threatening diseases such as malaria and self-

limited or benign conditions (such as most of viral infections), which hinders the initial management of these patients [50]. In addition, although malaria may develop specific clinical conditions related to organ damage (e.g. cerebral malaria), in non-endemic countries malaria usually presents as an undifferentiated febrile illness[51]. This ambiguous clinical presentation, along with human factors, can lead to a delay in diagnosis, which could be fatal[52,53]. Therefore, a high level of clinical suspicion is paramount, and laboratory tests are crucial to confirm or rule out the clinical impression.

The gold standard to diagnose a *Plasmodium spp.* infection is a microscopy examination of thick and thin blood films[38]. The test serves for multiple purposes. On the one hand, thick blood film is a concentration technique that provides enhanced sensitivity in case of low-level parasitemia (an estimated detection level of 50–200 parasites per µL of blood) and thin smear facilitates the identification of *Plasmodium* species. On the other hand, the thin blood film also permits the quantification of parasite density in peripheric blood, called parasitemia, which takes part of the malaria severity criteria and has prognostic value. The sensitivity and specificity for microscopy are 95% and 98%, respectively, when the polymerase chain reaction (PCR) is used for comparison[54,55]. Moreover, treatment success can be monitored after its initiation with this technique, and 3 negative determinations are generally required to rule out malaria in patients returning with fever or compatible symptoms from a malaria-endemic region[56]. Notwithstanding, this technique requires the presence of an expert microbiologist, usually located in tertiary centers, and if this test is interpreted by non-expert personnel, reliability can be compromised. Also, ideally, the time from sample collection to result should not exceed 4 hours according to national and international guidelines in non-endemic countries[57–59]. These standards are only achievable by reference hospitals and although blood smear is a gold-standard test, universal access is not guaranteed. Taking into account the potentially lethal nature of this disease, non-negligible time is needed to transfer patients where these laboratories are located[55].

To overcome the difficulties of expert laboratory capacities, rapid diagnostic tests (RDT) are a valuable alternative for *Plasmodium* detection. In fact, around the globe, 75% of diagnoses are based on this technique[14]. One of the main reasons is because it is a point-of-care (POC) test. This means that is manufactured to provide immediate results (within 15 to 20 minutes) that can be used to make timely clinical decisions[60–62]. POC tests are specifically designed to be simple, rapid, and portable. Another important aspect is that they are user-friendly, often requiring minimal training. The fact that RDT are portable devices make them suitable to be used in various settings, without the need of a laboratory structure, as they also need minimal sample preparation. Nowadays available RDT for malaria diagnosis are lateral flow immuno-tests (LFA), which rely on the capture of dye-labelled antibodies to produce a visible band on a strip of nitro-cellulose, often encased in plastic housing, referred to as cassettes (**Figure 5).** LFA have been widely used in other fields of medicine as are used in home pregnancy tests and some COVID-19 antigen tests.

The antigens currently used in available RDTs are *Plasmodium falciparum*-specific histidine-rich protein 2 (HRP2), *Plasmodium* pan-malarial lactate dehydrogenase (pLDH, although specific LDH for *P.vivax* exists), and pan-malarial aldolase. HRP2 was the first antigen selected to develop an RDT because of its abundance in *P. falciparum*: it is produced exclusively by asexual stages and gametocytes of *Plasmodium falciparum*, it is expressed on red blood cells' (RBCs) surface and it can also be found as a soluble protein in the blood. Instead, pLDH is expressed at high level in asexual stages of *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae* human malaria parasites, but it is mostly an intracellular protein and it cannot be found as a soluble protein[63]. Aldolase is a pan-specific enzyme involved in the glycolytic pathway of the malaria parasites that shares same characteristics as pLDH as it cannot be found as a soluble protein[55]. Most of the tests available on the market nowadays are tests that combine the measurement of HRP2 together with pLDH or aldolase, with RDT detection limit of approximately 200–2000 parasites per µL of blood for *P.falciparum* [64]. The diagnostic performance of this technique in non-endemic areas varies depending on commercial tests but can achieve mean sensitivity and specificity of 91.8% and 97% respectively for *P. falciparum* [55,65] ,

thanks to HRP2 performance. However, false-negative results can be obtained due to low parasite density, non-falciparum species or “prozone” effect in severe cases, and positive results can be detected after treated infections.[55,62,66,67] In addition, some malaria-endemic areas (mainly the Amazon basin and the horn of Africa) are menaced by the deletion of the gene HRP2, which hinders the performance of RDTs[14]. Ideally, in non-endemic areas, RDTs should not replace the blood smear but rather serve as a supportive tool or first-line test when a microscopist is not available. Finally, PCR tests are at least 10-fold more sensitive than microscopy[68], the limit of detection for PCR being approximately 0.2–6 parasites per µL of blood [55,68]. Although PCR is the most sensitive tests, it is a time-consuming, expensive technique, only available in referral centers that is not suitable as a valid tool for early diagnosis.

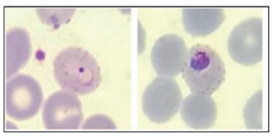
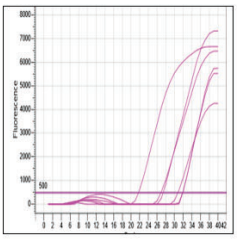
	RAPID DIAGNOSTIC TEST	MICROSCOPY	POLIMERASE CHAIN REACTION
Required time	15 to 30 minutes	4 hours	24 hours
Advantages	Minimal training	Gold standard	Most sensitive and specific
Disadvantages	Lack of sensitivity for non-falciparum HRP2 deletion	Expert microbiologist	Referral centers
			

Figure 5. Illustration of different diagnostic tests and their principal characteristics.[7,69–71]

Above all, regardless of the method used, testing should be available and performed 24 hours/day, 7 days/week due to the potentially life-threatening nature of the infection. If these standards along with the availability of an expert microbiologist are not achieved, access to diagnosis is jeopardized and physicians attending a returned traveler with fever must decide to wait an unacceptable time until results are available or need to transfer a patient with a diagnostic suspicion (any febrile patient returning from a

malaria endemic area). These obstacles might finally lead to diagnostic delay, which is one of the main pillars related to an increase in morbidity and mortality due to malaria in non-endemic regions[31,72–76].

Being aware of the lack of universal access to timely and reliable diagnosis of malaria, several studies identified the most relevant clinical and analytical characteristics associated with malaria, to guide clinicians during the first diagnostic approach to a patient after a stay in a malaria-endemic area. After a systematic review, Taylor S.M. et al.[77] concluded that among travelers the presence of splenomegaly, jaundice or pallor were clinical findings that significantly increased the risk of malaria, whereas thrombocytopenia and hyperbilirubinemia were the laboratory values demonstrating the highest likelihood ratios. Moreover, various scoring systems have been proposed to categorize patients in endemic areas as potentially high or low risk for malaria; however, no predictive scores have been suggested for non-endemic settings. Given the scarcity of tools for prioritizing malaria diagnosis in patients, new technologies could play a significant role. In this thesis, we will explore the use of Machine Learning (ML) for this knowledge gap.

ML is a branch of Artificial Intelligence (AI), and unlike traditional programming methods, ML algorithms are designed to autonomously discern patterns and rules from data [78,79]. ML models have gained widespread recognition and acceptance in various domains, including healthcare, demonstrating to be valuable resources for the unmet diagnostic needs in infectious diseases[80], as well as in chronic conditions[81]. Particularly in malaria, efforts have mainly been directed at improving laboratory diagnosis through the enhancement of microscopy reading [82,83], but the potential usefulness of ML in predicting imported malaria has not been explored.

Common ML types include supervised and unsupervised learning models (for example, clustering algorithms)[84]. Apart from logistic regression, which uses a linear approach, ML includes models enabling the interpretation of non-linear relationships (tree-based models), along with other models that assume an underlying probabilistic model[85,86]. The broader approach of ML, which incorporates models that employ various pathways, might improve the predictive capacity of conventional methods.

Therefore, in the absence of fully implemented diagnostic tests, Artificial Intelligence could help to overcome the diagnostic needs for malaria in non-endemic regions.

Second challenge for malaria in non-endemic settings: Case management and disease severity stratification

Once the diagnosis is achieved, the initial management of a patient with malaria is likely to determine the course of the subsequent infection. This underscores the importance of guidelines for practitioners; however, in managing malaria, notable differences exist between endemic and non-endemic regions.

Countries in non-endemic regions adopted in their guidelines the severity classification for imported malaria, taking as a reference the severity criteria established by the WHO. However, being aware of the historical evolution of the WHO classification, which prioritized sensitivity to target vulnerable populations, a more nuanced approach was taken by European guidelines[32] . To understand this is important to note that in non-endemic areas, population suffering from malaria differs as they are predominantly adult patients with no prior exposure to the infection or migrants whose last contact with the infection could be long time ago. Therefore, in European guidelines, published in 2012, the frequency of occurrence of severity criteria was differentiated from their prognostic value. For example, it was noted that hyperbilirubinemia was a criterion that occurred frequently but had little prognostic value for the patient (**Figure 6**). However, the European guidelines did not provide specific management recommendations based on these nuances of frequency and prognosis. It was defined that a patient had severe malaria if they met any severity criterion, meaning that all patients with severe malaria should be managed in ICU, despite huge differences in prognosis among cases.

Prognostic value	*Clinical manifestations and laboratory findings*	Frequency
(?) no data	**Prostration**	+++
+	**Impaired consciousness** (score <11 on the Glasgow Coma Scale)	++
+++	**Acute respiratory distress**	+
++	**Multiple seizures**	+
+++	**Circulatory collapse** (systolic blood pressure <80 mm Hg with features of peripheral circulatory failure)	+
+++	**Pulmonary oedema** (radiological)	+
++	**Abnormal bleeding** (clinically defined)	+
+	**Jaundice** (clinically defined or serum bilirubin >50 mol/L	+++
+	**Macroscopic haemoglobinuria**	+
+	**Severe anaemia** (haemoglobin <5 g/dL or haematocrit <15%)	+
+++	**Hypoglycaemia** (blood glucose concentration <2.2 mmol/L)	++
+++	**Acidosis** (pH <7.35 or plasma bicarbonate <15 mmol/L)	++
+++	**High plasma lactate** (>5 mmol/L)	++
++	**High parasitemia** (especially 2% in non-immune patients and 5% in semi-immune patients)	+
++	**Acute renal failure** (serum creatinine > 265 µmol/L and 24-hour urine output <400 mL)	+++

Figure 6. Frequency and prognostic value of severity criteria.[32]

Taking a step forward in refining patient management according to the prognostic value of severity criteria, French guidelines established the concepts of "very severe malaria" and "less severe malaria"[87], aiming to identify those patients who are more likely to require life-saving interventions and thus in a real close-to-death situation. In the same way, according to this guideline, those patients defined as “less severe malaria” could be monitored in a less intensive unit such as an infectious diseases ward (depending on local specificities).

Less severe malaria criteria from French guidelines included[87]:

- Uncomplicated confusion
- Isolated seizure

- Minor hemorrhage
- Isolated jaundice
- Isolated parasitemia > 4%
- Moderate renal failure
- Well-tolerated isolated anemia.

However, this approach has not been adopted by other non-endemic countries, resulting in considerable heterogeneity in malaria guidelines across these regions. [32,57,87]. Taking a deeper look into individual criteria and particularly for parasitemia, WHO establishes the cut-off value in 10% to consider it as a severity criterion. However, the threshold varies from 2% to 4% among European guidelines[32,57,59,87,88], and some of them differentiates between immune or non-immune patients to establish the cut-off. Also, the neurologic criteria became wider for some guidelines where confusion or any alteration in Glasgow Coma Scale could be included as a severity criterion [59], instead of Glasgow Coma Scale <11. UK malaria guidelines removed the prostration criterion in their 2016 guidelines[59], and Swiss guidelines include a duration of symptoms >3 days as a severity criterion[88].

Another important aspect to consider apart from the attributable prognostic value of criteria, is the mentioned difference between populations, as it also marks the frequency of appearance of severity criteria. As mentioned before, in non-endemic settings the majority of patients suffering from malaria are adults, and the clinical picture of an adult patient with severe malaria and a child is not the same. For children, coma, anemia and metabolic acidosis are the most frequent severe clinical presentations[89]. In contrast, adults usually do not present anemia, and instead kidney injury along with respiratory involvement can gain weight as common clinical presentations for severe malaria[39,43]. These differences are represented in **Figure 7.**

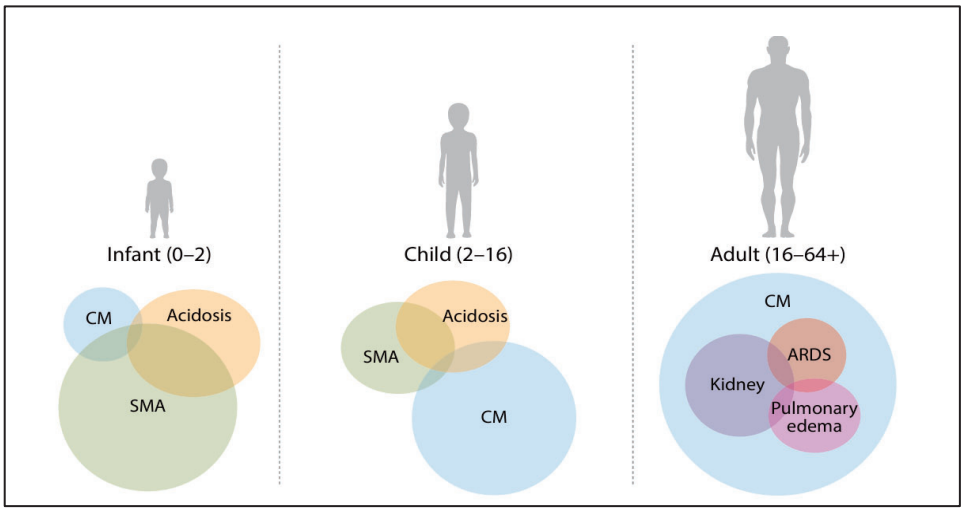


Figure 7: The circle sizes in the Venn diagrams depict the approximate relative frequency and degree of interaction between different severe malaria syndromes.[39]

As it can be seen, guidelines, as practical models for decision-making, are fundamental. Nevertheless, in clinical practice, the concern lies not only in detecting the presentations associated with SM but also in controlling the factors that may contribute to a worse outcome.

Again, it is presumable that if severe clinical pictures are different for adults and children, factors contributing to worse outcomes could differ. However, (logically) most of the evidence gathers around children in endemic areas, and the most representative study with this objective was published in 2012. This study was based on children who participated in large randomized controlled trials held in Africa, with a total sample size of 5426 participants[90]. In the study, authors described predictors of a poor outcome for SM using a multivariate logistic regression model. Interestingly, among the 20 indicators analyzed, base deficit, impaired consciousness, convulsions, elevated blood urea, and underlying chronic illness were associated independently with death. Except for chronic illness, all these indicators make part of severity criteria. Also, while for the entire cohort mortality was 9.8%, in children with acidosis and impaired consciousness, the mortality was 23%. Very similar results can be also found in other large cohorts of children in endemic countries. [91]

Conversely, looking for risk factors in imported malaria, age above 65 years was described as a risk factor for mortality with an adjusted odds ratio of 10.7 (95% CI 6.4 to 17.8) in large observational cohort of 25.054 malaria patients in UK[75]. In the same study, authors showed that there was an inverse correlation in mortality between region of presentation and number of cases seen in the region (RR=0.72, P<0.001). Most delay in fatal cases was in seeking care. These findings are also supported by a large national cohort study made in Sweden between 1995 to 2015, where risk factors associated with severe malaria were age >40 years, origin in non-endemic country, pregnancy, HIV, region of diagnosis within the country and health care delay[76]. In addition, in a multivariate analysis of SM patients, 3 variables evaluated at ICU admission showed to be independently associated to in-hospital death: older age, with OR 1.72 (95% CI 1.28-2.32, p < 0.001) per 10-year increment, Glasgow Coma Scale Score, with OR 1.32 (95%CI 1.20–1.45, p < 0.001) per 1-point decrease, and higher parasitemia, with OR 1.41 (95% CI 1.22–1.62, p < 0.001) per 5% increment. These findings highlight that, in addition to the classically described poor prognosis factors (such as deterioration of consciousness and increased parasitemia), other host-specific factors also play a significant role in determining patient outcomes, which are not currently reflected in existing guidelines. Therefore, there is a need to redefine severity criteria adapted to non-endemic areas and to optimize health resources.

In the search to improve malaria management, refining the criteria for severity is crucial. Additionally, understanding the relationship between the parasite's unique characteristics and the host's response could lead to objective, measurable markers. These markers might better correlate with organ damage, severity, and life-threatening outcomes, thus enhancing the clinician’s decision-making process.

Third challenge: Identification of severity biomarkers

Taking a deeper look at the mentioned host-parasite relationship, specific actors of different pathways could shed light on how the infection is progressing and serve as triage tools. On one side detecting the parasite biomass could have prognostic implications. Besides, if severity is understood as a disease spectrum, parasite's sequestration and endothelium activation can be the prelude of a point of no return for the host, being clinically beneficial to identify patients at that point.

Parasite's biomarkers: HRP2, pLDH and aldolase.

Based on the pathophysiology of malaria, a versatile biomarker emerges, as it has been already mentioned in the diagnostic section. HRP2 is not only an excellent diagnostic marker but can also serve as a prognostic marker. HRP2 can be measured in plasma due to its soluble component and is also found on the surface of infected red blood cells (iRBCs). Considering that the onset of the malaria virulence cascade begins with the cytoadherence of the parasite, the ability to quantify not only the parasites in peripheral blood but also those sequestered can have significant clinical utility (the so-called parasite biomass)[67,92]. In endemic areas, high HRP2 values have been widely correlated with death, cerebral malaria and severity [92–95]. However, there are only two studies that measure HRP2 in imported malaria; a retrospective study with 145 patients correlated HRP2 with disease severity[96], and a multicentric prospective study with 295 patients, where HRP2 was significantly higher in SM patients compared to UM patients (without cut-offs), and between very severe malaria patients and less severe malaria patients[97]. A significant limitation of this protein is the increasing prevalence in some geographic areas of *P. falciparum* strains that lack expression of HRP2 or its paralogue, histidine-rich protein 3 (encoded by the pfhrp2 and pfhrp3 genes). This trend poses a threat to the performance of rapid diagnostic tests and undermines the overall utility of this biomarker[14,67,98].

Glycolytic enzyme *Plasmodium* lactate dehydrogenase (pLDH) is present in all malaria species and have conserved amino acid sequences, which allows to detect individual species and/or the Plasmodium genus as a whole. It is considered less sensitive than HRP2 for diagnostic purposes[99] although to detect non-falciparum infections and has not been studied for its prognostic capabilities. Aldolase is also a glycolytic enzyme present in all Plasmodium species, playing the same role as pLDH in RDTs (be combined with HRP2 to increase sensitivity and specificity). Three retrospective studies have evaluated the relationship between the reactivity pattern of RDTs (HRP2 along with pLDH or aldolase) and parasitemia in imported malaria, which serves as a method to assess prognosis. This approach converts a qualitative result into a semi-quantitative one with prognostic implications. [100–102]

Host biomarkers: Angiopoietins, CRP, sTREM-1 and others.

As it has been recently explained, endothelium's activation is the final stage of different pathways, principally including mediators of endothelial cell function, the coagulation pathway, soluble cell surface adhesion molecules and regulators of vascular tone and permeability[103]. Among these pathways, Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are two biomarkers among the Angiopoietin/Tie-2 (Ang-Tie-2) axis (expressed primarily in endothelial cells) that merit special attention as they have been extensively studied in several infectious diseases. In steady situations, Ang-1 is inherently produced, and Ang-2 is stored in Weibel–Palade bodies for rapid release upon exposure to inflammatory stimuli[103]. As Ang-1 and Ang-2 are antagonistic ligands of the Tie-2 receptor, inflammation cascade allows the release of Ang-2 favoring its preferential bind the Tie-2 receptor. This, in turn, promotes proinflammatory and pro-thrombotic pathways, as well as microvascular leak. Therefore, angiopoietin dysregulation indicates endothelium activation and a variation of the normally low Ang-2: Ang-1 ratio, whether by decreased Ang-1, increased Ang-2, or both[104,105] .

Available data on Ang-Tie axis biomarkers suggest that they may predict strong outcomes such as death or cerebral malaria in malaria-infected children in endemic areas[95,106]. Moreover, a systematic review describes a correlation between Ang-1 and Ang-2 levels and malaria severity[107]. However, it has not been studied profoundly in imported malaria cases and presumably its performance could be altered as population differs. There is only one study on imported malaria measuring Ang-Tie axis biomarkers, which described higher levels in severe cases but lacked a cut-off point that would support clinicians in decision-making process and serve as a triage tool. [110]

Apart from biomarkers reflecting endothelium's activation, inflammatory response has also been a classic target for infectious diseases. Probably the flagship among acute phase proteins is the C-reactive protein (CRP). CRP is primarily produced in the liver, triggered by cytokines, particularly interleukin-6 (IL-6) and, while it has several applications outside the infectious diseases field, it could be a potential tool for malaria in non-endemic settings, where it has been shown to correlate with severity and as a disease-monitoring tool.[109–111]

Soluble triggering receptor expressed on myeloid cells (sTREM-1) is expressed on neutrophils and monocytes and is detected when myeloid cell activation occurs. It is hypothesized that in severe infection, an imbalance in TREM-1 signaling results occurs, leading to excess immune effector cells death and immunosuppression, resulting in poor clinical outcomes[112]. Therefore, sTREM-1 could reflect the inflammatory response triggered by *Plasmodium* spp. infection, as described in other works with pediatric patients in endemic areas[113,114]. In imported malaria, two studies measured this molecule with divergent results; in a retrospective study with 78 *P. falciparum* patients, sTREM-1 did not show significant differences when comparing uncomplicated and severe cases and had the worst performance regarding other measured biomarkers[115].In a French prospective study, SM patients showed higher sTREM-1 values compared with uncomplicated patients, in a cohort of 295 patients[97].

Following with host biomarkers, there is a laboratory value that has classically been associated with the presence of malaria: thrombocytopenia[77]. This finding is common due to several speculated mechanisms[116]: increased platelet destruction by the spleen, coagulation disturbances, bone marrow alterations, antibody-mediated platelet destruction, oxidative stress and the role of platelets as cofactors in triggering severe malaria (microvascular thrombosis and consumption of platelets). Apart from being a diagnostic marker, platelet count on admission in endemic areas has also been associated with disease severity and death[117–119]. Finally, procalcitonin has also been proposed as a biomarker for disease severity. This protein serves as a sepsis, widely implemented in high income settings. For malaria, two studies evaluated it as a marker for severity. Although in one study bacterial co-infection was excluded, information regarding this aspect was lacking in the other one. [115,120]

As a summary, imported malaria management has room for improvement. Starting with the diagnosis, universal access to gold-standard diagnostic tests is not guaranteed across the healthcare system and patients at high risk of having malaria should be promptly identified in order to achieve an early diagnosis. For this challenge, ML could be a helpful tool and we have evaluated this approach in this thesis. Once the patient is diagnosed, redefining severity criteria could help optimizing health care resources and prioritizing expert ICU management in those cases where bad prognostic factors or life-threatening clinical presentations are present.

In this thesis, we aimed to address the challenge of early diagnosis and the objective use of measurable prognostic biomarkers for patient stratification. We explored the innovation of using LFA RDT for both diagnostic and prognostic purposes. By employing a qualitative test to measure parasite biomarkers with the assistance of a smartphone, this approach has the potential to significantly enhance the management of imported malaria by providing fast and accurate results.

V.HYPOTHESIS

The assessment of new strategies for early identification and stratification of patients with malaria would optimize the management of malaria patients in non-endemic regions.

VI.OBJECTIVES

1. Develop a machine-learning-based tool to predict the risk of presenting malaria in returning travelers with fever.

2. Describe the prevalence of life-threatening conditions including deaths and life-saving interventions, as well as the prevalence of co-infections in patients with imported malaria.

3. Evaluate a modified classification of severe malaria for non-endemic regions, to identify patients at higher risk of developing life-threatening conditions.

4. Identify the predictive factors associated with organ failure and death in patients with malaria in a non-endemic region.

5. Identify host biomarkers associated with severity and organ failure in patients with imported malaria.

6. Identify parasite biomarkers associated with severity in patients with imported malaria.

7. Evaluate the capacity of a conventional Histidin-Rich-Protein-2 and Lactate dehydrogenase lateral flow assay to identify patients with severe imported malaria.

VII.MATHERIAL AND METHODS, AND RESULTS

ARTICLE 1

MALrisk: a machine-learning–based tool to predict imported malaria in returned travellers with fever

Journal of Travel Medicine, 2024, taae054
https://doi.org/10.1093/jtm/taae054
Original Article

Original Article

MALrisk: a machine-learning–based tool to predict imported malaria in returned travellers with fever

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Submitted 19 December 2023; Revised 13 March 2024; Editorial Decision 26 March 2024; Accepted 3 April 2024

Abstract

Background: Early diagnosis is key to reducing the morbi-mortality associated with *P. falciparum* malaria among international travellers. However, access to microbiological tests can be challenging for some healthcare settings. Artificial Intelligence could improve the management of febrile travellers.
Methods: Data from a multicentric prospective study of febrile travellers were obtained to build a machine-learning model to predict malaria cases among travellers presenting with fever. Demographic characteristics, clinical and laboratory variables were leveraged as features. Eleven machine-learning classification models were evaluated by 50-fold cross-validation in a Training set. Then, the model with the best performance, defined by the Area Under the Curve (AUC), was chosen for parameter optimization and evaluation in the Test set. Finally, a reduced model was elaborated with those features that contributed most to the model.
Results: Out of 11 machine-learning models, XGBoost presented the best performance (mean AUC of 0.98 and a mean F1 score of 0.78). A reduced model (MALrisk) was developed using only six features: Africa as a travel destination, platelet count, rash, respiratory symptoms, hyperbilirubinemia and chemoprophylaxis intake. MALrisk predicted malaria cases with 100% (95%CI 96–100) sensitivity and 72% (95%CI 68–75) specificity.
Conclusions: The MALrisk can aid in the timely identification of malaria in non-endemic settings, allowing the initiation of empiric antimalarials and reinforcing the need for urgent transfer in healthcare facilities with no access to malaria diagnostic tests. This resource could be easily scalable to a digital application and could reduce the morbidity associated with late diagnosis.

Key words: Malaria, travellers, machine-learning

Background

Unravelling the aetiology of febrile illnesses after international travel is challenging: practitioners in non-endemic areas should consider cosmopolitan pathogens and microorganisms of imported origin, which are less familiar to them.[1,2] Besides, there is frequently a clinical overlap between life-threatening diseases and self-limited or benign conditions, which hinders the management of these patients.[3]

Plasmodium falciparum malaria is one of the deadliest infection among travellers and migrants presenting with acute undifferentiated febrile illnesses.[3,4] The introduction of artesunate in endemic regions has led to an improved management of severe malaria and a significant reduction in mortality rates.[5] However, despite the availability and introduction of artesunate, the mortality attributable to severe imported cases still remains unacceptably high, up to 5%.[6,7] Hence, early diagnosis is the cornerstone to minimize morbidity and mortality associated with this infection.

The gold standard to diagnose *Plasmodium* infection is the thick and thin blood smear. Ideally, the time since sample

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collection to result should not exceed 4 hours according to national and international guidelines in non-endemic countries.[8–10] Notwithstanding, this technique requires the presence of an expert microbiologist, usually located in tertiary centres. Rapid diagnostic tests based on antigen detection are an alternative for *Plasmodium* detection when the laboratory capacities are not feasible, but its availability is not generalized in non-endemic regions. Thus, access to microbiological tests for malaria diagnosis is demanding for some clinicians, who have to wait an unacceptable time for laboratory results or are forced to transfer the patient elsewhere with an undiagnosed febrile illness.

To overcome the diagnostic difficulties, several studies identified the most relevant clinical and analytical characteristics associated with malaria, and Taylor SM et al. systematically reviewed them,[11] concluding that among travellers, the presence of splenomegaly, jaundice or pallor were clinical findings significantly increasing the risk of malaria, whereas thrombocytopenia and hyperbilirubinemia were the laboratory values demonstrating the highest likelihood ratios. Moreover, different scoring systems were proposed for endemic areas, but no predictive score was suggested for non-endemic settings.

Machine learning (ML) is a branch of artificial intelligence, and unlike traditional programming methods, ML algorithms are designed to autonomously discern patterns and rules from data.[12,13] ML models have gained widespread recognition and acceptance in various domains, including healthcare, demonstrating to be valuable resources for the unmet diagnostic needs in infectious diseases,[14] as well as in chronic conditions.[15] Particularly in malaria, efforts have mainly been directed at improving laboratory diagnosis,[16,17] but the potential usefulness of ML in predicting imported malaria has not been explored.

Common ML types include supervised and unsupervised learning models (e.g. clustering algorithms).[18] Apart from logistic regression, which uses a linear approach, ML includes models enabling the interpretation of non-linear relationships (tree-based models), along with other models that assume an underlying probabilistic model.[19,20] The broader approach of ML, which incorporates models that employ various pathways, might improve the predictive capacity of conventional methods.

Our goal was to develop an ML-based predictive model designed to serve as a clinical decision support system, in order to supply a probability assessment for suspected malaria in patients exhibiting fever following international travel. Its purpose is to offer guidance to healthcare professionals working in settings where rapid malaria diagnostics are not readily available.

Methods

Study site and data

This study was conducted as an ancillary study within a prospective multicentre cohort study of international returning travellers or recently arrived migrants with fever, that enrolled patients from November 2017 to November 2019.[21] The participants were recruited in three referral centres: Hospital Clinic of Barcelona/Barcelona Institute for Global Health, Spain, Tropical Medicine in Antwerp, Belgium and Primary Care and Public Health, University of Lausanne, Switzerland. Patients above 18 years presenting with axillary temperature $\geq$37.5°C (or equivalent symptoms of fever) were eligible to participate. After obtaining informed consent on the day of inclusion, a predefined clinical algorithm was applied with defined diagnostic procedures, published elsewhere.[21] Briefly, a blood smear was performed in all patients returning from malaria endemic areas. In patients with acute fever with no focal signs, once malaria was ruled out, targeted polymerase chain reaction (PCR) test and paired specific antibody tests against dengue, chikungunya and Zika viruses were performed in all patients. Serologies and targeted PCRs against Leptospira spp. and Rickettsia spp., blood cultures, HIV tests, as well as other microbiological test were performed according to the clinician's suspicion. Diagnosis of *Plasmodium* infection was defined by a positive microscopy of stained thick and thin blood smear. In febrile patients with previous antimalarial intake and a negative blood smear, diagnosis of malaria was also assumed if rapid diagnostic antigen test (Bioline™ Malaria Ag P.f/pan) or targeted PCR resulted positive, in absence of alternative diagnosis. For the non-malarial diagnoses, microbiologically confirmed cases were collected. These consisted of arboviral diseases and other viral diagnoses, bacterial and fungal infections. Also, specific syndromic diagnoses were included: travellers' diarrhoea, respiratory infections, skin and soft tissue infections and urinary tract infections. Finally, the group of undiagnosed non-malarial fevers was integrated as well.

A total of 764 patients with fever were included in the study period and used as dataset. For the design of the model, 70 features of the participants were used, including demographics, previous medical conditions, travel history and exposures, as well as symptoms, physical examination and laboratory data. The complete list of features used to build the model is shown in Supplementary Table 1.

Pearson χ^2 test or Fisher's exact test was used to compare categorical variables between groups, and for quantitative variables with non-normal distribution Mann–Whitney U test or Kruskal–Wallis tests were applied.

Model development

We employed an ML approach to develop a predictive model for malaria in patients with imported fever. Missing data were completed by multiple imputation without including the malaria infection variable. In the supervised learning workflow, all models undergo validation using a portion of the data known as the training set, typically comprising 80% of the dataset. Subsequently, they are evaluated on the test set, which constitutes the remaining 20% of the data. The model exhibiting the highest performance is selected and further optimized to enhance accuracy. The Training set was used to train 11 machine-learning classification models: Support vector machines (SVM), Radial Basis Function SVM (RBF-SVM), Gaussian Process Classifier (GPC), Decision Tree (DT), Random Forest (RF), Multi-layer Perceptron classifier (MLPC), AdaBoost (AB), Gaussian Naive Bayes classifier (GNBC), Quadratic Discriminant Analysis (QDA), XGBoost (XGB) and Logistic Regression (LR) whose performance was compared by 50-fold cross-validation using the area under the ROC curve (AUC) and F1 score (F1) as the scoring metric. In each

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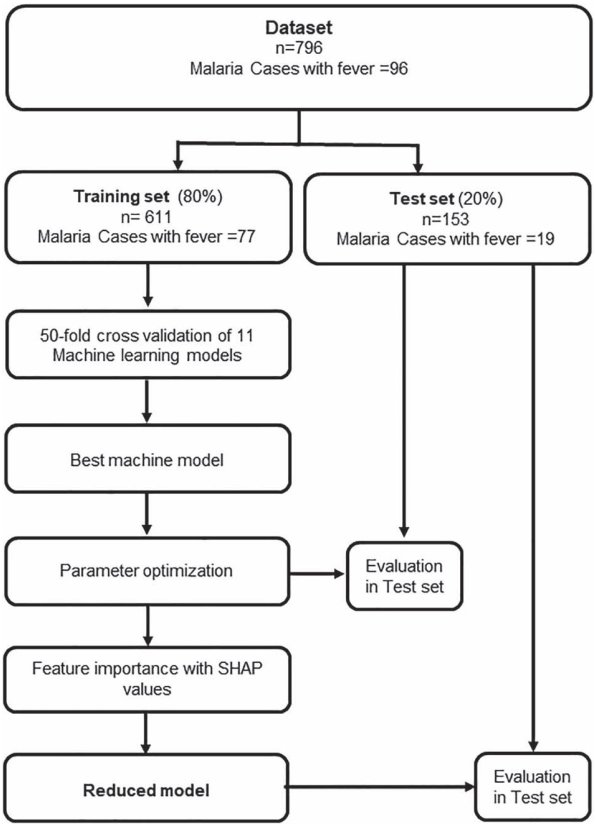

Figure 1 Model building

validation fold, 70% of training was used to train the model and 30% of the Training set was used to evaluate it. For the cross-validation procedure, we used all the variables and the default parameters of each model according to the scikit-learn Python library.[22] Figure 1 shows a visual representation of model's construction. For each model, the mean and standard deviation of AUC and F1 in the cross-validation were determined. The model with the best AUC and F1 values was chosen for parameter optimization.

The features were ranked according to their predictive contribution to the model by SHAP values,[23,24] taking into account that some features increased the likelihood of malaria and others decreased it. Those that contributed most to the model were selected to generate a reduced model, called MALrisk. As the classification model was designed to serve as a screening tool, a probability threshold that achieved high sensitivity was prioritized. Finally, a description of misclassified cases was done to assess its distribution between different aetiologies and the potential clinical impact. In this subgroup of misclassified patients, arboviral diseases were comprehensively analysed due to their high prevalence among undifferentiated febrile illnesses. The machine learning model was developed out with the scikit-learn Python library.[22]

Ethics

The study was approved by the Institutional Review Board and the Ethics Committee of the Hospital Clinic of Barcelona (HCB/2017/0612), the Institutional Review Board of the Institute of Tropical Medicine and the Ethics Committee of the University Hospital in Antwerp (ITG 1235/18) and the Ethics Committee of the canton of Vaud of Switzerland (CER-VD2018–00672). Written informed consent was obtained from all study participants. The study was designed in compliance with Good Clinical Practice and following the Declaration of Helsinki.

Results

Dataset

Regarding the 764 patients included in the cohort, the median age was 36 years (IQR: 28–47) and 405 (53.0%) were men. The main reason for travelling was visiting friends and family, in 133 (17.4%) patients. A total of 96 (12.6%) patients were diagnosed with malaria, of whom 32 (33.3%) had severe malaria, with no fatalities. Concerning to diagnostic methods, in all malaria cases a blood smear was performed. In 8/96 patients the blood smear was negative at presentation. Among them, 6/8 had a positive PCR and 2/8 had a positive RDT. Both patients were repatriated after initiating anti-malarial treatment and had <100.000 10^9/L platelet count and no alternative diagnosis. The remaining 668 (87.4%) patients had fever with a non-malarial diagnosis. The most common diagnoses in this last group were traveller's diarrhoea, respiratory infections and arboviral diseases, with 160 (20.9%), 154 (20.2%) and 85 (11.1%) participants, respectively. Besides, 155 (23.2%) patients remained with an undiagnosed condition (after excluding malaria) at the end of the diagnosis work-up. Relevant demographic and clinical characteristics of patients with malaria as well as non-malaria cases are summarized in Table 1. Regarding malaria cases, 31 (32.2%) had a previous medical condition, and among them, 10 (32.6%) were immunosuppressed. With regard to the visited continent, 94 (97.9%) came from Africa. Apart from having fever, which was an inclusion criterion for the study, clinical presentation was characterized by headache in 75 (78.1%) cases, followed by gastrointestinal symptoms in 57 (59.4%) and myalgia in 45 (46.9%) participants. These three nonspecific symptoms were also present in patients without malaria, accounting for 439 (65.7%), 403 (60.3%) and 361 (54.0%), respectively.

Predictive model: MALrisk

After assessing all 11 models, the XGBoost model had the best performance in the cross-validation, with a mean AUC of 0.98 and a mean F1 of 0.78 in the Training set (Table 2). After parameters' optimization, we obtained a XGBoost model with 70 features and an AUC of 0.92 in the Test set (Figure 2a). To enable the clinical application of the model, we selected the most contributing features to create a reduced model (MALrisk). The model is based on six features: Africa as a travel destination, platelet value, presence or absence of rash, presence or absence of respiratory symptoms, bilirubin value and chemoprophylaxis intake. Regarding its performance, MALrisk showed a high-predictive capacity, with an AUC of 0.95 in Test set evaluation (Figure 2a). Within the model, the contribution of each feature in the MALrisk was calculated with the SHAP values, and were

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Table 1 Baseline characteristics of patients with malaria and non-malarial fever

	Non-malarial fevers ($n = 668$)	Malaria ($n = 96$)	*P* value
Age Median (IQR)	35.5 (28–47)	40.5 (31–50.5)	0.006
Men, *n* (%)	334(50.0)	71 (74.0)	<0.001
Previous medical condition, *n* (%)	191 (28.6)	31 (32.3)	0.450
Travel destination:			<0.001
Africa, *n* (%)	241(36.1)	94 (97.9)	
Asia, *n* (%)	257(38.5)	0	
America, *n* (%)	152(22.8)	1 (1.0)	
Oceania, *n* (%)	6(0.9)	0	
Europe, *n* (%)	12(1.8)	1(1.0)*	
Travel reason			
Tourism, *n* (%)	429 (64.1)	9 (9.4)	<0.001
Business, *n* (%)	81 (12.1)	9 (9.4)	0.437
Cooperation, *n* (%)	70(10.5)	20 (20.8)	0.003
VFR and recent arrived migrants *n* (%)	86 (12.9)	57 (59.4)	<0.001
Antimalarial chemoprophylaxis *n* (%)	61 (9,1)	1 (1.04)	0.007
SYMPTOMS & SIGNS			
Seizures, *n* (%)	15 (2.3)	2 (2.1)	0.920
Headache, *n* (%)	439 (65.7)	75 (78.1)	0.015
Retro-orbital pain, *n* (%)	147 (22.0)	9 (9.4)	0.004
Myalgia, *n* (%)	361 (54.0)	45 (46.9)	0.188
Arthralgia, *n* (%)	216 (32.3)	35 (36.5)	0.421
Sore throat, *n* (%)	176 (26.3)	10 (10.4)	0.001
Rhinorrhoea, *n* (%)	140 (21.0)	5 (5.2)	<0.001
Ear pain, *n* (%)	32 (4.8)	0	0.028
Respiratory symptoms, *n* (%)**	340 (50.9)	23 (24.0)	<0.001
Gastrointestinal symptoms, *n* (%)**	403 (60.3)	57 (59.4)	0.858
Hypotension, *n* (%)	28 (4.2)	12 (12.5)	0.001
Tachycardia (>100 bpm), *n* (%)	80 (12.0)	30 (31.3)	<0.001
Tachypnoea (>25 bpm), *n* (%)	32 (4.8)	13 (13.5)	0.001
Glasgow coma scale alteration, *n* (%)	4 (0.60)	4 (4.2)	0.001
Haemorrhagic sign, *n* (%)	6 (1.0)	0	0.351
Conjunctival suffusion/Conjunctivitis, *n* (%)	34 (5.09)	1 (1.0)	0.076
Jaundice, *n* (%)	7 (1.1)	16 (16.7)	<0.001
Crackles, *n* (%)	18 (2.7)	4 (4.2)	0.420
Hepatomegaly, *n* (%)	22 (3.3)	12 (12.5)	<0.001
Splenomegaly, *n* (%)	7 (1.1)	6 (6.3)	<0.001
Rash, *n* (%)	163 (24.4)	3 (3.1)	<0.001
Tache noire, *n* (%)	19 2.84	0	0.094
LABORATORY PARAMETERS			
Neutrophils 10^9/L***	4.0 (2.5–5.9)	3.2 (2.5–4.3)	0.001
Lymphocytes 10^9/L	1.3 (0.8–1.7)	0.9 (0.5–1.2)	<0.001
Platelet count 10^9/L****	214.5 (172.0–270.0)	97.5 (58.0–163.0)	<0.001
Haemoglobin, g/dL***	141 (131–150)	132 (118–145)	<0.001
Creatinine, mg/dL***	0.84(0.71–0.99)	0.99 (0.8–1.16)	<0.001
ASAT/GOT, U/L***	26 (20–40)	37.5 (28–71)	<0.001
LDH, U/L	201.5 (172–251)	308 (230–457)	<0.001
Bilirubin, mg/dL	0.5 (0.4–0.7)	1.40 (0.76–2.1)	<0.001
C-reactive protein, mg/dL	2.26 (0.6–6.09)	9.1 (3.5–16.1)	<0.001

*Heart transplanted patient, did not travel. **Respiratory symptoms include cough, dyspnoea and pleural effusion. Gastrointestinal symptoms include nausea, vomiting, diarrhoea and abdominal pain. ***Statistically significant, without clinical value. ****Thrombocytopenia was defined as a platelet value lower than $150x10^9$/L.

as follows: Africa was the feature with the highest contribution (41%), followed by the quantitative platelet value (26%), rash (10%), respiratory symptoms (9%) and bilirubin values above 1.2 mg/dL (9%). The feature that contributed least to the model was the correct chemoprophylaxis intake (5%). The presence of rash and respiratory symptoms, as well as the chemoprophylaxis intake reduced the risk of malaria. Coming from Africa and high-bilirubin value increased the risk of having malaria. For platelets, normal value decreased the risk whereas thrombocytopenia increased the risk of having malaria (Figure 3) Based on

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Table 2 Performance of machine learning models in 50-folds cross validation in the Training set

Model	Mean AUC in cross-validation	SD AUC in cross- validation	Mean F1 in cross-validation	SD F1 in cross-validation
XGBoost (XGB)	0.98	0.01	0.78	0.06
Logistic Regression (LR)	0.98	0.01	0.77	0.05
Support vector machines (SVM)	0.97	0.01	0.74	0.05
AdaBoost (AB)	0.93	0.02	0.74	0.05
Gaussian Naïve Bayes Classifier (GNBC)	0.92	0.03	0.66	0.07
Random Forest (RF)	0.91	0.03	0.09	0.16
MLPC	0.86	0.08	0.57	0.1
Decision Tree (DT)	0.84	0.04	0.63	0.07
Quadratic Discriminant Analysis (QDA)	0.66	0.06	0.04	0.05
Radial Basis Function SVM (RBF-SVM)	0.5	0.01	0.1	0
Gaussian Process Classifier (GPC)	0.5	0.01	0.14	0.08

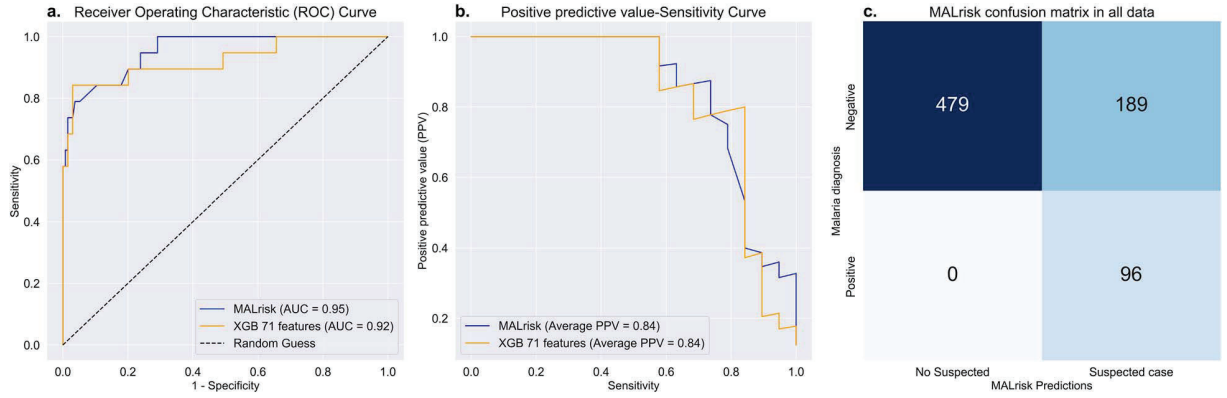

Figure 2 Performance of XGBoost models with 70 features and MALrisk. a. AUC in the Test set. b. Positive predictive value- Sensitivity curve. c. Confusion matrix of MALrisk applied to the whole data set

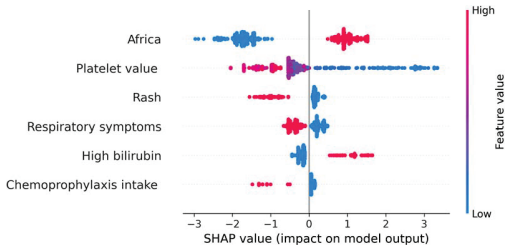

Figure 3 MALrisk SHAP values. Each point represents a patient. For quantitative features, high values are represented in red and low values in blue. For dichotomic features, affirmative answers are represented in red and negative answers in blue. The corresponding SHAP value is observed on the x-axis

AUC of Figure 2a, a cut-off value that showed a sensitivity of 100% (95% CI 96–100) with a specificity of 72% (95% CI 68–75) was obtained. Consequently, MALrisk labelled as suspected malaria cases all severe and non-complicated malaria cases, as well as all *P. falciparum* and non-falciparum malaria infections.

MALrisk model showed a low range of error among the main diagnoses for the non-malarial fevers. Overall, a total of 189 (28%) of non-malarial fevers were incorrectly classified as malaria cases (Figure 2c). All misclassified patients had at least one of the following conditions, regardless of their final diagnosis: 162 of 189 (85.7%) came from African countries and 108 (57.1%) presented with thrombocytopenia at first evaluation. Furthermore, 81 (42.9%) patients shared both conditions. Table 3 details the non-malarial fever diagnoses and the incorrectly classified patients in each category. Regarding patients with diarrhoea, 43 of 160 (27%) were misclassified, as well as 38 of 154 (25%) patients with respiratory infections. Regarding arboviruses, MALrisk incorrectly classified as malaria cases, 13 of the 77 (17%) dengue cases. As for the dengue cases, it is important to note that individuals incorrectly classified as suspected malaria cases had a significantly lower median platelet count compared to those correctly classified ($66x10^9$/L *vs.* $83x10^9$/L, $P = 0.045$). Among bacterial infections with no focal signs, the MALrisk incorrectly classified 20 of the 57 (35%) *Rickettsia* spp. infections, seven (41%) of the 17 (41%) *Coxiella* spp., as well as two of the 13 (15%) Leptospirosis cases. Moreover, 50 of the 155 (32.3%) undiagnosed non-malarial fevers were misclassified.

Discussion

MALrisk is a clinical decision support system to promptly suspect malaria in patients with imported fever. With only six variables that are easy to obtain through medical history (travel

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Table 3 Causes of fever in non-malarial fevers and percentage of incorrectly classified cases as suspected malaria

Cause of fever	Incorrectly classified as a suspected malaria case*
BACTERIAL INFECTIONS with no focal signs	
Rickettsia spp. ($n = 57$)	20 (35%)
Coxiella spp. ($n = 17$)	7 (41%)
Bartonella spp. ($n = 15$)	2 (13%)
Leptospirosis ($n = 13$)	2 (15%)
Anaplasma spp. ($n = 10$)	1 (10%)
Typhoid fever ($n = 6$)	N/A
Non-typhoid salmonella bacteraemia ($n = 4$)	N/A
Orientia spp. ($n = 3$)	N/A
Melioidosis ($n = 1$)	N/A
Syphilis ($n = 5$)	N/A
Lyme ($n = 1$)	N/A
ARBOVIRUS**	
Dengue ($n = 77$)	13 (17%)
Chikungunya ($n = 7$)	N/A
Zika ($n = 2$)	N/A
OTHER VIRAL DISEASES	
New HIV diagnosis ($n = 4$)	N/A
CMV ($n = 3$)	N/A
Hepatitis A ($n = 2$)	N/A
Hantavirus ($n = 1$)	N/A
OTHER INFECTIONS	
Histoplasma ($n = 3$)	N/A
Amebiasis ($n = 4$)	N/A
Mycobacteria ($n = 3$)	N/A
Aseptic meningitis ($n = 2$)	N/A
Katayama syndrome ($n = 1$)	N/A
SYNDROMIC DIAGNOSIS	
Traveller's diarrhoea ($n = 160$)	43 (27%)
Respiratory tract infection ($n = 154$)	38 (25%)
Skin and soft tissue infection ($n = 22$)	7 (32%)
Urinary tract infection ($n = 18$)	6 (33%)
UNDIAGNOSED NON-MALARIAL FEVERS ($n = 155$)	50 (32.3%)

All diagnoses were microbiologically confirmed, except from syndromic diagnosis. Among traveller's diarrhoea, 145(90.6%) had a positive stool test. *Diagnoses with $n < 10$ patients were excluded from the statistical analysis. **There was one co-infection among arboviral infections.

destination, chemoprophylaxis intake, presence of rash and respiratory symptoms) and basic laboratory results (platelet count and bilirubin level), MALrisk achieved 100% sensitivity and 72% specificity in the Test set. This ML tool aims to offer guidance to clinicians working in healthcare settings without rapid access to malaria diagnostic tests. In the evaluated population, all malaria cases, regardless malaria species or severity, were correctly classified by MALrisk.

Apart from the study of Taylor SM et al., other studies have also described afterwards the most frequent variables associated with imported malaria.[3,11,21] Consistently with previous reports, the MALrisk included epidemiological variables such as returning from Africa and chemoprophylaxis intake, while symptoms classically associated with malaria such as abdominal pain, vomiting, headache or myalgia did not show enough discriminatory power (as they are also common symptoms in other infections). In addition to hyperbilirubinemia, thrombocytopenia is the main laboratory value associated with malaria in literature, and a normal platelet count has a high-negative predictive value.[3,25] For MALrisk, platelet value was the second most important feature for risk prediction. On the other hand, arboviruses and respiratory infections were two of the main diagnoses in travellers with non-malarial fevers. Not surprisingly, features, such as rash (classically associated with arboviral infections) and respiratory symptoms, were negatively associated with malaria.[26]

Identifying characteristics that increase diagnostic probabilities of malaria is a fundamental and necessary step when assessing a patient with fever. One effective way to incorporate these variables into clinical practice is through algorithms or score systems. Although such systems have been proposed for malaria in endemic areas or for arbovirus infections in non-endemic regions,[11,26] there is currently a lack of scores specifically tailored for travel-related malaria. At the same time, ML involves an interesting step forward in terms of considering clinical variables of predictive value. ML contributes to the development of these scores by providing a comparable level of accuracy while offering adaptability. This adaptability stems from the model's capacity to be trained with diverse populations, enabling it to handle large sets of variables and demographics, while maintaining an easy-to-use approach for the MALrisk application by a clinician: A digital application that asks 5 'yes or no' questions and needs a number to be registered for the platelet value.

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On the other hand, ML has been mainly applied to optimize the blood smear interpretation.[17] Only two studies have addressed malaria diagnosis with a ML-based tool: One of these studies extracts information from PubMed case reports and abstracts, but its practical approach needs to be defined.[27] The other study was not developed for the prediction of imported malaria and uses laboratory parameters (platelet values, haemoglobin and leucocytes) to predict malaria in endemic settings.[28] To the best of our knowledge to date, this is the first predictive model using both clinical and laboratory features, designed for imported malaria diagnosis.

Despite the wax and wanes of malaria incidence observed in endemic countries during the last years, most countries are geared towards malaria control and eradication.[29] Notwithstanding, imported malaria cases barely have changed in the past, and during pandemics, a higher risk for severe malaria was described due to delay in diagnosis.[30] Also, COVID-19's irruption has changed the diagnostic probabilities of acute fever, and clinicians should be again prepared to evaluate febrile patients after international travel. In this sense, self-limited viral illnesses may be indistinguishable from malaria, which could lead to underestimate the odds of severe outcomes. At the same time, the key to reducing morbidity and mortality from imported malaria is early diagnosis. Since MALrisk identifies patients with high risk of having malaria, the result should encourage clinicians to start an urgent package of measures, which may range from transferring patients to a referral centre where the workup can be completed to initiating empirical treatment while awaiting results. Albeit the use of empirical antibiotic therapy is widely implemented, the prescription of empirical antimalarials is not widespread, despite the safety of antimalarials have been demonstrated over time.[31] Therefore, MALrisk could support clinicians to start an urgent treatment, if diagnostic tests are unavailable within a few hours or if the clinical severity of the patient requires it.

On the other hand, the model misclassified 28% of non-malarial febrile patients as suspected malaria cases. This implies that, in less than one-third of patients, the model recommends urgent testing and supports a decision to start an empirical treatment. To understand this outcome, it is important to point out that for MALrisk, platelet value and coming from an African country were the features that contributed most to the model, and this is the reason why in the incorrectly classified cases these two features were often present. However, we believe that emphasizing the need to quickly rule out malaria in febrile travellers returning from Africa or presenting with thrombocytopenia, is still key in clinical practice, as the main problem for malaria in non-endemic countries is delayed diagnosis.

However, this study presents several limitations. Further studies allowing robust external validation in an independent cohort with large populations would be necessary to evaluate the performance of MALrisk, especially in different populations, presenting not only with fever but a wider variety of symptoms (e.g. diarrhoea or joint pain only). This fact could also alter the contribution of the selected features to the model, making hypothetically Africa as a travel destination and platelet value less decisive. However, if these steps could be executed, the model could be trained to upgrade the performance and could be easily integrated into computers' software or into a digital application. Finally, studies evaluating the feasibility should be performed to make possible a real and practical impact in different healthcare settings.

To summarize, the MALrisk is a promising tool to promptly identify suspected malaria cases in patients with imported fever in all clinical settings, allowing the initiation of empiric antimalarials and reinforcing the need for urgent transfer. This resource could be easily scalable to a digital application and could help clinicians in the decision-making process of the patient. Finally, this measure could reduce the morbi-mortality associated with this infection, pending universal access to microbiological tests.

Supplementary data

Supplementary data are available at *JTM* online.

Funding

The ISGlobal authors acknowledge support from the Spanish Ministry of Science and Innovation and State Research Agency through the 'Centro de Excelencia Severo Ochoa 2019–2023' Program (CEX2018–000806-S), the CIBER-Consorcio Centro de Investigación Biomédica en Red-(CB 2021) and the Generalitat de Catalunya through the CERCA Program and AGAUR program (2021 SGR 01558).

Author Contributions

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Conflict of interest: None declared.

Data and code availability

The study deidentified participant data and data dictionary will be made available upon request to the corresponding author after approval of a proposal and signed data access agreement.

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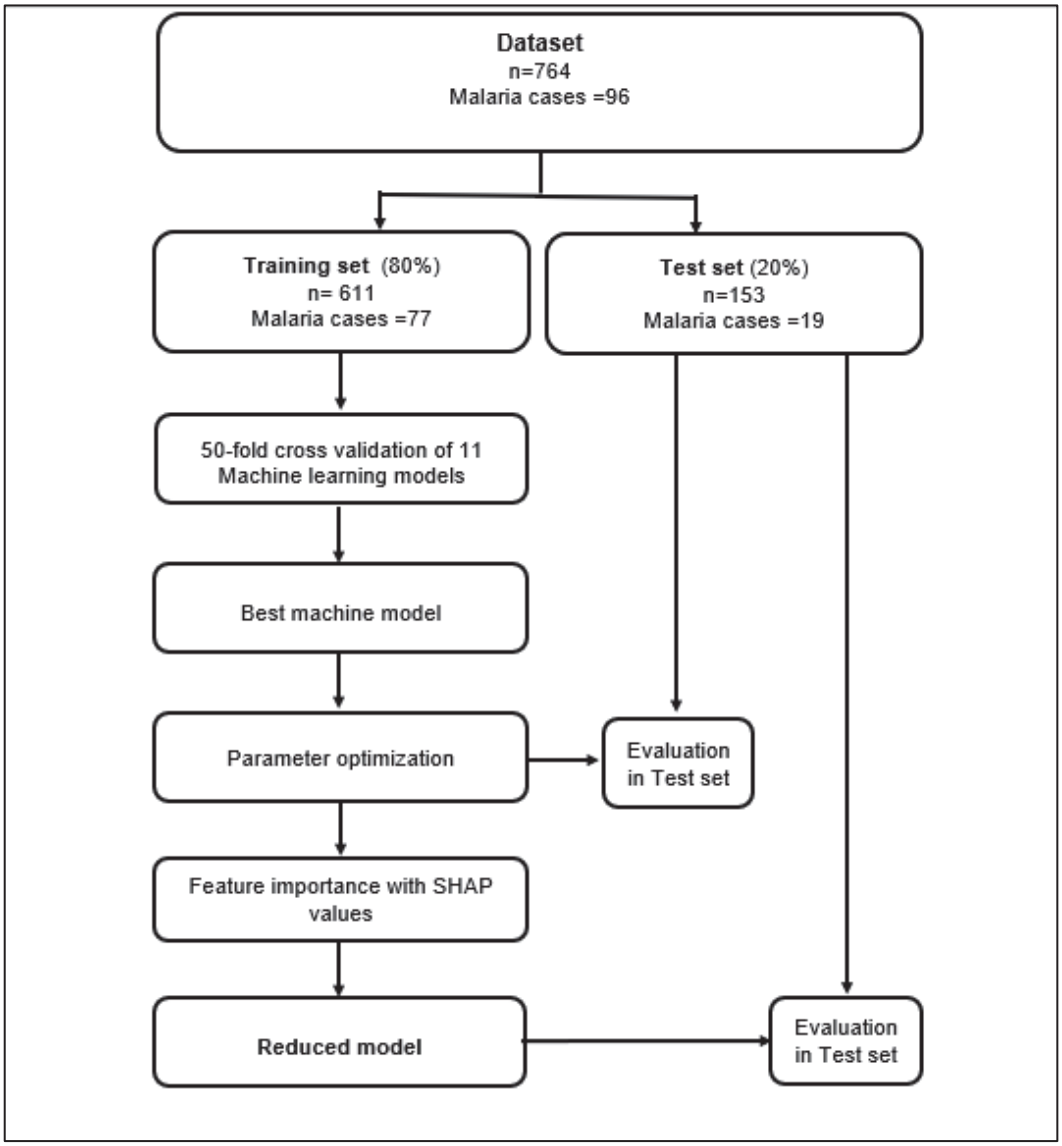
SUPPLEMENTARY TABLES

Supplementary table 1. List of all features used to build the model.

DEMOGRAPHIC AND TRAVEL RELATED VARIABLES	SYMPTOMS AND SIGNS	PHYSICAL EXAMINATION	LABORATORY VALUES***
Age	Duration of fever before consultation	Fever	Leucocyte count
Sex	Minimum incubation period	Hypotension	Neutrophil count
Born in a non-endemic country	Neurologic impairment	Tachycardia	Lymphocyte count
Visiting Friends and Relatives	Chills	Tachypnea	Eosinophil count
Previous malaria episode	Seizures	Glasgow coma scale alteration	Platelet count
Correct chemoprophylaxis intake	Headache	Coma	Hemoglobin
Travel destination Asia	Retro-orbital pain	Hemorrhagic signs	Creatinine
Travel destination Africa	Myalgia	Conjunctivitis	ASAT
Travel destination America	Arthralgia	Abscess	ALAT
Travel destination Europe	Sore throat*	Jaundice	GGT
Travel destination Oceania	Rhinorrhea*	Crackles	Alkaline phosphatase
Number of countries visited/stayed	Ear pain	Heart murmur	LDH
Travel duration (days)	Cough*	Abdominal tenderness	Bilirrubin
Contact with rural area	Pleuritic pain*	Painful hepatic percussion	C-reactive protein
Contact with animals	Dyspnea*	Hepatomegaly	Glucose
	Nausea**	Splenomegaly	
	Vomiting**	Lymphadenopathies	
	Abdominal pain**	Rash	
	Diarrhea**	Tache noire	

* Respiratory symptoms were grouped together to create a new feature** Abdominal symptoms were were grouped together to create a new feature.*** Laboratory values were quantitative variables, the rest of variables were categorical variables.

Supplementary figure 1. Model building.



ARTICLE 2

Not all severe malaria cases are severe: Is it time to redefine severity criteria for malaria in non-endemic regions?

Travel Medicine and Infectious Disease 60 (2024) 102740

Contents lists available at ScienceDirect

Travel Medicine and Infectious Disease

journal homepage: www.elsevier.com/locate/tmaid

Not all severe malaria cases are severe: Is it time to redefine severity criteria for malaria in non-endemic regions?

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ARTICLE INFO

Keywords:
Severe malaria
Imported malaria
Travel medicine

ABSTRACT

Background: The current definition of severe malaria in non-endemic areas follows WHO criteria, which mainly target children in malaria-endemic areas, potentially misclassifying cases in non-endemic regions. We assessed the performance of a modified severe malaria classification criteria within our patient cohort.
Methods: A cohort study of patients managed for malaria in a non-endemic setting (2005–2023) was analyzed. We classified patients into severe malaria (SM) using WHO 2013 criteria except for hyperparasitemia, where 2 % threshold was applied. Patients with SM were distinguished as very severe malaria (VSM) when presenting at least one of the following conditions: parasitemia >10 %, pulmonary edema, impaired consciousness, seizures, renal failure, metabolic acidosis or hyperlactatemia, shock or hypoglycemia. In patients with SM and no criteria for VSM, less severe malaria (LSM) was defined by: 2–10 % parasitemia, hyperbilirubinemia, prostration, anemia or minor bleeding. The primary composite outcome was death or the need for a life-saving intervention, as analyzed in the three comparative groups. Secondary outcome was the prevalence of co-infections.
Results: Among 506 patients with malaria, 176 (34.8 %) presented with SM. A total of 37 (7.3 %) patients developed a life-threatening condition, namely death (n = 4) and/or the need for life-saving interventions (n = 34). All fatalities and 33 out of the 34 life-saving interventions occurred in the VSM group. Patients in LSM group did not develop any life-threatening conditions. As to co-infections, 28 (5.5 %) patients had a community-acquired co-infection, with no differences between groups (p = 0.763).
Conclusions: Severity criteria definitions would benefit from a review when assessing patients with malaria in non-endemic areas. Within the spectrum of SM, patients reclassified as LSM have a low risk of developing a life-threatening condition and present low co-infection incidence and could benefit from management out of intensive care units and a restrictive use of empirical antibiotics.

1. Introduction

Despite the advances in control strategies, the global picture of malaria by the end of 2022 included an estimated 249 million malaria cases (an increase of 5 million cases compared with 2021) and 608.000 deaths (fewer cases than the previous year but still more cases than in 2019) [1]. Apart from the enormous public health impact that represents malaria in endemic regions, its incidence in non-endemic regions remains unchanged or has increased in many European countries [2–4]. Besides, the arrival of the COVID-19 pandemic raised the alarming

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https://doi.org/10.1016/j.tmaid.2024.102740
Received 9 May 2024; Received in revised form 14 June 2024; Accepted 16 June 2024
Available online 14 July 2024
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concern of an increase in severe malaria cases [5,6]. Although large cohorts of imported severe malaria cases in non-endemic regions are scarce, some studies before the artesunate era reported mortality of severe cases of 10.5 % [7]. The introduction of artesunate shifted the paradigm in the management of malaria, but despite its widespread use, mortality of imported cases remains unacceptably high, up to 5 % [8,9].

In an attempt to identify patients at higher risk of developing severe disease and enhance the management, WHO defined clinical, analytical and parasitological criteria to establish a severity classification. Since 1995 and in its last meeting in 2013, WHO aimed to prioritize sensitivity in recognizing potentially severe malaria cases and thus, inclusiveness of severity criteria was given precedence [10]. However, the contrasting needs for more specific criteria were also highlighted [11].

The main target of this criteria were children from malaria endemic areas, the most vulnerable population for severe malaria (SM). WHO severity criteria were therefore adopted for malaria guidelines in non-endemic regions, although some patients' key characteristics such as age and previous malaria immunity may differ between patients in endemic and non-endemic areas. Being aware of these potential differences, heterogeneity between malaria guidelines can be found across non-endemic countries [7,12,13]. The differences between guidelines rely on the efforts made towards a selection of those criteria that could make a difference in patient's prognosis and management. Particularly for parasitemia, WHO establishes the cut-off value in 10 % to consider it as a severity criterion. However, the threshold varies from 2 % to 4 % among European guidelines [7,12–15]. Also, WHO describes another category, "uncomplicated hyperparasitemia", which is not a severity criterion but a precaution for closer management [16]. Notwithstanding, peripheric parasite count does not entirely reflect the *P. falciparum* pathogenic mechanisms [11], and markers that could better correlate with organ damage, severity and life-threatening outcomes are needed. Moreover, prostration criterion in adults becomes difficult to assess and other criteria such as anemia are uncommon presentations compared with children. Lastly, several risk factors for mortality and worse clinical outcomes such as age and duration of fever have been described in imported malaria [17,18], but are underrepresented in current severity classifications. In clinical practice, it is not infrequent to attend patients with high parasitemia that do not develop organ damage and respond swiftly to treatment without complications, and also patients with very severe malaria (including organ damage) with an initial low parasitemia, possibly influenced by intense parasite cytoadherence. Therefore, there is a need to reevaluate criteria for SM adapted to non-endemic areas. In this sense, the French guidelines created two subgroups of severe patients: less severe malaria (LSM) and very severe malaria (VSM) groups [7]. However, this classification has been scarcely evaluated across different cohorts of imported malaria.

The objective of this study is to apply a modified classification of severe imported malaria cases to identify those patients at lower risk of death and severe complications (classified as LSM) that would benefit from a less intense clinical management.

2. Methods

2.1. Study design and workflow

We conducted a retrospective cohort study of adults over 16 years of age consecutively admitted with malaria in the International Health Department of the Hospital Clinic of Barcelona (Spain), from 2005 to 2023.

We diagnosed *Plasmodium* spp. infection by microscopy of stained thick and thin blood smear or polymerase chain reaction (PCR). We also considered those patients with a negative blood smear who received antimalarial treatment after a positive rapid diagnostic antigen test (RDT) (Bioline™ Malaria Ag P. f/pan), when clinical manifestations and laboratory findings were compatible. Patients without microbiologic confirmation of *Plasmodium* spp. infection and participants not managed at Hospital Clinic were not included. Patients were followed up for 28 days, or until hospital discharge if their admission exceeded 28 days.

We classified patients into severe malaria (SM) and uncomplicated malaria (UM) according to the WHO 2013 severity definition, except for the parasitemia criterion, in which we applied the parasite density threshold >2 % defined by European and Spanish guidelines [12,13].

Given the potential inaccuracies of the WHO criteria for identifying SM, we distinguished patients who met severe malaria criteria between very severe malaria (VSM) and less severe malaria (LSM) cases, based on the French malaria guidelines [7]. VSM was defined by the presence of at least one of the following conditions: parasitemia >10 %, pulmonary edema (radiologic finding or SpO2 <92 %), impaired consciousness (Glasgow coma scale <11 points), seizures, renal failure (creatinine >3 mg/dL), metabolic acidosis (bicarbonate <15 mmol/L) or hyperlactatemia (>5 mmol/L), shock (Systolic Blood Pressure <80 mmHg) and hypoglycemia (<40 mg/dL). In patients without criteria for VSM, LSM was defined by: 2–10 % parasitemia, hyperbilirubinemia (>3 mg/dL) or jaundice, prostration, anemia (Hemoglobin <7 g/dL) or minor bleeding. Our primary composite outcome was death and the need for life-saving interventions (i.e., life-threatening conditions), such as mechanical or non-mechanical ventilation, vasoactive agents, hemodialysis, and red blood cell exchange. Secondary outcomes were prevalence of co-infections and prevalence of complicated malaria (defined by those additional conditions beyond the malaria infection that could worsen the prognosis). We collected demographics, previous medical conditions, travel history and exposures, clinical manifestations, complementary test results, life-saving interventions required during the hospitalization, and clinical outcomes at the end of the follow-up. We carried out analysis by comparing SM and UM groups, as well as stratifying by VSM and LSM. All outcomes were compared between groups (SM *vs.* UM and VSM *vs.* LSM). Finally, we developed an exploratory analysis to evaluate predictive factors for life-threatening conditions.

2.2. Statistical analysis

Qualitative variables were expressed in percentages, while quantitative variables were reported as medians and interquartile ranges. Pearson $\chi 2$ test or Fisher's exact test was used to compare categorical variables between groups, and Mann–Whitney *U* test or Kruskal–Wallis tests were carried out for quantitative variables. For factors associated with life-threatening conditions, all significant variables from the bivariate analysis and those considered clinically relevant were included in a multivariate logistic regression model, allowing estimating adjusted odds ratio (aOR) along with its confidence interval (CI) for variables identified through backward stepwise selection. The statistical analysis was performed using Stata16.1 (StataCorp LLC, College Station, TX).

2.3. Ethics

The study was approved by the Institutional Review Board and the Ethics Committee of the Hospital Clinic of Barcelona (HCB/2023/1117). The study was designed in compliance with Good Clinical Practice and following the Declaration of Helsinki.

3. Results

3.1. Study population and baseline characteristics

Our cohort included 506 patients with malaria. Patients' median age was 38 years (IQR: 30–47), 164 (32.4 %) were female, and 129 (25.5 %) presented previous medical conditions. Most patients (429, 84.8 %) visited the Africa WHO region, and almost half of them (239, 47.2 %) traveled to visit friends and relatives. Only 24 (4.7 %) patients developing malaria reported having done antimalarial chemoprophylaxis correctly. Within the cohort, solely one patient did not travel: the patient

developed malaria as a consequence of a heart transplantation. Detailed baseline characteristics of patients as well as their travel characteristics can be found in Table 1.

Concerning microbiological diagnosis, 491 (97.0 %) patients had a positive blood smear. In the remaining 16 patients with a negative blood smear, the diagnosis was made with RDT (n = 10) and/or PCR (n = 7). Overall, 438 (86.6 %) patients were diagnosed with *P. falciparum* infections. Of the 12 patients with mixed *Plasmodium* infections, nine had *P. falciparum* mixed infections and the other three had non-falciparum mixed infections. In 30 (5.9 %) cases the first blood smear resulted negative. Supplementary Table A shows *Plasmodium* spp. species distribution and each species's parasitemia.

Clinical and laboratory presentations of malaria patients are shown in Table 2 and Supplementary Table B, respectively.

3.2. Severe malaria

Among the 506 patients with malaria, 330 (65.2 %) presented with UM and 176 (34.8 %) with SM, according to WHO severity criteria. Four (50 %) of the 8 pregnant developed SM. Regarding clinical presentation, diarrhea, jaundice and impaired consciousness were more common in SM cases and occurred in 29.0 %, 7.3 % and 6.8 % of SM cases. In total,

Table 1
Baseline and travel characteristics of patients.

Characteristics	TOTAL n = 506	UM group n = 330	SM group n = 176	p value
Baseline characteristics				
Age, years	38 (30–47)	36.5 (29–47)	40 (32–49)	0.106
Sex, female	164 (32.4)	113 (34.2)	51 (29.0)	0.228
Pregnant women	8 (4.9)	4 (3.5)	4 (7.8)	0.236
Previous malaria episodes	175 (34.6)	132 (40.0)	43 (24.4)	**0.002**
Previous medical condition	129 (25.5)	27 (23.3)	52 (29.6)	0.127
Cardiovascular risk factor[1]	54 (10.7)	29 (8.8)	25 (14.2)	0.060
Heart disease	8 (1.6)	6 (1.8)	2 (1.1)	0.720
Lung disease	7 (1.4)	6 (1.8)	1 (0.6)	0.430
Liver disease	18 (3.6)	9 (2.7)	9 (5.1)	0.167
Renal disease	2 (0.4)	1 (0.3)	1 (0.6)	0.575
Neurological disease	7 (1.4)	4 (1.2)	3 (1.7)	0.463
Non-immunosuppressive haematologic disease	6 (1.2)	2 (0.6)	4 (2.3)	0.114
Immunosuppression[2]	33 (6.5)	19 (5.8)	14 (8.0)	0.340
Other medical conditions	32 (6.3)	23 (7.0)	9 (5.1)	0.414
Travel characteristics				
Travel destination: WHO regions				
Africa	429 (84.8)	274 (83.0)	155 (88.1)	0.133
America	16 (3.2)	14 (4.2)	2 (1.1)	0.057
South-East Asia region	13 (2.6)	12 (3.6)	1 (0.6)	0.038
Eastern Mediterranean region	15 (3.0)	9 (2.7)	6 (3.4)	0.667
Western Pacific	7 (1.4)	7 (2.1)	0	0.052
Travel reason (n = 392)				
Cooperation	42 (8.3)	26 (7.9)	16 (9.1)	0.638
Migration	38 (7.5)	31 (9.4)	7 (4.0)	0.033
Business or tourism	161 (31.8)	94 (28.5)	67 (38.1)	0.027
Visiting friends-relatives	201 (39.7)	132 (40.0)	69 (39.2)	0.862
Travel duration, days[3]	33 (21–109)	42.5 (21–144)	30.5 (19–91)	**0.013**
Pre-travel advice	112 (22.1)	76 (23.0)	36 (20.5)	0.506
Correct chemoprophylaxis	24 (4.7)	18 (5.5)	6 (3.4)	0.303

UM = Uncomplicated malaria. SM = Severe Malaria. Categorical variables are expressed as number (percentage) and quantitative variables are expressed as median (interquartile range).

[1] 39 patients with arterial hypertension, 15 with obesity, 13 with Diabetes Mellitus and 6 with dyslipemia.

[2] 1 patient had congenital immune deficiency, 1 had a B type lymphoma under chemotherapy treatment, 4 patients had asplenia (all in SM group), 23 patients were people living with HIV (9 with CD4 count below 450 cells/mm^3). 4 patients had solid organ transplantation (Heart and liver), 2 patients had autoimmune diseases treated with immunosuppressive drugs.

[3] Newly arrived migrants were excluded.

Table 2
Clinical presentation.

	TOTAL n = 506	UM group n = 330	SM group n = 176	p value
Symptoms duration until diagnosis (days)	4 (2–7)	4 (2–7)	4 (2–6)	0.994
>5 days of symptoms until diagnosis	185 (40.9)	120 (40.8)	65 (41.1)	0.947
Asymptomatic[1]	1 (0.2)	1 (0.3)	0	0.465
General symptoms				
Fever	377 (74.5)	251 (76.1)	126 (71.6)	0.272
Chills	262 (51.8)	171 (51.8)	91 (51.7)	0.981
Malaise/Asthenia	56 (11.1)	39 (11.8)	17 (9.7)	0.461
Headache	285 (56.3)	193 (58.5)	92 (52.3)	0.180
Arthromyalgia	197 (38.9)	134 (40.6)	63 (35.8)	0.291
Back pain	18 (3.6)	10 (3.0)	8 (4.6)	0.381
Gastrointestinal symptoms				
Nausea and/or vomits	137 (27.1)	85 (25.8)	52 (29.6)	0.361
Diarrhea	116 (22.9)	65 (19.7)	51 (29.0)	**0.018**
Abdominal pain	89 (17.6)	54 (16.4)	35 (19.9)	0.322
Respiratory symptoms	35 (8.0)	23 (8.0)	12 (8.1)	0.965
Other symptoms	6 (1.2)	4 (1.2)	2 (1.1)	
Physical examination				
Hepatomegaly	28 (5.5)	15 (4.6)	13 (7.4)	0.183
Splenomegaly	26 (5.1)	16 (4.9)	10 (5.7)	0.686
Jaundice	20 (4.0)	7 (2.1)	13 (7.4)	**0.004**
Impaired consciousness	17 (3.4)	5 (1.5)	12 (6.8)	**0.002**

UM = Uncomplicated malaria. SM = Severe Malaria. Categorical variables are expressed as number (percentage) and quantitative variables are expressed as median (interquartile range).

[1] A patient that was diagnosed because of thrombocytopenia in a control blood test.

185 (40.9 %) patients had more than 5 days of symptoms before diagnosis, with no differences between groups. Moreover, C-reactive protein (CRP) > 10 mg/dL ($p < 0.001$), platelet count <50 × 10^9/L ($p < 0.001$) and LDH >500 U/L ($p = 0.002$) showed significant differences between groups. Overall, 232 (56.0 %) patients within the cohort presented at diagnosis a score> 2 in SOFA scale; 112 (43.4 %) patients from the UM group and 120 (76.9 %) patients from the SM group ($p < 0.001$). The median parasitemia at diagnosis for severe cases was 4.50 % (IQR 2.20–9).

Table 3 shows the distribution of severity criteria among patients with SM. The most common criterion was the presence of parasitemia >2 % (140 patients, 79.65 %), while parasitemia >10 % was detected in almost one-fifth of patients (41, 23.3 %). Over half of patients with SM (101, 57.4 %) presented a single severity criterion. Hyperparasitemia was the most frequent criterion (75, 42.65), followed by jaundice (66, 37.5 %). In contrast, only 2 (1.1 %) patients presented with seizures.

After applying the modified WHO criteria, out of 176 SM patients,

Table 3
Distribution of severity criteria among patients with SM.

SEVERITY CRITERIA	Patients [n = 176, n (%)]
Parasitemia >2 %	140 (79.6)
• Parasitemia 2–10 %	99 (56.3)
• Parasitemia >10 %	41 (23.3)
Jaundice or hyperbilirubinemia	66 (37.5)
Prostration	29 (16.5)
Shock	23 (13.1)
Metabolic acidosis	23 (13.1)
Pulmonary edema	19 (10.8)
Acute kidney injury	19 (10.8)
Impaired consciousness	17 (9.7)
Anemia	8 (4.6)
Hypoglycemia	4 (2.3)
Bleed	4 (2.3)
Seizures	2 (1.1)
1 criterion	101 (57.4)
Parasitemia >2 % as only criterion	63 (35.8)
≥2 criteria	75 (42.6)

104 (59.1 %) were classified as LSM and 72 (40.9 %) as VSM (Fig. 1).

Referring to treatments, 112 (63.6 %) patients received artesunate, being the median number of doses 3(IQR 3–4), while quinine was used in 51 (30.0 %) of patients, being 41/51 (80.4 %) used before 2014. After 8 h of treatment, there were no differences between treatments regarding the negativization of blood smears, but there were significant differences at 24 h (97 % vs. 77 %), with a difference of 16 h in time to blood smear negativization. Among patients who received artesunate, 32/112 (28.6 %) developed port-artesunate delayed hemolysis, and of these almost one-fifth (6/32) needed hospitalization. Although all severe malaria cases should have been managed in an Intensive Care Unit (ICU) per protocol, 54/176 (30.7 %) SM cases were managed in a conventional hospitalization ward. Among them, 48/54 (88.9 %) were LSM cases. None of the 48 patients developed a life-threatening condition.

3.3. Outcomes

In our cohort, death or need for life-saving intervention (the primary outcome) occurred in 37 (7.3 %) patients: one (0.3 %) patient in the UM group, 36 (50 %) patients in the VSM group, and none in the LSM group ($p < 0.001$) presented it (Table 4).

The four patients who died, all in the VSM group, presented clinical and radiological findings of cerebral malaria and died before or <24 h after admission. In addition, 34 patients needed life-saving interventions such as vasopressors (n = 25), mechanical ventilation (n = 14), renal replacement therapy (n = 13) or automated red blood cell exchange (n = 13), being 33/34 VSM cases (Table 4). The only UM case that ended with a life-threatening condition was a pregnant woman that 48 h after the initiation of oral treatment, developed shock and respiratory failure that required vasoactive drugs and non-invasive ventilation, with the subsequent diagnosis of cytomegalovirus pneumonia.

As to secondary outcomes, within the cohort, 28 (5.5 %) patients had a community-acquired co-infection detected at admission, with no differences between UM or SM groups ($p = 0.763$) nor between LSM and VSM groups ($p = 0.739$). Nonetheless, 16 (4.0 %) patients developed a nosocomial infection during the hospital stay, the majority in SM patients (n = 13, $p < 0.001$). In addition, VSM patients had more nosocomial infections 11 (15.3 %), comparing to LSM patients ($p = 0.002$). Details can be found in Table 5. Overall, 199 (39.4 %) patients received empiric antibiotherapy, 77 (23.4 %) being UM patients and 122 (69.3 %) being SM patients. Equally, 178/199 (89.5 %) of patients that received empiric antibiotherapy did not have a coinfection. Supplementary Table C summarizes those scenarios typically referred as "complicated malaria".

After multivariate analysis, severe thrombocytopenia ($\leq 50 \times 10^9$/L) with an aOR 6.02 (2.32–15.68), CRP >10 mg/dL with an aOR 13.79 (1.76–108.15), LDH value > 500 U/L with an aOR of 10.11 (1.11–92.37), and *P. falciparum* presented as factors that increased the risk of life-threatening conditions (Table 6).

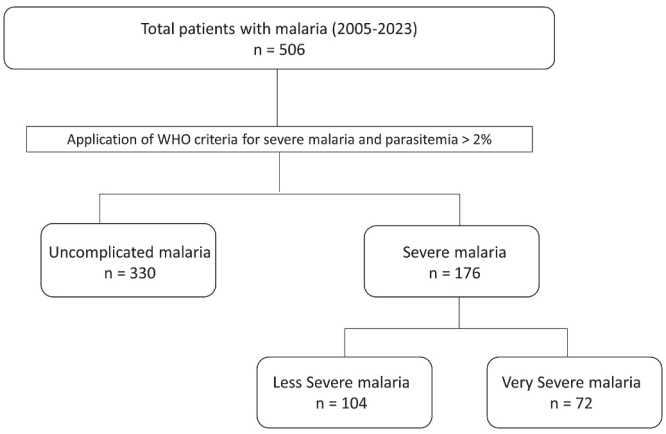

Fig. 1. Flowchart for the classification of patients with malaria

4. Discussion

In our cohort, 35 % of patients had a SM episode based on classical WHO criteria for non-endemic areas, however no patient belonging to the LSM group developed a life-threatening condition. That means that almost 60 % of cases labeled initially as severe did not present severe organ damage. By contrary, 50 % of patients with VSM developed life-threatening conditions. A nuanced selection of severe malaria cases would allow accurate management of patients with imported malaria and optimize healthcare resources, restricting ICU for those patients with VSM.

In 1985 the first official definition of severe malaria was formulated [19]. The creation of a classification that allowed protocolizing the management of malaria cases supposed an enormous step forward [20]. However, it has been observed that the severity of malaria presents differently in adults and children: children more frequently present with anemia, acidosis, and convulsions, whereas in adults, renal failure and pulmonary involvement are more prevalent characteristics of severe malaria [20–23], as were in our cohort. Additionally, prostration is an easily detectable and definable criterion in children, but it is subjective and imprecise for adults. Despite that, what is indisputable is that the presentation with the highest morbidity and mortality, regardless of age, is cerebral malaria. In our cohort, all patients died from cerebral malaria, which is primarily defined as a Glasgow Coma Scale score of <11 points [16]. This criterion does not allow early detection of neurological deterioration in malaria-infected patients. Therefore, some guidelines include any neurological impairment as a severity criterion [7,14]. Another parameter that triggers debate as a defining factor of severity is parasitemia. It is historically described the increase in mortality with the increase of parasite load [11,24]. However, this is only an indirect measure of the real pathogenesis in target organs (whose failure is the origin of morbidity and mortality). In our cohort, parasitemia between 2 and 10 %, jaundice and prostration were the most common criteria, accounting for 56 %, 37 % and 17 % of patients, all belonging to LSM group. Based on these differences, some guidelines in non-endemic areas attempt to weight the prognostic value of these criteria, which contributes to heterogeneity [12]. On the other hand, guidelines in non-endemic areas do not include the assumption of "uncomplicated hyperparasitemia" [16], a clinical scenario commonly found in this cohort (36 % of patients with SM).

Despite the need to redefine severity criteria in non-endemic areas [25], VSM and LSM groups are described only in French guidelines, and they have not been adopted by other non-endemic recommendations. This study, although is based on these concepts, does not completely share the same criteria. In French guidelines, the severity threshold for parasitemia in LSM is 4 %, contrarily to our range of 2–10 %. Isolated seizure as well as moderate renal failure are included in the French LSM group, but they have not been contemplated in this study. Prospective studies could finally define the spectrum of these subgroups.

On the other hand, large cohorts in non-endemic areas describe risk factors (different from those collected by the WHO) associated with mortality [17,18,25]. Easy to obtain variables such as age and reason for travelling could be considered when classifying patients, as well as the duration of fever, reflected in some guidelines [15], albeit in our cohort symptoms' duration has not been found as a risk factor. Another special population at risk could be pregnant women; in our cohort, 4/8 (50 %) had severe malaria, and the only patient with UM who needed ICU intervention was pregnant. Additionally, in our cohort, severe thrombocytopenia and elevated CRP were consistently a risk factor after the multivariate analysis. The inclusion of these biomarkers, as well as HRP2 (histidine-rich-protein 2), which has been associated with mortality in both endemic and non-endemic areas may be considered to aid in future decision-making algorithms [26,27].

Table 4
Primary outcome: life-saving interventions and deaths among groups.

Outcome n (%)	Total n = 506	Uncomplicated malaria n = 330	Less severe malaria n = 104	Very severe malaria n = 72	p-value[2]
Life-threatening conditions[1]	37 (7.3)	1 (0.3)	0	36 (50.0)[3]	<0.001
Deaths	4 (0.8)	0	0	4 (5.6)	0.017
Life-saving interventions	35 (6.9)	1 (0.3)	0	34 (47.2)	<0.001
Vasopressors	25 (4.9)	1 (0.3)	0	24 (33.3)	<0.001
Mechanical ventilation	14 (2.8)	1 (0.3)	0	13 (18.1)	<0.001
Renal replacement therapy	13 (2.6)	0	0	13 (18.1)	<0.001
Automated red blood cell exchange	13 (2.6)	0	0	13 (18.1)	<0.001

[1] Composite outcome including deaths and life-saving interventions.
[2] p-value between less-severe and very severe malaria.
[3] Two patients requiring life-saving interventions died.

Table 5
Community-acquired and nosocomial infections.

Infection, n (%)	Total n = 506	UM group n = 330	SM group n = 176	p-value	LSM group n = 104	VSM group n = 72	p-value
Community-acquired infections	28 (5.5)	19 (5.8)	9 (5.1)	0.763	6 (5.8)	3 (4.2)	0.739
Nosocomial infections	16 (4.0)	3 (1.3)	13 (7.7)	0.001	2 (1.9)	11 (15.3)	0.002
Antibiotic use without confirmed infection	178 (37.3)	65 (21.0)	113 (67.7)	<0.001	57 (58.2)	56 (81.2)	<0.001
Community acquired infections (n = 28)							
Travelers' diarrhea[1]	8 (28.6)	6 (21.4)	2 (7.1)	0.558	1 (3.6)	1 (3.6)	0.652
Pneumonia[2]	7 (25.0)	3 (10.7)	4 (14.3)	0.195	2 (7.1)	2 (7.1)	0.542
Urinary tract infection	3 (10.7)	3 (10.7)	0	0.277	0	0	–
Typhoid fever	2 (7.1)	1 (3.6)	1 (3.6)	0.575	1 (3.6)	0	0.591
Blood stream infection[3]	2 (7.1)	1 (3.6)	1 (3.6)	0.575	1 (3.6)	0	0.591
Upper respiratory infection[4]	3 (10.7)	3 (10.7)	0	0.205	0	0	–
Dengue	2 (7.1)	2 (7.1)	0	0.425	0	0	–
Rickettsia	1 (3.6)	1 (3.6)	0	0.465	0	0	–
Skin and soft tissue infection	1 (3.6)	0	1 (3.6)	0.170	1 (3.6)	0	0.591
Nosocomial infections (n = 16)							
Pneumonia[5,6,7]	10 (62.5)	2 (6.3)	8 (50.0)	0.017	1 (6.3)	7 (43.8)	0.009
Catheter related blood-stream infection	5 (31.3)	0	5 (31.3)	0.096	1 (6.3)	4 (25.0)	0.160
Urinary tract infection[8]	2 (12.5)	0	2 (12.5)	0.099	0	2 (12.5)	0.166
Ventilator-associated tracheobronchitis	1 (6.3)	0	1 (6.3)	0.243		1 (6.3)	0.409

UM: Uncomplicated malaria, SM: severe malaria, LSM: Less severe malaria, VSM: Very severe malaria.
[1] Isolated microorganisms: 4 *E. coli* spp, 1 *Shigella* spp, 2 *Clostridioides difficile,* 1 *Criptosporium parvum* (this patient presented also a bacteriemia due to *S. aureus*).
[2] In one patient pneumonia due to Coxiella was suspected.
[3] Included primary blood stream infection due to *Moraxella osloensis and S. aureus* bacteriemia related to intramuscular injections.
[4] Two patients were diagnosed with symptomatic COVID-19 infection.
[5] Among patients with nosocomial pneumonia the following microorganisms were isolated: Meticilin resistant *S. aureus*, CMV, Influenza A, Adenovirus, Aspergillus spp.
[6] 1 patient had pneumonia due to *E. coli*, catheter related blood-stream infection (*E.faecium*), and urinary infection (Pseudomonas).
[7] 1 patient had catheter related blood stream infection due to *E. faecalis* and a pneumonia.
[8] The urinary infections were due to *K. oxytoca* and *Pseudomonas aeruginosa* [7].

The most effective intervention against malaria is the early administration of artesunate, allowing for a 39 % decrease in adults mortality in endemic areas [28,29]. This tendency can be seen in imported malaria also: in a large cohort of SM patients in the "pre-artesunate" era mortality was 10.5 % [18]. A systematic review of imported SM showed a pooled prevalence for death of 8 % between 2000 and 2009, descending to 4.4 % between 2010 and 2021 [8]. In Spain, the availability of artesunate was related to a better prognosis [30], and the duration of ICU and hospital admission was shorter according to a European multicentric retrospective study [31]. What is more, artesunate shows a safety profile that permits its use outside of ICUs [32], and should be initiated empirically while awaiting additional tests if the patient presents with severe clinical conditions or if the results of the malaria test is expected to take time [20]. Interestingly, a study made in our hospital from 1991 through 2007 reported a mortality rate of 25 % among SM patients [33]. In this cohort, overall mortality was of 0.79 % and 2.3 % among patients with SM. Therefore, establishing different severity groups such as LSM and VSM would allow for the optimization of very valuable resources such as ICU admission, provided that access to artesunate treatment is straightforward.

Another important factor to consider in the management of patients with malaria is the possibility of co-infection. The WHO recommends having a low threshold to initiate antibiotic therapy and specifically recommends administering it to children with SM in areas of high malaria transmission due to the high prevalence described of *Salmonella* spp. infections and the increased predisposition to bacterial translocation associated to malaria from capillary sequestration [16,34]. As indicated by local protocol, broad use of antibiotics was described in our cohort. However, the frequency of co-infections was only 5.5 %, with no differences between groups (p = 0.763), slightly lower than other cohorts in non-endemic areas [35,36]. Therefore, a more restrictive use of antibiotics could optimize patient management and fit into antibiotic stewardship strategies.

As to limitations, prognostic value of some severity criteria can be difficult to determine due to their infrequent appearance along the cohort. Also, prospective studies are needed including different populations such as children, to verify if results can be extrapolatable. In the same line, some classical risk factors (e.g. duration of travel or pregnancy) may not have statistical significance due to low statistical power.

To summarize, there is a need to review the severity criteria definitions for patients with malaria in non-endemic areas. It may also be beneficial to include new variables for severity, which could potentially lead to standardization of guidelines across countries. Within the spectrum of severe malaria, patients with LSM have a low risk of developing

Table 6
Bivariate and multivariate analysis of factors significantly associated with life-threatening conditions.

	Bivariate analysis				Multivariate analysis	
	Absence of life-threatening condition	Life-threatening condition	OR (95% CI)	*p-value*	aOR (95% CI)	*p-value*
	Total n = 468 n (%)	Total n = 37 n (%)				
Age >50 years	86 (18.3)	12 (32.4)	2.13 (1.03–4.42)	0.037	2.68 (0.98–7.28)	0.054
Pregnancy	7 (3.8)	1 (9.1)	2.56 (0.29–22.85)	0.384		
Previous malaria episodes	171 (36.5)	4 (10.8)	0.21 (0.07–0.61)	0.003	0.33 (0.10–1.16)	*0.085*
Cardiovascular risk factors	49 (10.5)	5 (13.5)	1.34 (0.50–3.60)	0.561	–	
Travel reason: Visiting friends-relatives or migration	231 (49.3)	8 (21.6)	0.28 (0.13–0.63)	0.001	0.63 (0.18–2.15)	0.456
Travel reason: Work or tourism	141 (30.1)	20 (54.1)	2.74 (1.39–5.38)	0.003	1.22 (0.43–3.48)	0.706
>5 days of symptoms before diagnosis	162 (39.0)	23 (63.9)	2.77 (1.36–5.63)	0.003	2.02 (0.82–4.99)	0.127
Diarrhea	107 (22.8)	9 (24.3)	1.08 (0.50–2.38)	0.833		
Jaundice	15 (3.2)	5 (13.5)	4.73 (1.62–13.84)	0.002	2.67 (0.56–12.75)	0.219
Impaired consciousness	12 (2.6)	5 (13.5)	5.95 (1.97–17.93)	<0.001	4.00 (0.85–18.81)	0.079
Severe thrombocytopenia (<50 × 10^9/L)	**89 (19.0)**	**28 (75.7)**	**13.28 (6.05–29.14)**	**<0.001**	**6.02 (2.32–15.68)**	**0.000**
LDH (>500 U/L)	**308 (65.7)**	**36 (97.3)**	**18.82 (2.56–138.51)**	**<0.001**	**10.11 (1.11–92.37)**	**0.040**
C-reactive protein (>10 mg/dL)	**249 (53.1)**	**35 (94.6)**	**15.46 (3.68–65.03)**	**<0.001**	**13.79 (1.76–108.15)**	**0.012**
Plasmodium falciparum	405 (85.5)	37 (100)	–	0.013	–	
Bacterial co-infection	27 (5.8)	1 2.70	0.45 (0.06–3.44)	0.434		

*Life-threatening conditions included death and need for vasopressors, mechanical ventilation, renal replacement therapy and automated red blood cell exchange.

a life-threatening clinical outcome, and could benefit from a less intensive monitorization unit and a restrictive use of empirical antibiotics.

Funding

The ISGlobal authors acknowledge support from the Spanish Ministry of Science and Innovation and State Research Agency through the "Centro de Excelencia Severo Ochoa 2019–2023" Program (CEX2018-000806-S), CIBER-Consorcio Centro de Investigación Biomédica en Red-(CB 2021), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea and support from the Generalitat de Catalunya through the CERCA Program. L.B., C.P., J.M. and D.C. also acknowledge support from the Hospital Clinic of Barcelona and the Spanish Ministry of Science and Innovation through the program PID2020-116770RJ-I00 and CPP2021-008658.

CRediT authorship contribution statement

Leire Balerdi-Sarasola: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jose Muñoz:** Writing – review & editing, Validation, Supervision, Conceptualization. **Pedro Fleitas:** Writing – review & editing, Methodology, Formal analysis. **Natalia Rodriguez-Valero:** Writing – review & editing, Visualization, Supervision. **Alex Almuedo-Riera:** Writing – review & editing, Validation, Supervision. **Alba Antequera:** Writing – review & editing, Supervision, Conceptualization. **Carme Subirà:** Supervision, Project administration, Data curation. **Ignacio Grafia-Perez:** Writing – review & editing, Validation. **Maria Ortiz-Fernández:** Writing – review & editing, Visualization, Validation. **Tessa de Alba:** Writing – review & editing, Validation. **Miriam J. Álvarez-Martínez:** Writing – review & editing, Validation. **M Eugenia Valls:** Writing – review & editing, Validation. **Claudio Parolo:** Writing – review & editing, Visualization, Validation, Supervision. **Pedro Castro:** Writing – review & editing, Visualization, Validation. **Daniel Camprubí-Ferrer:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The ISGlobal authors acknowledge support from the Spanish Ministry of Science and Innovation and State Research Agency through the "Centro de Excelencia Severo Ochoa 2019–2023" Program (CEX2018-000806-S), the CIBER-Consorcio Centro de Investigación Biomédica en Red-(CB 2021), and the Generalitat de Catalunya through the CERCA Program and AGAUR program (2021 SGR 01558).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tmaid.2024.102740.

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Supplementary table B. Laboratory parameters.

Parameters, median (IQR)	TOTAL n = 506	UM group n = 330	SM group n = 176	p value
Glucose mg/dL	102 (92-117)	102 (92-116)	103 (91.5-120.5)	0.641
Creatinine mg/dL	0.96 (0.8 - 1.2)	0.94 (0.8- 1.1)	1.0 (0.8 -1.4)	0.004
C-reactive protein mg/dL	10.4 (4.9-16.1)	7.5 (3.7-12.7)	15.2 (9.85- 20.9)	<0.001
Bilirubin mg/dL	1.3 (0.8- 2.3)	1.1 (0.7-1.6)	2.2 (1.2-3.9)	<0.001
Alanine aminotransferase U/L	37 (22- 73)	34 (21- 60)	49 (29- 92)	<0.001
Alkaline phosphatase U/L	109 (71- 175)	112.5 (71- 173)	105 (71-182)	0.971
Gamma-glutamyltransferase U/L	47 (26 – 98)	40.5 (24-79)	64 (33- 123)	<0.001
LDH U/L	520 (364 - 765)	444.5 (320 - 609)	656 (429 - 1138)	<0.001
Platelets 10^9/L	87 (51-140)	108 (66- 155.5)	55 (30- 90)	<0.001
Hemoglobin g/dL	13.2 (11.9 - 14.4)	13.6 (12.2 - 14.5)	12.6 (11- 14.2)	<0.001
Leukocytes 10^9/L	5 (3.7- 6.3)	4.6 (3.5- 5.9)	5.45 (4.12-7.6)	<0.001
Neutrophils 10^9/L	3.4 (2.3 - 4.7)	3.1 (2.1-4.2)	4 (2.8 - 5.4)	<0.001
Lymphocytes 10^9/L	0.9 (0.5 - 1.7)	0.98 (0.5- 1.6)	0.9 (0.5 - 1.9)	0.6650
Prothrombin %	77 (68.4 - 85)	79 (71.3 - 88)	71 (65- 80)	<0.001

UM = Uncomplicated malaria. SM = Severe Malaria.

SUPPLEMENTARY TABLES

Supplementary Table A. Plasmodium species distribution and parasitemia.

Species	TOTAL n = 506	UM group n = 330	SM group n = 176	*p-value*
Plasmodium falciparum	438 (86.6)	265 (80.3)	173 (98.3)	<0.001
Non-falciparum[1]	77 (15.2)	72 (21.8)	5 (2.8)	<0.001
P.vivax	48 (9.5)	45 (13.6)	3 (1.7)	<0.001
P. ovale	23 (4.6)	22 (6.7)	1 (0.6) [2]	0.001
P. malariae	9 (1.8)	8 (2.4)	1 (0.6) [2]	0.172
Parasitemia at diagnosis	0.075 (0.60 – 3)	0.03 (0.15 – 0.70)	4.50 (2.20 – 9)	<0.001
***P.falciparum* parasitaemia**	0.1 (0.7 – 3)	0.03 (0.18 - 0.78)	4.96 (2.4 – 10)	<0.001
P.vivax parasitaemia	0.064 (0.15 -0.55)	0.067 (0.15 - 0.23)	0.55 (0.01 – 3)	0.555

[1]Patients infected with more than 1 *Plasmodium* species were allowed to classify in more than one category. There were no cases due to *Plasmodium knowlesi*.

[2] Co-infection with *P.falciparum*

UM = Uncomplicated malaria. SM = Severe Malaria. Categorical variables are expressed as numbers (percentage) and quantitative variables are expressed as median (interquartile range).

Supplementary table C. Complicated malaria among SM and UM malaria groups, and also referring LSM and VSM groups.

Complications, n (%)	TOTAL n = 506	UM group n = 330	SM group n = 176	p value	LSM group n = 104	VSM group n = 72	p value
Spleen rupture	1 (0.2)	0	1 (0.6)	0.348	0	1 (1.4)	0.409
Pancreatitis	3 (0.6)	0	3 (1.9)	0.042	1(1.0)	2 (2.8)	0.568
Neurologic post-malaria syndrome	2 (0.4)	0	2 (1.1)	0.121	1 (1.0)	1 (1.4)	0.652
Community- acquired infections	28 (5.5)	19 (5.8)	9(5.1)	0.763	6(5.8)	3(4.2)	0.739
SIADH	1 (0.2)	0	1 (0.6)	0.339	0	1 (1.4)	0.409
Atrial fibrillation	2 (0.2)	0	2 (1.1)	0.121	1(1.0)	1 (1.4)	0.652
Rhabdomyolysis or lower limb ischemia	3 (0.2)	0	3 (1.9)	0.042	1 (1.0)	2 (2.8)	0.568
Cholecystitis	1 (0.2)	0	1 (0.6)	0.339	0	1 (1.4)	0.409
Cerebral venous thrombosis	1 (0.2)	1 (0.3)	0	0.652	0	0	-

UM: Uncomplicated malaria, SM: severe malaria, LSM: Less severe malaria, VSM: Very severe malaria, SIADH: Syndrome of inappropriate secretion of antidiuretic hormone.

ARTICLE 3

Host biomarkers for early identification of severe imported Plasmodium falciparum malaria

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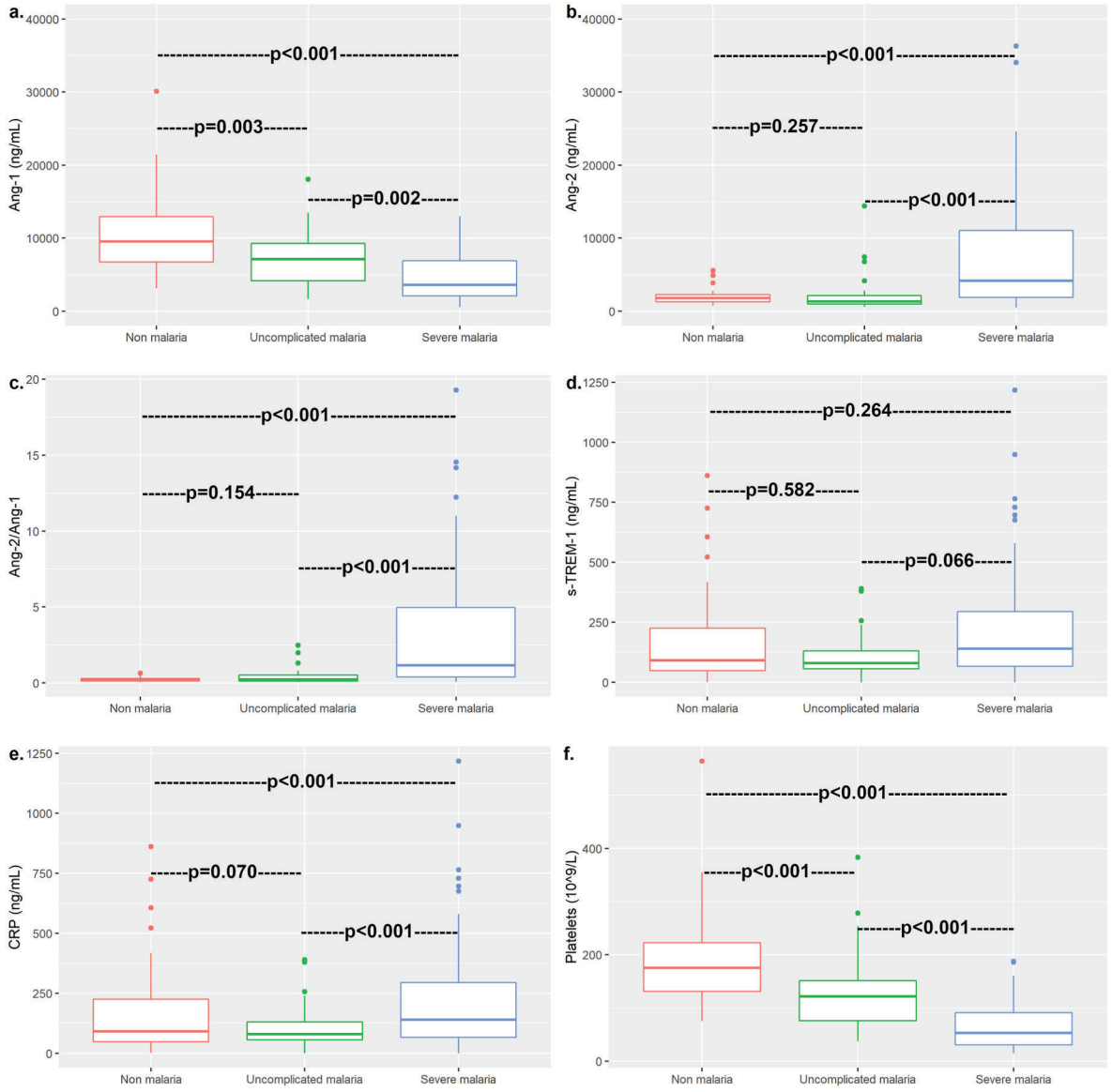
OnlaDetltaPfidHlp clDr-clc-rIPracfilaDclmoIclo:licdlDotalseov rnicmlenl aDclcrmidleficnaeyfraeonlo:ltc-cmclv rIrmerlenlnonScnficv eftcaaenxtIl

e. hs3Ru2l

2.1. MDBaírahl,PHrGHarnbnBSGDbH

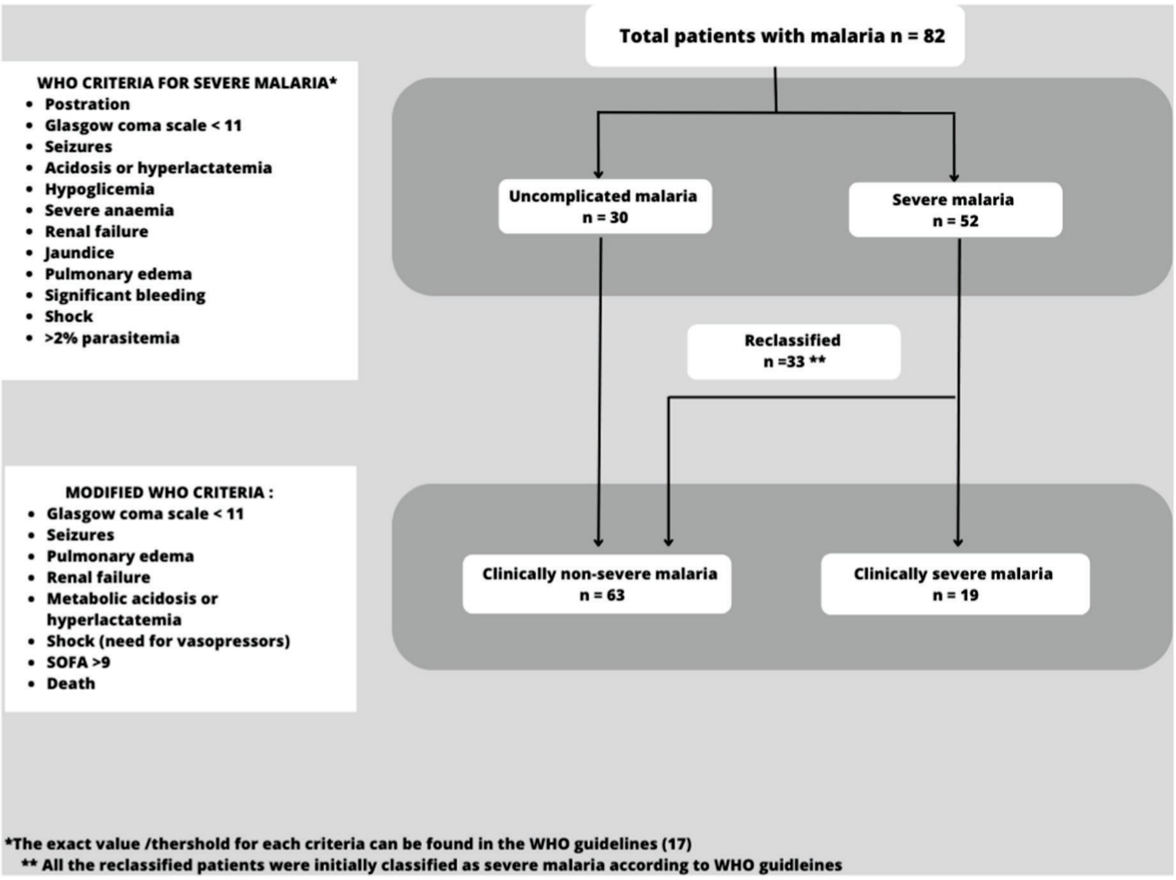
znlostcmraeonrIlfrtcSfonaoIltaPfidlprtlfonfiPfacfilenlaDcl0nacmrS aeonrIlNcrIaDlTcgrmav cnalo:laDclNotgearIlJIeneflo:lBrmficIonrHl, grenHl :novlCrnPrmdlwWHHlaolCrnPrmdlwWWHlzfiPIalgraecnatlpeaDl:c-cmhrnfil vefmoseoIoxefrIIdlfonymv cfilI.rcG&2,nGpBoren:cfaeonlpcmdlfIrtteycfilrfS fonfiienxlaolE Ní lfmeacmerlenaoltc-cmclv rIrmerl(,q 2lrnfilPnfov gIefracfil v rIrmerl(kq 2lpeaDlaDclc0fcgaeonlo:lgrmcteacrcv erl(grmcteaclficnteadl>w4 l



Tao.MI. Bo0gIoalo:lDotalseov rnicmh-rIPctlscap ccnl, q hlkq lrnfilnonSv rIrmerIlfrtctIll

2.2.

2.3.


Total patients with malaria n = 82
WHO CRITERIA FOR SEVERE MALARIA*
• Postration
• Glasgow coma scale < 11
• Seizures
• Acidosis or hyperlactatemia
• Hypoglicemia
• Severe anaemia
• Renal failure
• Jaundice
• Pulmonary edema
• Significant bleeding
• Shock
• >2% parasitemia
Uncomplicated malaria
n = 30
Severe malaria
n = 52
Reclassified
n =33 **
MODIFIED WHO CRITERIA :
• Glasgow coma scale < 11
• Seizures
• Pulmonary edema
• Renal failure
• Metabolic acidosis or hyperlactatemia
• Shock (need for vasopressors)
• SOFA >9
• Death
Clinically non-severe malaria
n = 63
Clinically severe malaria
n = 19
*The exact value /thershold for each criteria can be found in the WHO guidelines (17)
** All the reclassified patients were initially classified as severe malaria according to WHO guidleines

v oficltlp cmlf Dotcnl:onlaDclogaev eFraeonlo:lgr mv cacmll

uenr lIdHaDcl- r lPctlo:ltcnteae- eadHtgcf ey f eadHncxrae- cl r nfil goteae- cl gmfief ae- cl- r lPctlp cmlctaev racfil:onlcr f Dls eov rnicmlrnfilaDclv r f Dencl lcr mnenxlv oficltlp eaDlr l%) 4 J Oll

z Ill aDcl r nr ldtctl p cml r nr ldFcfil p eaDl Vl to:ap r ml 95w[H c0f cgal aDcl v r f Dencl lcr mnenxl r nr ldtetl aDral p r tl f r mcfil oPal p eaDl aDcl tf eSi eal lcr ml MdaDonlles mml 955 [Il

2.L. vDf,elr

. Dcl taPfidlp r tlr ggmo- cfils dlaDclOntaaPaonr llVc- ecp lBor mfilr nfilaDcl GaDef tlJ ov v eacclo:laDclNotgear llJ Ienflo:lBr nficlonr l(NJ B] wWH%][W5%2H r nfilp maacnlen:onv cfilf ontcnalp r tlos ar encfil:mv lr llltaPfidlgr mef egr natll . Dcl taPfidlp r tlfictexncfilenlf ov gler nf clp eaDlUoofillJ Ienf r llMmf aef clr nfil :olIop enxlaDclT cf lr mnaeonlo:lNclteni ell

,. Ví BGltar acv cnal:onlmgomaenxlf r tcSfonamlltaPfiectlp r tl:olIop cfil b, PggIcv cnar mll. rs Iclz 0.M

8. 4slcDl

3.1. uGlhS,Hhref GpGeDhp,lDel

z v onxlaDcl5H/lgr aecnatlfier xnotcfilp eaDlv r lr mr l:mv lCrnPr mllwWHH aolCrnPr mllwWwHl/wlgr mef egr natlp cmlenf lPficfilo:lp Def Dl5Wgmtcnacfil k q lr nfil) wlp cml, q lf r tctlr f f onfienxlaolE Ní lf lr ttey f raeonlr nfilPtenxl aDclw4 lgr mteacv er laDmtDoIfil9wH7 [llz fifieaeonr lldH1%:cs melclam- clcmtl p cmlenf lPficfilenlaDclnonSv r lr mr lxmoPgl(H5Hlgr mef egr natlenlaoar l2llMS aecnat' s r tclenclf Dr mf acmtaef tlr mlficar elcfilenl. rs IclHr. Dcenlv cfier nlr xcl p r tl571(QQVlw–182ldcr mtHr nfil/11(811H4 2lp cmlv r lclIz laoar llo:l551 (w) Iw4 2lgr mef egr natlDr filr lgmo- eoPtlv cfief r llf onfieaeonll. Dclv r enl- eteacfil E Ní lmxeontlp cmlz :mf r l(r llls PaloncIv r lr mr lf r tclp Dolr f f lPenfilaDcl en:cf aeonlaDmoPxDlDcr mlaamtgr na2lr nfil, oPaDSGr talz ter lr f f oPnaenxl:onl wwl (1) I%4 2l nonSv r lr mr lgr aecnatll Onl aoar lH) W(5/Iw4 2lgr aecnatlp cml aoPmtatlr nfil551(w) Iw4 2l- eteacfil:mcnfitlr nfilmIrae- ctllRonclo:laDclgr mS aef egr natlPnficmp cnalr naev r lr mr llf Dcv ogmgDdlr 0etll

. Dclv r enlfier xnotetl:onlaDclnonSv r lr mr llxmoPgl(nl= 1%2lp cmc3H71 (51174 2l r msv o- emIlfietcr tctlH5l(w8l) 4 2lPnfie:cmnaer acfil:c- cmlHr nfil) l (HWIw4 2lgr aecnatlDr filmtgemaomlden:cf aeontlb, PggIcv cnar mll. rs IclB0.Ml aDetlxmoPglH5l(w8l) 4 2lgr aecnatlnccficfilDotgear leFraeonlls PalaDcmlp cml nceaDcrlficr aDtlnonlOJ k lr fiv etteontll

Vcxr mfienxltgcf ey f ls r tclenclf Dr mf acmtaef tlr laoar llo:lwl (8174 2lgr S aecnatlenlaDclk q lxmoPglrnfilwl(51%4 2lenlaDcl, q lxmoPglDr filr lfiof Pv cnacfil f ov v PneadSr f flPenfilf oSen:cf aeonlnonclo:laDclgr aecnatlfic- clogcfiltcgtetll z ltoHv cfier nlfiPmaeonlo:ltdv gaov tls c:omlv r lr mr lfier xnotetlp r tl5lfir dtl (QQV3l w–82H p eaDl nol fie:cmcnf ctl s cap ccnl Pnf ov glef racfil r nfil tc- cmcl v r lr mr lf r tctl(gl= W81121l, PggIcv cnar mll. rs IclJ lfictf mes ctlaDclf Ienef r ll gmotcnar aeonlo:lgr aecnatlp eaDlv r lr mr ll. Dclq r enllrs omaomlly nfienxtlr ml tPv v r mlFcfilenl. rs IclwlIz v onxl5Wgr aecnatlp eaDlPnf ov glef racfilv r lr mr H w/(%54 2lp cmlamr racfilp eaDlomllr macv etenenlf ov s encfilaDcmgectHr nfil rv onxl) wl, q lf r tctH1/l(%w4 2lp cmlamr racfilp eaDlr mactPnr acllz lllgr aecnatl aDralmf ce- cfilenam- cnoPtlamr av cnalPnficmp cnalr ltPs tcflPcnalf oPmclo:l omllamr av cnall

3.2. I GDhHDrk ,Djrl hThphro GSGp,Gr

z v onxlaDcl) wlgr mef egr natlp cmlgmotcnacfilp eaDltc- cmadlf mecmr H wl1 (18Iw4 2l gmotcnacfil p eaDl v oml aDrnl oncl tc- cmadl f mecmr ll . Dcl v otal f ov v onlf mecmr lficacf acfilp cm3181(//I) 4 2lgr mteacv er l>w4 lrnfilH%4 (58I) 4 2l DdgcmselenPs encv er H :olIop cfil s dl HW (H%w4 2l gr aecnatl p eaDl tDof i llNdgoxldf cv er l(nl= w2HtceFPmtl(nl= H2lr nfilr nr cv er l(nl= H2lp cml lcttlf ov v onldl fier xnotcfill Rol gr aecnal gmotcnacfil p eaDl v r ;onls lccfienxl b, PggIcv cnar mll. rs Icl T0.Ml onclo:laDclwl1l(w%154 2lgr aecnatlp Dol gmoS tcnacfilgr mteacv er >w4 lrtlaDclonldltc- cmadlf mecmr lnccficfil:PmaDcmlenS acmcnaeontls cdonflf lenef r llv oneaomFraeonlenlaDclOJ k llz v onxlaDcl) wl gr aecnatlp eaDltc- cmlv r lr mr Hlecs tPggomalacf DneflPctlp cmlr ggIecfilenlwH (1WI14 2l gr aecnat3l H5l (w) IW4 2l nccficfil- r torf ae- cl finPxtH /1 (H) I14 2l

FngDsMlM
Mraecnat' s r tclenclf Dr mf acmtaef tllll

	z ??1 UVí k M,l nl= H5Hl	Rí RSl q z ?z VQ:l xmoPglnl= 1%l	k q l xmoPgl nl= 5Wl	, q l xmoPgl nl=) wl	gSl - r lPc[:l]
z xclq cfier nl(QQV2l	571(w/lS 182l	5W9w8–5%[l	5/I) l (5WS 182l	1W) l (5wS 1%2l	WIWW5l
q cnHnl(4 2l	/1l(811H2l	w8l() 5IW2l	wwl (75152l	58l (8%Iw2l	WIHH8l
E ov cnHnl(4 2l	171(5) I%2l	w5l(181%2l	/l (w8172l	H8l (5W/2l	WIHH8l
Mm- eoPtlv r lr mr l cgetoficlHnl(4 2l	H%(H11) 2l	H(wIW2l	/l (w8172l	HW (H%Iw2l	WIWWwl
Mm- eoPtlv cfief r ll fonfieaeonHnl(4 2l	55l(w) Iw2l	71(H1152l	%l (5WIW2l [xl]	H71 (5w172l [xl]	WIW wl
Gr v PnotPggmatteonl nl(4 2l	8l(1182l	H(wIW2l	wl(8172l	51() 172l	WI81%l
. m- clIfictaenraeon3E Ní lmxeontl[rl]					
z :mf r lnl(4 2l	%/l(71I/2l	H71(51172l	5W (HWW2l	) H (%/IW2l	<WIWWHl
, oPaDSGr talz ter lnl (4 2l	wwl(H8l/2l	ww(1) IW2l	W	W	<WIWWHl
z v cmf r lnl(4 2l	71() I512l	71(H1152l	W	W	WIWWHl
E ctacmlM f ey f lnl (4 2l	8l(1I) /2l	8l(HwIw2l	W	W	WIWWj1
Grtacml q cfieacmmncr nlnl (4 2l	wl(HI) 52l	w(1IH2l	W	W	WIH/%l
. m- clIncr tonl[s]					<WIWWHl
. oPmtv lnl(4 2l	) W(5/Iw2l	5/l(77182l	5l (HWIW2l	%l (H7152l	Sl
b eteaenxl:mcnfitSl melrae- ctlnl(4 2l	55l(w) Iw2l	5l(81H2l	H8l () 5152l	H1l (w81%2l	Sl
E omlnl(4 2l	wHl(H8IW2l	wl(1IH2l	5l (HWIW2l	H8l (5W/2l	Sl
J oogcmaeonlnl(4 2l	H/l(H5172l	) l(HWIw2l	8l (wWIW2l	71 (H5I) 2l	Sl
q exmaeonlnl(4 2l	8l(1182l	H(wIW2l	H(5152l	1l(7172l	Sl
. m- clIfiPmaeonlfir dtl q cfier nl(QQV2l	wwl(H1S) W2l	H819H5–w5[l	511(H/1 S18H2l	w) l(H) Sl %w2l	WIWW8l
MmSam- clIr fi- ef cHnl (4 2l	111(55182l	w8l() 5IH2l	8l (wWIW2l	Hwl (w5IH2l	WIWWHl
NotgearleFraeonHnl (4 2[fl]	%W(8/172l	H5l(w8I) 2l	w8l (/8172l	) Hl (%/IH2l	<WIWWHl
NotgearleFcfilfir dtl q cfier nl(QQV2l	119w–7[l	W(W552l	519w5[l	8l 91–HW[l	<WIWWHl
z fiv etteonlaolr nlOJ k H nl(4 2[fil]	171(5) I%2l	W	H(5152l	18l (//I) 2l	<WIWWHl
OJ k lfir dtlq cfier nl (QQV2l	519w–) [l	Sl	1l 95–5[l	5l 9w–) [l	WI/) Hl
J ov v PnealIr f flPenfil foSen:cf aeonHnl(4 2[cl]	1l(1I%2l	W	wl(8172l	wl(51%2l	WI5w8l
í ntcalo:ltdv gaov tH v cfier nlfir dtH(QQVl	519w–) [l	519w–) [l	5l 9w–7[l	5l 9w–) [l	WI8W5ll

[r] HlgraecnalDr filr lf r mier f lamntgIr nalr nfilfiefilnoalDr- clrlam- clIDetaomlllOnlaDcl nonSv r lr mr lxmoPgl) lgr aecnatl- eteacfilv omclaDr nlHf onaencnalenlaDcltr v clam- clI(wl , Gz l& gr f ey f lHHl, Gz l& z :mf r Hwlz :mf r l& q cfieacmmncr n2ll
[s] 5lgr aecnatlp eaDlv r lr mr lp eaDlPni nop nlam- cllncr tonll
[f] NotgearIl gmaoaf oll mef ov v cnfitl r fiv etteonl aol r lll gr aecnatl p eaDl r l v r lr mr l fier xnotetll
[fi] , c- cmlv r lr mr lgr aecnatlr mlv r nr xcfilenlOJ k lonlGv cmxcnf dlficgr mav cnalr al lcr talaDclyl mtalwl Dlgcnlgmaof oIlllMraecnalp eaDlr lnonSc- cmlv r lr mr lp cmlr f f cgacfil enlr nlOJ k lfiPclaoletolr acfillDdgcms elenPs encv er l:onlwl DIl
[c] enlaDcl k q 3l gmos rs Icl mfi caateotetlr nfil r lam- clcmtl fier mnDcr l fiPcl aol cnacmS oen- r te- clGIf oIeHenl, q lrls r f acmv er lfiPclaolA bpGfihSGrblSbhH,lrnfilr lam- clcmtl fier mnDcr lfiPclaolt SbIDp,a,Bo ra,cxe,ShIl
[:] J ov gr mtonls cap ccnlaDclaDmclxmoPgtll
[x] J r nfieo- r tf PIr nlmati l:r f aom3lk q l= 5(HWIW4 2H, q l= HW(H%w4 2Hgl= WIw7Wl Ncr malfietcr tc3lk q l= H(5154 2H, q l= H(H1%4 2Hgl= WI8WHlNcgr aef lfietcr tct3lk q l= WH, q l=) (%184 2Hgl= WH) 51lRcPmoloxef r llfietcr tct3lk q l= WH, q l= H(H1%4 2Hgl= WI8511lRolgr aecnatlp eaDlIPnxlfietcr tclonlnonr llfietcr tcll

FngDs MM
?rsomaomdlynfienxtlo:lgraecnatlp eaDlv rIrmarIll

?zBí Vz. í Xl MzVzqG. GV,l (v cfiernlQQV2l	.í.z?l qz?zVQal Jz,G,lnl= /wl	kqlxmoPglnl = 5W	,qlxmoPglnl =)wl	gS-rIPcl
UlPfotclvx]fi?l	HW)l)l (%5L)–Hwl2l	HWwL)l(%5lSl HHP%2l	HW7l(%lSl Hw%2l	W)/Hl
Jmrraenenclvx]fi?l	HLW5l(WL/)–Hlw2l	HLW (WL/H–HLw2l	HLW%4 (W%-HL)2l	WLW7W
JSmrrfae-clgmoacenl vx]fi?l	HwLW(1l8–HP4)2l	1L)l(HL/–HH2l	H8l8l (%4)–wwL)2l	<WLWWH
BelenPsenlvx]fi?l	Hl%4(W%-5l52l	HIH(W7–w2l	wl5l (HIH–1L/2l	WLWWH
MrracIcatlHW?]?l	7Hl(1WSlHw)2l	HwHL)l(78lSl H)12l	)5l(5WSl %4HL)2l	<WLWWH
Ncvoxlosenlx]fi?l	H5lwl (HHl%-HlL/2l	H5L)l (HwL1–HlL/2l	H5lHl (HHlw-H)lW2l	W)%8l
Mrmrtearcverli vcfiern(QQV2l	wL)l(W58–8l52l	Wlw)l (WLW7w-W82l	)l(5lw)–HW2l	<WLWWH
Befrmsonracllc-cll vvol]?[r1]	H)Ll1 (HHL)–HP472l	W	H)Ll1 (HHL)–HP472l	Sl
?rfaraclvx]fi?[r1]	8wL)l(5/lSl/%L)2l	W	8wL)l(5/Sl /%L)2l	Sl
,íuzl>%l	%l(HHLW2l	W	%(H7152l	WLWH8ll

[r] í nIdlr-relrsIclenl/l,qlgraecnatIl

cmdaDmogDcmttctHl8l(HHL)4 2lsIoofilamnnt:PteonlH)l(%l84 2lmrnrIlmgIrfcS vcnalaDcmgdlrnfil1l(7l74 2lvcfDrnefrIl-cnaeIraeonIl

zlaoarIlo:l5l()I%4 2lgraecnatlfiecfilHrIIlo:laDcv lscIonxenxlaolaDcltc-cmdl vrIrmarlxmoPgIl. DcdlpcmdrIIlvcnlpeaDlrnlrxclmrnxclo:lwH–1wldcrmtHlpeaDl nolfov onsefieacctlonlgmr-eoPtlvrIrmarlcgetofictlrnfilgrmrtearcverIc-cItlo:l 1WLW4 hHlLW4 lrnfil)l14 llzIIlgmrtcnacfilpeaDlfcmrsmIlvrIrmarlrnfiltc-cmdl fIenefrIlpomtcnenxlsc:omdlamrravcnalrfivenetamaeonIl

3.3. *yblDQbo GpEhpl'a,cdhphHehlrGobHPrMAgrCArGHarHbHso GSGp,GrnGDhHDr*

uexIlH1tDop tlznxSHHznxSwHt. VGqSHHgIracIcatlrnfilJVMlfonfcnamrSaeontlenl,qHkqlrnfilnonSvrIrmarlgraecnatIlBeovrnicmlfonfcnamraontlfiefilnoaltDoplfie:cmrnfctlscapccnlnonSvrIrmarlrnfilkqlgraecnatHc0fcgal:omhznxSH1(gl= WLWW52lrnfilgIracIcatl(gl< WLWWH2llznxSH1rnfilgIracIcatlgmrStcnacfiltexneyfrnaIdlIopcmlfonfcnamraontlenl,qlfrtctlfovgrmrofilaolkqlfrtctl(znxSH1gl= WLWWWHgIracIcatlgl< WLWWH2H1rnfilnonSvrIrmarl(znxSH1rnfilgIracIcatlgl< WLWWH2llznxSwlrnfilJVMlgmrtcnacfilDexDcmlfonfcnamrSaeontlenlfrtctlo:l,qlfov grmrofilaolkql(znxSwlgl< WLWWHHrnfilJVMlgl< WLWWH2lrnfilnonSvrIrmarlfrtctl(znxSwlgl< WLWWHHrnfilJVMlgl< WLWWH2llJon-cmtcIdHt. VGqSH1fiefilnoalgmrtcnaltexneyfrnalfie:cmrnfctlrvonxlxmoPgtIl

3.L. *Aba,xhar0y8reSGll,xeGD,bHrGHarmpha,eDIHrGO,SIDrbcrfblDr Q,bo GpEhplr*

uexIlwltDop tlaDclmrfIrtteyfraeonlo:lgraecnatlrffonfienxlaolaDclv ofieycfilE Nílfirtteyfraeonl:omhevgomacfilvrIrmar3íPalo:l/wlvrIrmarlfrtctlH85l(78L/4 2lpcmdmrfIrtteycfillrtlfIenefrIIdlnonStc-cmdlvrIrmarlrnfilHP4(w5lw4 2lrtlfIenefrIIdltc-cmdlvrIrmarlINcnfc:omaDH55lgraecnatlpcmdmrfIrtteycfil:movlaDcltc-cmdlaolnonStc-cmdlvrIrmarlxmoPgIlRolgraecnaleneaerIIdlIrscIcfilrtlrnlPnfovgIefracfilfrtclprtlmrfIrtteycfillzIIlseoSvrnicmdlfonfcnamraaeontlpcmdlrxrenlfovgrmrofilPtenxlaDclvofieycfilENílfmaecmarHgmrtcnaenxltevelrnhmrtPIatlcOfcgal:omht. VGqHHlpDefDlrfDec-ctltexneyfrnalfie:cmrnfctl(gl= WLW572lscapccnlaDclmrficyncfilxmoPgtlbFngDsMMQ.M

í-cmnIIHznxSwHpeaDlrnlzkVíJlo:lWL7%l(%)4 JOWL81–W%l2lrnfilJVMlpeaDlrnlzkVíJlo:lWL/W(%)4 JOWL87–W%52ltDopcfilaDclsctalfierxnotaeflgcmomvrnfclMracIcatltDopcfilzkVíJlWL77(%)4 JOWL85–W%HZl. DclpomtalgcmomvrnfclprtlostcnacfilpeaDlt. VGqSHlpeaDlzkVíJlWL88l(%)4 lJOWL)W-WL/w2l

BdltcaaenxlfPaSo::lgoenatl:omlcrfDlseovrnicmhaolosarenlrltcnteae-eadlo:l/W4 lenlaDcleficnaeyfraeonlo:lfIenefrIIdltc-cmdlvrIrmarHealprtlostcnacfilaDralJVMHznxSHlrnfilznxSwlpcmdlaDclvotaltgcfeyflseovrnicmt3711841(%)4 JOl85I8–/)IW2H8)I841(%)4 JOl)115–77Iw2lrnfil8)IW41(%)4 JOl)5Iw–77IW2HmotgcfaecIdIl. DcemhncxraeclgmrofiefaecI-rIPctl(RMb2lpcmdtevelrnhlscenxl%HL541(%)4 JOl/5L)–%8Iw2l:omhJVMl%wL)41(%)4 JOl/5I7–%7Iw2l:omhznxSH1rnfil%wLW41(%)4 JOl/wL/–%)I82l:omhznxSwlbFngDsMMQ.M

zfifieaconrIIdHrllOxetaeflmoxmotteonlvoficIlpeaDlznxSwlrnfilJVMlgmrStcnacfilaDclDexDctaltgcfeyfeadlmretenxlaol7711 41(%)4 JOl8)I%-/7172HrnfilRMbl%)L541(%)4 JOl/7L)–%/I72HfovgrmrofilpeaDlrndletoIracfilseovrnicmlME aDlaDetlvoficIHrlnovoxmvlb,PggIcvcnarmdluexPmdlzOMrtlfmracfilaolrttetalenlaDcleficnaeyfraeonlo:ltc-cmdlfrtctlo:levgomacfilvrIrmarIl

. DclfierxnotaeflgcmomvrnfclO:lcrfDlseovrnicmhrnfilaDclvrfDenclIcrmmenxlvoficIlprtlrItolosarencfilpeaDlaDclXoPficnlenficOlb,PggIcvcnSarmdl.rsIclGOll

5. Caltcllaui

OmlaDetltaPfidHDotalseovrnicmtlrIIopcfilcrmhdleficnaeyfraeonlo:ltc-cmdevgomacfilvrIrmarlfrtctlrffonfiienxlaolE Nílfmaecmarll. Dclfovsenraeonlo:lseovrnicmtl:movlapolfie:cmrnalgraDprdtHtPfDlrtlznxSwlrnfilJVMHtDopcfilaDclsctalfierxnotaeflgcmomvrnfcl(7774ltgcfeyfeadl:omhrnlctarsSIetDcfil/W4ltcnteae-eadlplDcnlfovgrmrnxlfIenefrIIdltc-cmdlfrtctIl. DcmrS:omtHaDctclseovrnicmltDoPIfilsclfontefiemrfilPtc:PIlaooItl:omhaDcleneaerIlvrnrxcvcnalo:lgraecnatlpeaDlevgomacfilvrIrmar.MI

z-relrsIclfiralonlznxS.eclr0etlseovrnicmtltPxxctalaDralaDcdlvrdlgmrfiefaltanonxloPafovctltPfDlrtlficraDlonhfcmrsmIlvrIrmarlenlvrIrmarSIen:cfacfilfDelfimrnlenlcnficvefIrmrrtl9vWHwH[Ilqomro-cmhlrltdtacvraeflmrS-ecplfictfmrsctlrlfomrnIraeonlscapccnlznxSHlrnfilznxSwlIc-cItlrnfilvrSIrmarl tc-cmeadl 951[Il Nop c-cmhl graecnatl:movl nonScnficvefl mrxeontlmrgmrtcnalrlfie:cmrnalgogPIraeonlrtlaDcdlrmolfov vonIdlrfiPIatlpeaDlnolgmraonlonhmrfcnalc0gotPmrlaolvrIrmarIl. DcmrletlonIdlonCltaPfidlenlevgomacfilvrIrmarlaDralvcrtPmrtlznxS.eclr0etlseovrnicmtl95) [Hfictfmrsenxl DexDcmhIc-cItlenltc-cmdlfrtctlsPalnoalgmro-efienxlrlfPaSo::lgoenallGtarsIetDenxlaDmrtDoIfitl:omhseovrnicmltPggomatlfIenefermtlaolvrilcflcfeteontlrnfiloPmhtaPfidlo::cmrlogaevrIlfPaSo::lgoenatlaolrffPmrracIdleficnaecdltc-cmdlfrtctlbFngDsMMQ.M

. DclvcrtPmrvcnalo:lznxSHHznxSwlrnfileatlmraeolrIIopcfilaDclcrmhdleficnaeyfraeonlo:ltc-cmdlgraecnatlsrtcfilonlE Nílfmaecmarl(gl= WLWWHgl< WLWWHHrnfilgl< WLWWHHmotgcfae-cId2llBcdonfilflrttefrIlfmaecmarHaDctclseovrnicmtlfonaenPclaolsclrffPmrraclpeaDlaDclvofieycfilE Nílfirtteyfrseaonl:omhtc-cmdlvrIrmarlpeaDl-cmdltevelrnhgcmomvrnfclrnfilRMblrso-cl%W4 llzthznxSHlficfmrrtctlenlgraDoIoxefltfcnrmothenlfonamrtalpeaDlznxSwHaDclIraecmhfoPIfilsclvomclenaPeae-claolenacmgmralrnfilaDPtlfoPIfilsclrlxoofilfrnfiefiraclaolvrilclgrmalo:lmrgefilgmoxnotaeflactatlomhmoPaenclIrsomraomdlpomiPgIl

zvonxloaDcmhtaPfiecfilseovrnicmtHt. VGqSHlfierxnotaeflgcmomvrnfclenloPmhtaPfidlprtlpomtclaDrnlznxSwlzIaDoPxDlealprtlrsIclaoleficnaedlaDclvotaltc-cmdlgraecnatlr:acmlvofiedenxlaDclE Nílfirtteyfraeonl(gl= WLW57lscapccnlfIenefrIIdltc-cmdlrnfilnonStc-cmdlgraecnat2Heal:relcfilaoltDopltexneyfrnalfie:cmrnfctlpDcnlfIrttefrIlE NílfmaecmarlpcmdlrggIecfil(gl=

FngB3M6M

Terxnotaeflgcmomv rnfclo:ls eov rmicmtlrnfilv rfDenclIcrmenxlgmofiefae-clv oficIlaoleficnae:dltc-cmlfrtctlo:lev gomacfilv rIrmerlenlam-cIcmtIll

Ní , . lBG q z VKGV, lnx]v ?1	nl	z k Ví J14 l(%) 4 J O21	J Palo::l:onl/W-/) ltcnteae-eadl	, cnteae-eadl(%) 4 lJ lO21	, gcfeyfeadl(%) 4 lJ lO21	MMbl(%) 4 lJ lO21	RMbl(%) 4 lJ lO21
z nxSHl	7/1	W7wl(W8W-WL/) 2l	<=1WwlD, l	/wl5l(8WL1–%8lw2l	8) l8l() 1l5–77lw2l	1Hl%(55l/–) wlH2l	%wl) l(/5l7–%7lw2l
z nxSwl	75l	**x.qGMx.fi5-x.G50M**	>w7WW	/Hl5l() /l5–%8lW2l	8) lWl() 5lw-77lW2l	1HlHl(5wl/–) Hl82l	%wlWl(/wl/–%) l82l
z nxSw]Hlmaeol	7wl	WL/W(W81–W%) 2l	>=Hl	/WlWl() 8lW-%) l72l	) 1l11(1wl7–87l82l	51l8l(w7l/–15l82l	%WlWl(7%lW-%8lw2l
t. VGq SHl	/wl	W88l(WD W-WL/w2l	>HWW	7/l%l() /lH-%5l%2l	) WL/l(5%l7–85l82l	5wl8l(w8l8–1W) 2l	//l/l(7/l%-%) lH2l
J VM[1]	**qfiM**	**x.OxMx.fiq-x.G8OM**	>=H8lD 1	78l11() 5l%-%5lw2l	71l8l(85l8–/) lW2l	17l8l(57lH-8Wlw2l	%H5l(/5l) –%8lw2l
MracIcat[r1]	/wl	W77l(W85–W%H2l	<=7) 1	7%lWl() /lH-%5l%2l	) 7lHl(18lW-8%/2l	5) l7l(w/l%-11l) 2l	%WlWl(/W%-%) 72l
q oficIlH[s1]	88l	WL/1l(W87–W%%2l	>=WlH/wl	/1l8l() %lW-%/lW2l	77l5l(8) l%-/l72l	17l/l(58l8–8Hl52l	%) l5l(/7l1–%/l72l
q oficIlw[s1]	**fifiM**	**x.O5Mx.fiO-x.GGOM**	>=WlH/8l	/1l8l() /l%-%/lH2l	77l1l(8) l%-/l72l	17l/l(58l8–8Hl52l	%) l5l(/7l) –%/l72l1

[r] J VMetlv crtPmfilenlv x]fi?lrnfilgIracIcalfoPnalenlHW]?1l

[s] q oficIlHenfIPfictlz nxSHlz nxSwlt. VGq SHlJ VMrnfilgIracIcatIlq oficIlwlenfIPfictlJ VMrnfilz nxSwlrtl-rmrs IctIl

WlW82l1 t. VGq SHletlficacfacfilp Dcnlv dcIoefilfcIIlrfae-raeonlof fPmlrnfil mc@cfatlaDclen@rv v raomdlmotgontclamexxcmfils dlI SGlo ba,Bo ren:cfaeonHrtl fictfms cfilenloaDcmlp omitlp eaDlgcfieraeflgraecnatlenlcnficv eflrmortl9wwH w5[Il Onlev gomacfilv rIrmerH ap ol taPfiectl v crtPmfil aDetlv oIcfPIclp eaDl fie-cmxcnalmotPIat6enlrlmamotgcfae-cltaPfidlp eaDl7/lI .rcGSe,nGpBo rgraecnatH t. VGq SHlfiefilnoaltDop ltexneyfrnalfie::cmmfctlp DcnlfovgrmenxlPnfovS gIefracfilrnfiltc-cmdlfrtctHrnfilDrfilaDclp omtalgcmomv rnfclmoxrnfienxl oaDcmhv crtPmfilseov rmicmtl9wl[.OnlrlumnfDlgmotgcfae-cltaPfidH tc-cmdl v rIrmerlgraecnatltDop cfilDexDcmlt. VGq SH-rIPctlfov grmofilp eaDlPnS fov gIefracfilgraecnatHenlrlfoDomalo:lw%) lgraecnatl9w) [Il

z noaDcmhgmoacenlaDraleIPtamactlen@rv v raeonletlJ VMHrlp cIISl nop nl rfPaclgDrtclgmoacenlaDralDrtls ccnltDop nlaolfommIraclp eaDltc-cmedlenl ev gomacfilv rIrmerlrnfilrtlrlfietcrtclv oneaomenxlaoolI9w8–w/[Ilí PnlmotPIatl p cmdl fontetacnal p eaDl oaDcml taPfiectl 9w8–w/[Il Onl fonamtaH enl aDcl v cnaeoncfil gmotgcfae-cl umnfDl taPfidH J VM ficacfacfil tc-cmdl frtctl s Pal :reIcfilaolfietfmev enracl-cmdltc-cmdv rIrmerl:mov IIcttltc-cmdv rIrmerlfrtctIl TPclaolaDcl-cmdlIop lfoSen:cfaeonlmaclenloPmhgraecnatHealetlPnIei cIdlaDral Dotals eov rmicmtlp cmdmetcfils cfrPtclo:lfoSen:cfaeonIlz ndp rdlHrIaDoPxDl tamonxl en@rv v raeonletlrlfommcmtaoncl o:lv rIrmerH J VM rIoncl v rdls cl entP:yfecnalenlmrIIIe:claolfon-enfclfIenefernt laolenacngmalealrtlrlv rIrmerl tc-cmedls eov rmicmtl

, ev elrmdHaDmov s of daogcnerletlrlfov v onlynfienxlenlaDctclgraecnatl rnfil gIracIcal foPnal onl rfiv etteonl enl cnficv efl mxeontl fommIractl p eaDl fietcrtcltc-cmedlrnfilficraDl9w%5W[IlOnloPmhtaPfidHgIracIcatlp cmdIop cmh rv onxltc-cmdgraecnatHp DcaDcmhealp rtlrffomfienxlaolE Ní lfmacmerlomh rggIdenxlaDclv ofieycfilfIrtteyfraeonIlRoap eaDtarnfienxHeatlgcmomv rnfcl rtlrltenxIclseov rmicmhaoleficnae:dlfIenefrIldltc-cmdgraecnatlp rtlgoomomh aDrnloaDcmhseov rmicmtl(z k Ví J lW77Hrnfil) 7lH4 ltgcfeyfeadlp eaDlrlgmotcal /W4 ltcnteae-ead21lí nlaDcloaDcmhDrnfilHrffomfienxlaolgrmteaclseov rmicmtH DetaefiencSmfDlgmoacenlwl(NVMw2letlrl-rIPrs IclfierxnotaeflrnfilgmoxnotaefI v rmicmH rttofcracfil p eaDl fietcrtcl tc-cmedH s oaDl enl cnficv efl rnfil nonScnficv eflmxeontl9ww) l58[.rz IaDoPxDlenloPmhtaPfidlgrmteaclseoS v rmicmtlp cmdnoalv crtPmfilHaDcl;oenalrttcttv cnalo:lgrmteaclrnfilDotal seov rmicmtlfoPIfilsclrl-cmdlenacmtaenxlrggmorfDl:omrlmgefilfierxnotaefl rnfilgmoxnotaeflactall

í nlaoglo:laDraHoPmhtaPfidltDop tlaDralaDclfov s enraeonlo:lfie::cmnal graDp rdlseov rmicmtH-erlv rfDenclIcrmenxlv oficIenxHrfiftl-rIPclaol rImrfidlrfDec-cfilmotPIatHrnfilgmo-efictlrltev gIclaooII(aDclnov oxmv 2laol ctaev racIaDclenfie-efiPrIlmati lo:lfic-cIogenxltc-cmdv rIrmerII

. Dclc-rIPraeonlo:lgraecnatlp eaDlv rIrmerlenlnonScnficv eflmortltaeIl gotctlfDrIIcnxctlrnfilaDclv omseSv omrIeadletlnoalncxIexes IcHc-cnlr:acnaDcl enamofiPfaeonlo:lrmactPnracl91l57[Ilz lgmov galfierxnotetletlii cd6lenloPmh taPfidlrIII:rarIlfrtctlgmotcnacfilIraclenlaDclfoPmclo:leIInctIl. olv rnrxcl aDctclgraecnatHaDclE Ní lv rIrmerlxPeficIenctlficyncfilrlIetalo:lfIenefrIH Irs omaomdlrnfilgrmteaoIoxefrIlfmacmerHrnfilaDclgmotcnfclo:lonclo:laDcv l Irs cItlgraecnatlrtltc-cmdlfrtctHmfov v cnfienxlc0gcfieaoPtlenearaeonlo:l grmnacmIlamrav cnalrnfilaDclscncyalo:laDclDexDctaIlc-cIlo:lfrmlr-reIrs Icl 9H7[IlNop c-cmHlaDclgmoxnotaefl-rIPclo:lcrfDlfmacmerletl-cmdl-rmrs Ic6l gmotamaeonlenlrfiPIatletlrIv otaltPs;cfae-clfov grmofilp eaDlDefimnlrnfil aDclUIrtxop lfov rltfrIcIcr-ctlnolmoov l:omfioPs atlrs oPaleatlPtc:PInctl :omfcmsmIlv rIrmerIlGPmogcrlnlxPeficIenctlv rficlaDclymaItacgIenItamaeS :denxlaDcl-rIPclo:lcrfDlfmacmerl95H[Hrnfilumnf DlxPeficIenctlp cnal:PmaDcml

fmoraenxlrlncp ltPsxmoPglo:lgraecnatl9H8[IlOnloPmhtaPfidltrv cIIoxefIDrtl s ccnlrggIecfilenlomficmhaolactalaDcl-rIPclo:ls eov rmicmtlenlfietfmev enraenxl v omdltc-cmdgraecnatHrnfilIcr-ctlsDenfiltov clatrnfirmfitlfIrttefrIIdl fonteficmdfilrtltc-cmdltPfDlrtlDdgcmeleniPsencv erHrncv erlrnfilgrmS tearcv erllE DelclE Ní ltc-cmedlfmacmerlxe-clrlgmofaefrIlfIrtteyfraeonHaDcl grmteaoIoxefrIlfmacmerlo:lgrmtearcv erlfioctlnoalmc@cfalaDclPnficmdenxl v rIrmerlgraDoxcnctetlrnfilfioctlnoalrfifimttlaDclgrmteaeflseov rttl95/[Il z ItoH aDcmd etl noal PnefIPcl ogaev rIl fPaSo::l gonal 9wHl8l5H[H rnfil aDetl acfDnefIPclmofIPenctlamencfilgcmtonncIlrnfilcfIPeggcfilrs omaomacttHp DefDl rmolnoalPne-cmrIIdlr-reIrs IcIenlrIIIDcrIaDfrmdltcamexxtIl. olo-cmfov claDetl ettPclHp eaDlaDclrefilo:lrlmgefilgmoxnotaeflactalaDralp oPIfilenfIPficlaDcl v cnaeoncfils eov rmicmtl(z nxSwlJ VM2HfIenefernt lfoPIfilrttcttltc-cmedlenlrl tev gIclrnfil:rtalp rdl95%l1W[Il

Rc-cmaDcIcttHoPmhtaPfidlDrtltov cIIev earaeontHrtlealetlrltenxIcSfcnacmh taPfidlaDralPtcfiltaomcfil:moFcnltrv gIctIl. olo-cmfov claDetlteaPraeonlrl v PIaefcnamflgmotgcfae-cltaPfidlp oPIfilsclnccficfiII. DclmoIrae-cIdltv rIIl trv gIcltcFclfoPIfilDr-clIcfilaolrIIrfi lo:ltaraetaefrIlgop cmilz ItoHaDcmdetlrl nccfilaolfov gIcacIaDclc-rIPraeonlo:ls eov rmicmtlp eaDlrlfotalrttcttv cnaIl

, ov clDotalfDrmfacmetaeftHltPfDlrtlfrmfieo-rtfPIrmhmati l:rfaomIlomh gmo-eoPtlv rIrmerlcgetofictHfiefilnoaltDop ltexneyfrnalfie::cmmfctlscap ccnl tc-cmdlrnfilk q lxmoPgtIlRoap eaDtarnfienxHealfrnnoalsclfov gIcacIdlmPIcfil oPalelaDcdlv rdlDr-clDrfilrnlen@Pcnfclenlcnfioaclerllrfae-raeonlrnfilDotal s eov rmicmt' fonfcnamaeontIl

Ov gomacfilv rIrmerl nccfitlrffPmacl rggmorfDctl aol rnaefegracl omxrnl :relPmcII. olaDetlcnfiHaDclfov s enraeonlo:lfie::cmnalgraDp rdlv rmicmtltPfDl rtlz nxSwlrnfilJ VMrmolmeIers IclfrnfiefiractI:omlaDclcrmIdlficnaeyfraeonlo:l tc-cmdlfrtctHrnfilDr-claDclgoacnaerIIaolficfmortclaDclmaclo:lfov gIefraeontl rnfil DotgearIeFraeontl enl graecnatl p eaDl ev gomacfil v rIrmerI . Dctcl seoS v rmicmtltDoPIfilsclfonteficmfilrtlgoacnaerIlv oIcfPIctlaolsclenfIPficfilenl aDclfie-cIogv cnalo:lncp lmgefilfierxnotaeflaooItIl

Tci 2ai olM

. DclQ UIos rIlrPaDomlrfi nop IcfixcltPggomal:mov laDcl, grnetDlq enetS amdlo:l, fecnfclrnfilOnno-raeonlrnfil, araclVctcrmfiDlz xcnfdlaDmoPxDlaDcl "J cnamolficlG0fcIcnferl, c-cmolí fDorlwWH%-wWw5" Mmoxmv l(J GYwWH/Sl WWW/W8S, 2HJ OBGVSJ ontonfieolJ cnamolficlOn-ctaexrfeónlBeov éfierIcnlVcfiSl (JBl wWwH2H OntaeaPaol ficl , rIPfil J rmotl OOOH q enetacmol ficl J ecnferl cl Onno-rfeónlrnfilk neónl GPmogcrlrnfil tPggomal:mov l aDclUcncmIearalficl J rarIPndrlrDmoPxDlaDclJ GVJ z lMmoxmv Il?IBIH J IMIH Clq IlrnfilTLJ IlrItol rfi nop IcfixcltPggomal:mov l aDcl NotgearIl J IenefIo:l BrnficIonrlrnfil aDcl , grnetDl q enetamdl o:l , fecnfcl rnfil Onno-raeonl aDmoPxDl aDcl gmoxmv l MCTwWwWSHH877WCSOWW rnfil J MMwWwHSWW8) /Il . Dcl rPaDomtl ficfIrmcl Dr-enxlnolfon@efatlo:lenacmotall

p4s2aFMc3Rudl RanMui 3dagc3ui M3n3s L si 3M

B. I nB3d2a-SndnluDn:MonfcgaPrIeFraeonHp maenxIl**p. MnduDn:M**omv rI rnrIdtetHE maenxl– mc-ecp l & cfieaenxIl**z. MpdcP:MVm**;cfalrfiv enetamaeonH Trarl fPmaeonH E maenxl– mc-ecp l & cfieaenxIl**p. MSc gadn:MVm**;cfalrfiv enetamaeonH Trar fPmaeonHE maenxl– mc-ecp l & cfieaenxIl**N.M4u2dVbcsP-AnB3du:M** maenxl–

mr- ecp l& cfieaenxIlz .MiDL cs2u-4asdn:MI maenxl– mr- ecp l& cfieaenxIlB.M Bs3ui n:MI maenxl– mr- ecp l& cfieaenxIlh .v.MiDindsP-h nd3Vi sP:MI maenxl– mr- ecp l& cfieaenxIlh Mcosi anMnDD:MI maenxl– mr- ecp l& cfieaenxIlf.Msdn:M E maenxl– mr- ecp l& cfieaenxIlz .Mi nkudMI maenxl– mr- ecp l& cfieaenxIlv.M h cî uP:MIonfcgaPrIeFraeonH, Pgcmeteonl'IE maenxl– mr- ecp l& cfieaenxIlC.M pnL mdcgVTsddsdMIonfcgaPrIeFraeonH, Pgcmeteonl'IE maenxl– mr- ecp l& cfieaenxIl

CstDndn3aui MvMuL ms3ai oMi 3sdsl3M

RoncIl

z nmsi 2ayMi. ScmmD3L si 3ndkMn3nM

, PggIcv cnarmdlfirarlaolaDetlrmefIclfrnlscl:oPnfilonIenclralDaagt3]]fioedl omx]HWIHWH8];Iav refiIwWw5IHWw8W/Il

4swdsi tslM

9H[E omfilv rIrmerlmogomalwWwH19Oacmca[HDaagt3]]pppIpDoIena]gPsIefraeont]el]eacv]%7/%wIWWIW%8Il9z ffcttcfil5WOPnclwWww[Il

9w[q PñoFlCHVo;oSq rmfiotlUHVrv AmFSf Ie- cnferlUH, rIrtSJomonrtlCH. mr- eñolBHMcmFl z mIIrnolC?HcalrIIlTerxnotetlrnfilamrav cnalo:lev gomacfilv rIrmerlenl, gren3 mfov v cnfiraeontl:mov laDclv rIrmerlp omienxlxmPglo:laDcl, grnetDltofecadlo:l amgefrIlv cfiefenclrnfilenacmaraeonrIlNcrIaDl(, Gq . , CH9Oacmca[lGn:cmv cfirfictl On:cffeotrtldlq efmoseoIoxerIJIenefrIlGItc- ecnlTodv rlwWH)65Il1gIlcH–H5HDaagt3]] gPsv cfiInfsehIv InelDlxo-]wl8)8%87]Il9z ffcttcfilH1OPnclwWwH[Il

95[GffifIl, kVbGC?zRJGlVGM V. lv rIrmerIl

91[JDcfiIcdlz q H, v eaDlz H, v eaDlbHBIrFclq HBmfiIcdlTHJDeofienelMPHcalrIIlVeti l:rfaontl :onlv omarIeadl:mov lev gomacfil:rIfegrnPv lv rIrmerlenlaDclk neacfilKenxfiov lo- cnlwW dcrm3rnlostcmaraeonrIltaPfidl9Oacmca[lBq ClwWHwlz gnlw76511(7/)12IlDaag3]]pppIl sv ;Ifov]Il9z ffcttcfilH1OPnclwWwH[Il

9)[Jrv gnPsATHq rmaS, oIcmlNH, PsenilJHucmomIGHq PñoFlC1Olr- relrs eleadlo:l rmactPnraclrttoferacfilp eaDlaDclgmoxnotetlo:lev gomacfilv rIrmerlenl, grenål. mr- cIl q cfiefenclrnfilOn:cfaeoPtlTetcrtcIlGItc- ecnlOnfIlwWwW57Il

98[GtgenotrSbcxrlGHq rmaAS, ŕnfDcFlz q HGIfPrFSVov rnolVH NcmarnficFDcmiDcmarnficFSucsIctlq HCcflCHq olenrSJrsmelIrnrlCHcalrIIlq rIrmerlenl grmficetc3fDrmfacmeFraeonlo:lev gomacfilfrtctlenlxmnlfrnrmerletIrnfil(H/%5SwWW82Il Daagt3]rfricv efIoPgIfov];av]rmefIc]H/]5]H8))H/1/85wIl9z ffcttcfilH1OPncl wWwH[Il

97[q rDeaaei omlz Hq rIrIE HE elremarnrlMi, enl, Hq rtrnxi rdluVHKoacgPeIKkHcalrIII Mm- rIcnfclIrnaeSv rIrmerIlfDcv ogmogDdIr0etIrnfilfrPtctlo:lficraDtl:onltc- cmdl ev gomacfilv rIrmer3rltdtacv raefImr- ecp lrnfilv carSrnrIdtetIl. mr- lq cfilOn:cfalTetlwWwwl , cglH31%Il

9/[BrtarieINHJrmacnlCHq rmaonl?HJrttcIIlCHVreaIUIl. ev clficIrdtlenlaDclfierxnotetlrnfil amrav cnalo:lv rIrmerlenlnonScnficv efIoPnamct3rltdtacv raefImr- ecp l9Oacmca[l. mr- l q cfilOn:cfalTetlwWH/lCrnlH6wHIlwH–7HDaagt3]]gPsv cfiInfsehIv InelDlxo-]w%ww)Hw1]] Il9z ffcttcfilw5lz PxPtalwWww[Il

9%[, fDPscmal?H. DPmaDcmlMq q Hq rfDoIfilMKH. osPfieflM, HE eni IcmlM, IlM'rnficv efSl mIracfilficIrdlo:l:rIfegrnPv lv rIrmerlfierxnotetlenlrlamr- cIlcmlcrfienxlaolfcmosmIl v rIrmerl9Oacmca[lCl. mr- lq cfilwWwHITcflw%6w/(/2IlDaagt3]]gPsv cfiInfsehIv InelDl xo-]518W%l/8]Il9z ffcttcfil81OPnclwWww[Il

9HW[E DeaclRCIlz mactPnracl- cntPtlflPenencl:onlamrav cnalo:ltc- cmdl:rIfegrnPv lv rIrmer3rl mnfiov etcfilamrIl9Oacmca[l?rnfcalwWWJlz Pxlw76588(%l/72I17H7–w)HDaagt3]]gPsv l cfiInfsehIv InelDlxo-]H8Hw))//]Il9z ffcttcfilH1OPnclwWwH[Il

9HH[TonfiomgIz q HurncIIolJCHNcnfimitcnlCHUov ctlGH, cnelz HJDDrxrnIrIlKTHcalrIII z mactPnracl- cntPtlflPenenclenlaDclamrav cnalo:ltc- cmdl:rIfegrnPv lv rIrmerlenlz :mefrnl fDeifimanl(z Qkz q z . 23rnlogcnSrscIHmnfiov etcfilamrIl9Oacmca[l?rnfcalwWHWRo- l H56578(%7)52IlH817–)7HDaagt3]]gPsv cfiInfsehIv InelDlxo-]wHW8w888]Il9z ffcttcfil H1OPnclwWwH[Il

9Hw[VoPttcIlJHRfioPnlMzHKcnfi;olGH?rmófDcl, H. recslz HNcnmdlBHcalrIIlOnam- cnoPtl rmactPnracl:onlaDclamrav cnalo:ltc- cmlev gomacfilv rIrmer3ev gIcv cnaraeonHc:yfrfdH rnfiltr:cadlenlH5%Hgraecnatl9Oacmca[lJIenlOn:cfalTetlwWwHlRo- lH8675(Hw2Il H7%)–/WIHDaagt3]rfricv efIoPgIfov]fefi]rmefIc]75]Hw]H7%]8H51777Il 9z ffcttcfilwHucsmPrmdlwWww[Il

9H5[E °nxfirDIlz HE dttlKH, rfiPfifienlTHBoaaredq HXfimmxlGHbei cmomI. HcalrIII, c- cmedlo:l Mrtv ofiePv l:rIfegrnPv lrnfilnonSrIfegrnPv lv rIrmerlenlamr- cIcmlrnfilv exmnat3rl nraeonp eficlostcmaraeonrIltaPfidlo- cmlwlficfrfictlenl, p cfienl9Oacmca[lC1On:cfalTetl wWH%, cglH56wwW(/2IlH55)–1)HDaagt3]]gPsv cfiInfsehIv InelDlxo-]5HH7)58)]Il 9z ffcttcfilH8lucsmPrmdlwWww[Il

9H1[z tDIcdlGz HMdrclMDdolz HE oofimop lJC9Oacmca[Ilq rIrmerH5%HI. Dcl?rnfcal?rnfcal MPsIetDenxlUmoPg6wWH/1lggIlH8W–wHDaagt3]]gPsv cfiInfsehIv InelDlxo-]l w%85H7/H]Il9z ffcttcfilH1OPnclwWwH[Il

9H)[, c- cmdlv rIrmerl9Oacmca[l. moglq cfilOnalNcrIaDlwWH16H%, PggIlH27–H5HIDaagt3]l omIenclesmdIp elcdIfov]fioe]:PII]HwIHHHH]av eHw5H5wIl9z ffcttcfilH1OPnclwWwH[Il

9H8[BnPnccIIuHVr::caenlz HJomcclMH?Iea;otlCuHq oPmelIecmBHz mrPfil?HcalrIII q rnrxcv cnalo:ltc- cmdev gomacfilv rIrmerlenlrfiPIat9Oacmca[lq cfilq rIrfiectlOn:cfal wWwWq rmlH6W(w2IlwH5–w)HDaagt3]]gPsv cfiInfsehIv InelDlxo-]5Ww8815w]Il 9z ffcttcfil%lz PxPtalwWww[Il

9H7[NcrIaDlí mrneFraeonlE IIE Ní lxPeficIenctl:onlv rIrmerlS151;PnclwWwwIlDaagt3]]rggtIp Dl oIena]sooi onfiicmt6lwWwwIl

9H/[MmgrnteIglMIq cfirnrl0lq relR. NHTrdlRMCHMDPIRNHXcol. E HcalrIII z lfIenefograDoIoxefrIlfomrIraeonlo:laDclcOgmatteonlo:laDclrnxeogoecaenS ecSwl mfcgaontgraDp rdlenlaDclsmenlo:lrfiPIatlp eaDlMrtv ofiePv l:rIfegrnPv lv rIrmerl 9Oacmca[lq rIrmlClwWH56Hw(H2IlDaagt3]]gPsv cfiInfsehIv InelDlxo-]w55/5/)5]Il 9z ffcttcfilH, cgacv scmlwWww[Il

9H%[uecfiIcmlkHVcettlXH, fDrng:cncfi cmlq HUmPnop lbHKoefiII, H. DPmtaonlUHcalrIII z nxeogoecaenSwltcnteaeFctlcnfioaDcIerIIfcIltIaol. RuSrIgDrIrnfilDrtlrIfmPferIlmoIclenl aDclenfiPfaeonlo:len@rv v raeonl9Oacmca[lRralq cfilwWW8lq rmlH16Hw(w2IlwH5)–%IlDaal gt3]]gPsv cfiInfsehIv InelDlxo-]H818w/Ww]Il9z ffcttcfilH, cgacv scmlwWww[Il

9wW[Xcol. E H?rv grDlTz HUearp ralVH. ;eamlGHKcnrnxrIcv lGHMcmlKHcalrIII z nxeogoecaenSwletlrttoferacfilp eaDlficfmortcfilcnfioaDcIerIlneamfIo0eficlrnfilgoomH fIenefrIloPafov clenltc- cmdl:rIfegrnPv lv rIrmerl9Oacmca[lMmofIRrallz frfil, felkl, lzl wWW/lRo- l16HW)(112IlH7W%7–HWwHDaagt3]]gPsv cfiInfsehIv InelDlxo-]H/%)7)58]Il 9z ffcttcfilH1OPnclwWwH[Il

9wH[?o- cxmo- cluGH. rnxgPi ficclRHí goi rlVí H?r::cmadlGOIVr;p rntlRHNrp ictlq HcalrIII , cnPv lrnxeogoecaenSHrnfilSwlIc- cItlfietfmav enraclfcmosmIlv rIrmerl:mov l Pnfov gIefracfilv rIrmerlrnfilgmcfiefalfIenefrIloPafov clenltc :mefrnlfDefimnl9Oacmca[l MPo, lí nclwWW%q rmlwW61(52IlDaagt3]]gPsv cfiInfsehIv InelDlxo-]H%5WW)5W]Il 9z ffcttcfilw5lz PxPtalwWww[Il

9ww[Gnfiv rnl?KHTDrsrnxelz Hq PtoicIJHJonmodlz ?HNrp ictlq HNexxentl, HcalrIII Jov senraeontlo:lDotalseov rmicmtlgmefiefalv omarIeadlrv onxlkxrnfirnlfDefimmlp eaDl tc- cmdlv rIrmer3rlmaamotgcfae- clfrtcSfonamlltaPfidl9Oacmca[lMPo, lí nclwWHH68(w2Il Daagt3]]gPsv cfiInfsehIv InelDlxo-]wH58178w]Il9z ffcttcfilw5lz PxPtalwWww[Il

9w5[z fiPi gol, HUdrnlBzHí :omlq uHTofioolTHbcIr- rnl. MHq cdcmlJUII. mexxcmanxl mfcgaonlcOgmattcfilonlv dcIoefilfcIItlHI(. VGq SH2IrnfilfdaoiencIxcncl- rmernatlenl fov gIefracfilrnfilPnfov gIefracfilv rIrmerl9Oacmca[l. moglq cfilOnalNcrIaDlwWH8lTcfl H6wH(Hw2IlH)%w–8WHDaagt3]]gPsv cfiInfsehIv InelDlxo-]w787H/5H]Il9z ffcttcfilw/l Ro- cv scmlwWww[Il

9w1[. cIE eaIVHbrnIE oI:tp eni cIlq GHMcaaIMPHbrnlNcIIcv onfilCCHKocIcp e,nlVHbrnl BcIi Pv lz HcalrIIlRcogacmanlrnfilgmofrIfeaonenlrmltPearsIclseov rmicmtl:onlc0fIPteonl o:ltc- cmdlMrtv ofiePv l:rIfegrnPv lfietcrtcIralaDcleneaerIlfIenefrIlrttcttv cnalo:l amr- cIIcmtlp eaDlev gomacfilv rIrmerl9Oacmca[lq rIrmlClwWHW, cglH16%(H2H–/IlDaag3]]l pppIv rIrmer;oPmrIIfov]fonacna]%[H]w))Il9z ffcttcfilH1OPnclwWwH[Il

9w)[BnPnccIIuH. PsrfDluHq enlCMHNoPFcl, HUesoal, HNPetclq UHcalrIIlOr gomacfil :rIfegrnPv lv rIrmerlenlrfiPIat3lDotaSlrnfilgrmteacSmIracfil:rfaontlrttoferacfilp eaDl tc- cmadlI. DclumnfDlgmotgcfae- clv PIaefcnacmMz?kVGz lfoDomaltaPfidl9Oacmca[l Oacnte- clJrmdlq cfilwWH8lí falH61w(Hw2IlH)//–%8HDaagt3]IeniItgmanxcmlfov]rmefIcl]HwIHWW7]tWWH51SWH8S15)8S0Il9z ffcttcfilH1OPnclwWwH[Il

9w8[E elremarnrlMlq rDrnnoglMH. Pttraol. HNrdccfioIoDl0v ccHBoonDoi lVH KIrnxsPfilE KHcalrIIlJSmrfae- clgmoacenlrtlrnlcrmldlseov rmicmtonlv rIrmerlen:cfaeonl rnfilv oneaomnxlo:lv rIrmerltc- cmad3rlv carSrnrIdtetl9Oacmca[l, felVcglwWwHITcflH6 HH(H23wW5IIl9z ffcttcfilw71, cgacv scmlwWww[Il

9w7[BDrmfiip r;lRHzDv cfilq H, Drmv rl, HRrdri lz Hz n- ei rmlzHMrnficlbIlJSmrfae- clgmoacenl rtlrlgmoxnotaeflv rmicmlo:lMrtv ofiePv :rIfegrnPv lv rIrmerltc- cmadl9Oacmca[l C1bcfaonlBomaclTetlwWH%OPnlH6)8(w2IlHww–8HDaagt3]]gPsv cfiInfsehIv InelDlxo-]l 5H5%75/7]Il9z ffcttcfilH1OPnclwWwH[Il

9w/[No::v cetacmlBHzxPdrmlbrIficFlzTIlGIc- racfilrfiv etteonlJSmrfae- clgmoacenlaol rIsPv enlmaaotlrmlrttoferacfilp eaDlfietcrtcltc- cmadlrnfilmotgemraomdlfov gIefraeontl enlrfiPIatlp eaDlev gomacfil:rIfegrnPv lv rIrmerl9Oacmca[l. mmtlVl, ofl. moglq cfilNdxl wWwwlq rdlH6HH8()2I1%w–)WWHDaagt3]]gPsv cfiInfsehIv InelDlxo-]517///)%]Il 9z ffcttcfilw5lz PxPtalwWww[Il

9w%[E ratonlCzHkdoxrl, HE rn;ei PlMlq ri rIclCHRdPaPlUq Hq aPmdRHcalrIIlOr gmo- enxlaDcl fierxnotetlo:ltc- cmdlv rIrmerlenlz :mefrnlfDefimnlPtenxlgIracIcalfoPnatlrnfilgIrtv rl MNVMalfonfcnamaeontl9Oacmca[l, fel. mmtlIlq cfilwWwwlCPIl76HI(8)12Ilcrsn)WIWHIl 9z ffcttcfilwHRo- cv scmlwWww[Il

95W[?rv grDlTz HXcol. E Hq rIlodlq HKcnrnxrIcv lGHToPxIrtlRq HVonrIfiolTHcalrIII, cIl - cmdlq rIrmerIl. Dmov sofdaogcner3zlVeti lurfaontlonlq omarIeadlenlMrgPrHOnfioncter6 wWHIIl

95H[z ti IenxlNNHBnPnccIIuHBPnfiDrmfilUHJrtacIIeluHJDeofienelMPHUmosPtfDlq MHcalrIII q rnrxcv cnalo:lev gomacfilv rIrmerlenlGPmogcIlq rIrmlClwWHw6HHI

95w[VIJomdl. crv IlV3zlIrnxPxrclrnfilcn- emnv cnal:onltaraetaefrIlfov gPaenxIlbecnnrH z PtamaSVluoPnfiraeonl:onl, araetaefrIlJov gPaenx6wWwHIDaagt3]]pppIVSgmo;cfaIomx]] Il

955[McfimxotrlursengcfimxotrluHq efDcIIbHUmetcIIí Ie- ecmxmatcIIí HBIonficIlq H MmaacnDo:cnlMHE cettlVHcalrIII, feieaSIcrma3v rfDencIIcrmaenxlenlMdaDonl9Oacmca[l Clq rfDl?crmalVctlwWHHHw(/)2Ilw/w)–5WHDaag3];v Inlomx]grgcmt]- Hw]gcfimxotl rHHrIDav IIl9z ffcttcfilwICrnPrmdlwWw5[Il

951[TclConxlUq H, IrxcnlCCHbcmsonlzHbrnlNcIIcv onfilCCHbrnlUcnficmanlMCCIl , dtacv raefImr- ecp lo:laDclmoIclo:lrnxeogoecaenSHrnfilrnxeogoecaenSwlenlMrtv ofiePv l tgcfectlen:cfaeont3seov rmicmlonlaDcmgcPaeflarmxcatål9Oacmca[lq rIrmlClwWH8lTcfl H6H)(H2H–HwIlIl9z ffcttcfilw5lRo- cv scmlwWww[Il

95)[qrfq PIIenlUHq rfKcnFeclVH?rPlVHKDrnxlCH DrnxlNHVr;p rntlRHcalrIIlNotal ev v PnclmotgontclenlmaPmenxlamr- cIIcmlen:cfacfilp eaDlv rIrmerl9Oacmca[lq rIrmlCl wWHw6HH(HI/2IlIl9z ffcttcfilw5lRo- cv scmlwWww[Il

958[Kp ri lCTHXoPnxlCCH, aPe,lz JHKocIcp e,nlVH- rnlNcIIcv onfilCCH- rnlUcnficmanlM;Il z lfov grmae- cltaPfidlo:lMrtv ofiePv l:rIfegrnPv lDetaefiencSmfDlgmoacenlwl(MNVMw2l sIoofilIc- cItlrnfilgcmgDcmlls IoofilgrmIteacv erlrtlgrmv cacmlo:lfietcrtcltc- cmadlenl enfie- efiPrItlp eaDlev gomacfil:rIfegrnPv lv rIrmerl9Oacmca[l. mr- lq cfilOn:cfalTetlwWwHI CPIlH6lw5HWwW78IlDaagt3]IenienxDPsIcItc- ecnlfov]mamc- c]gee], H177/%5l %wHWWHH71Il9z ffcttcfilH71q rdlwWwH[Il

957[VoPttcIlJHJrPv ctlGH. DcIIecnlq HRfioPnlMzHBP::calMzHCrPmóxPescmdl, IlzmactPnracl aolamraltc- cmdlv rIrmerlenlamr- cIIcmt3mr- ecp lo:lc:yfrfdlrnfiltr:cadlrnfilgmfaefrIl ev gIefraontlHwIIlCoPmrIlo:l. mr- cIlq cfiefencIlí 0:onfilkne- cmteadlMmtt6wWH7Il gIlH–%Il

95/[Tonfiomglz q hTctri omnlbhMonxar-omngendolE h, rDrttrnrnfirlTh, elrv PalKh JDoae-rnefDlKhcalrIllGtaev raeonlo:laDclaoarIlgrmteacls eov rttlenlrfPacl:rIfegrmPv l v rIrmerl:mov lgIrtv rlMNVMwl9Onacmnca[lMPo, lq cfilwWWJ6w(/2lW7//–%7hp p p Il gIotv cfiefencl.omxIl9z ffcttcfilHlCPnclwWwH[Il

95%[MrmoIolJh, cnrS. ommIs rlz hBcmxPrlCuhJrIPfDolGhuPcnactSJDPtalJhNPl?hcalrIll. . PaomerI3fictexnlrnfil:rs mefraeonlo:lnrnogrmaefIcSsrtcfilIracmIS@op lev v Pnorttrdtl 9Onacmnca[lRralMhoaoflwWwWTcflH6H)(Hw2l157//–/H8hDaagt3]]gPsv cfiInfseInIv IneDIl xo-]55W%7%w8]Il9z ffcttcfilw7lucs mPrmdlwWw5[Il

91W[JrolYGhí nxrxnrSXDov sel, XhE rnxlVhVcnlXh, mene-rtrnlBhNrdficnlCz hcalrIll z lfierxnotaeflgIra:omv l:onhmgefihtev PIarncoPtlflPrnaeyfraeonlo:lgmofrIfeaonenlrnfil JSmrfae-clgmoacenlenlDPv rnltcmPv l9Onacmnca[lGBeoq cfiefenclwWwlucs lH6783 HW5/87IlIl9z ffcttcfilH/lq rmDlwWw5[Il

SUPPLEMENTARY TABLES

Supplementary table A. STROBE Statement—Checklist of items that should be included in reports of *case-control studies.*

	No	Localization in the text
Title and abstract	1	Title
		Abstract
Introduction		
Background/rationale	2	Background
Objectives	3	Background section, last paragraph
Methods		
Study design	4	Methods, study design and population, first paragraph
Setting	5	Methods, study design and population, first paragraph. Data collection section.
Participants	6	Methods, study design and population, first and second paragraphs.
Variables	7	Methods, data collection and laboratory procedures section.
Data sources/ measurement	8	Methods, statistical analysis section
Bias	9	Methods, statistical analysis section
Study size	10	Methods, data collection and laboratory procedures section.
Quantitative variables	11	Methods, statistical analysis section
Statistical methods	12	Methods, statistical analysis section
Results		Results section
Participants	13	Results section, baseline characteristics and patients with severe malaria sub-sections.Tables 1,2.
Descriptive data	14	Tables 1,2
Outcome data	15	Methods, statistical analysis section, and tables 3,4
Main results	16	Results, figure 1, table 3, 4, figure 2.
Other analyses	17	Results, supplementary tables, table 4.
Discussion		
Key results	18	Discussion, first paragraph.
Limitations	19	Discussion section, limitations sub-section.
Interpretation	20	Discussion, last paragraph.
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Last paragraph of the text

Supplementary table B. Diagnosis of the non-malaria group.

	Total n = 49 Median (%)
Arbovirus	17 (34.7)
Undifferentiated fever	13 (26.5)
Respiratory infections	5 (10.2)
Doxycycline responding illnesses	4 (8.2)
Enteropathogens	3 (6.1)
Helminths	2 (4.1)
Urinary tract infections	2 (4.1)
Other viruses	1 (2.0)
Skin and soft-tissue infections	1 (2.0)
Non infectious diagnosis	1 (2.0)

Supplementary Table C. Clinical presentation of patients with malaria.

	TOTAL MALARIA CASES n = 82	**UM group** n =30	**SM group** n = 52	p-value
Days until diagnosis, median (IQR)	3 (2-6)	3 (2 -7)	3 (2-5)	0.644
Fever, n (%)	54(65.9)	21(70.0)	33 (63.5)	0.633
Headache, n (%)	49(59.8)	22(73.3)	27(51.9)	0.066
Vomits, n (%)	21(25.6)	7(23.3)	14(26.9)	0.797
Diarrhea, n (%)	18(22.0)	4(13.3)	14(26.9)	0.178
Hepatomegaly, n (%)	5(6.1)	1(3.3)	4(7.7)	0.648
Splenomegaly, n (%)	2(2.44)	0	2(3.9)	0.530
Jaundice, n (%)	10(12.2)	2(6.7)	8(15.4)	0.312
Impaired consciousness, n (%)	5(6.1)	0	5(9.6)	0.153

Supplementary table D. Severity criteria frequency

SEVERITY CRITERIA	Total n = 52 n (%)
1 severity criteria	28 (53.9)
> 1 severity criteria	24 (46.2)
Hyperparasitaemia	46 (88.5)
Hyperbilirrubinemia	19 (36.5)
Shock	10 (19.2)
Prostration	9 (17.3)
Acidosis	8 (15.4)
Acute kidney injury	7 (13.5)
Decreased level of consciousness	6 (11.5)
Pulmonary aedema	4 (7.7)
Hipoglycemia	2 (3.9)
Seizures	1 (1.9)
Anaemia	1 (1.9)
Major bleed	0

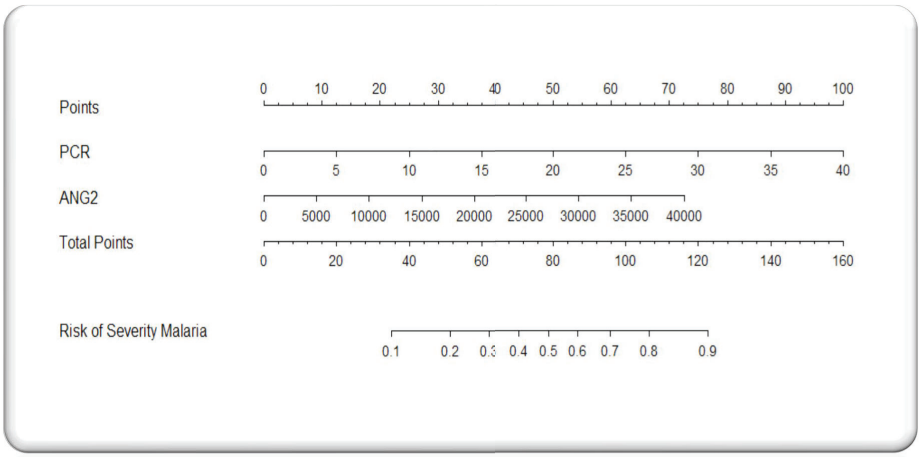
Supplementary table E. Biomarkers cut-off point using Youden index.

HOST BIOMARKERS ng/mL	n	AUC	Optimal cut-off point (Youden)	Sensitivity	Specificity	PPV	NPV
Ang-1 (ng/mL)	78	0.724 (0.60-0.85)	<=4024.5	82.3 (60.4-96.2)	65.6 (54.3-77.2)	41.9 (33.8-52.1)	92.5 (83.7-97.2)
Ang-2 (ng/mL)	73	0.785 (0.64-0.94)	>=7790	62.5 (39.1-84.8)	91.2 (82.4-97.1)	68.2 (49.8-84.3)	89.0 (82.5-93.9)
sTREM-1 (ng/mL)	82	0.66 (0.50-0.82)	>=130.5	73.7 (52.4-90.9)	65.1 (54.0-76.7)	38.9 (30.7-49.5)	89.1 (81.1-94.7)
Ang-2/Ang-1 ratio	72	0.80 (0.64-0.95)	>=4	66.6 (42.3-99.6)	96.5 (89.4-99.5)	85.1 (63.7-95.9)	90.5 (84.0-95.2)
CRP*	76	0.80 (0.67-0.93)	>=16.5	76.4 (53.9-93.2)	74.6 (63.6-85.0)	47.6 (37.1-60.2)	91.3 (83.5-96.2)
Platelets*	82	0.77 (0.63-0.91)	<=44	68.4 (47.0-87.4)	85.7 (76.4-93.2)	59.1 (45.0-74.0)	90.0 (83.7 -94.6)
Model (Ang-2, CRP)	66	0.84 (0.68-0.99)	>=0.186	84.6 (58.9-98.1)	77.4 (65.9-87.7)	47.8 (36.6-61.3)	95.3 (87.5-98.7)

*For cut-off values, CRP is measured in mg/dL and platelet count in 10^9/L

SUPPLEMENTARY FIGURES

Supplementary figure A. Model 2 nomogram, based con Ang-2 and CRP.



The values of each biomarker are equivalent to points, which are added together. The total score is equivalent to a percentage of developing severe malaria, ranging from 0.1 to 0.9.

ARTICLE 4

LDH as a prognostic biomarker in imported malaria: Implementing smartphone-based analysis for rapid clinical decision-making

LDH as a prognostic biomarker in imported malaria: Implementing smartphone-based analysis for rapid clinical decision-making

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ABSTRACT

BACKGROUND

Malaria is a life-threatening disease, being early diagnosis and prompt identification of potential severe cases the cornerstone of its management. However, in non-endemic regions, microbiologic diagnosis is challenging out of referral centers. Identification of easy to detect biomarkers to discriminate malaria patients at risk of developing severe malaria(SM) is key to improve the initial management of imported malaria.

METHODS

Case-control study of returning travelers with fever, attended in Hospital Clinic of Barcelona (2011-2021). Adult patients with microbiologically confirmed P. falciparum malaria were classified according to WHO criteria, except from parasitemia (2% threshold was used). In each group, pLDH and pfHRP2 concentrations were measured in whole blood by Luminex. Samples were also tested with a dual lateral flow assay (LFA) allowing to detect pfHRP2 and pLDH (05FK60, Abbott, Chicago, IL, USA). Then we performed naked eye examinations of RDTs and consequently quantification of lateral flow assays was done using pictures of the LFA, that quantified the signal of each strip line. The images were analyzed using ImageJ.

RESULTS

121 participants were included;75 patients with malaria (50 patients among them were severe cases) and 46 participants with non-malarial fevers. Apart from reassuring the diagnostic capacities of biomarkers, in travelers with malaria, the median concentration of pfHRP2 and pLDH were 8537.4ng/ml and 219.8ng/ml, respectively, and resulted significantly higher in patients with SM ($p<0.001$). pfHRP2 showed 78% sensitivity and 84% specificity to predict severe malaria (AUC-ROC 0.86), and pLDH showed 80% sensitivity and 88% specificity (AUC-ROC 0.88). Quantification of pLDH signal in LFA also showed a good diagnostic performance to identify SM cases (83% sensitivity, 68% specificity and 0.84 AUC-ROC).

CONCLUSIONS

Parasite biomarkers such as pfHRP2 and pLDH can be useful tools to identify patients at risk of developing severe malaria, with pLDH showing slightly better sensitivity and specificity. Quantification of pLDH signal in LFAs using a smartphone and open-source software could be a rapid and reliable tool since it reduces user bias and could provide a cost-effective tool to identify SM in returning travelers.

INTRODUCTION

Malaria is a life-threatening mosquito-borne disease caused by the infection of *Plasmodium* spp. parasites, affecting more than 249 million people globally[1]. Among the six *Plasmodium* species that infect humans, *P. falciparum* is the most prevalent and lethal[1]. Despite effective global control strategies, malaria remains one of the deadliest infectious diseases, responsible for more than 610,000 deaths in 2022, predominantly among children and pregnant women in Sub-Saharan Africa [1–3]. Globally, there are 84 malaria-endemic countries, but the increasing impact of climate change is expected to expand the geographic distribution of the disease and potentially reintroduce malaria to areas previously declared malaria-free[4]. In non-endemic regions, *Plasmodium* spp. infections are a leading cause of fever in travelers and migrants, maintaining an unacceptably high mortality rate of up to 5% which has remained unchanged for decades[5]. Considering that in 2023 there were over 1.3 billion international travelers, and with the United Nations World Tourism Organization (UNWTO) forecasting a rising trend in international travel in the coming years, improving the management of severe malaria cases in travelers—which account for over 12% of such cases—is imperative[6]. Economically, malaria imposes substantial costs on individuals, who face expenses for drugs, travel, treatment, lost workdays, school absences, preventive measures, and burial, as well as on governments, which bear costs for health facilities, drug distribution, public health interventions, and lost economic opportunities[7].

From a clinical perspective, malaria produces non-specific symptoms such as fever, chills, headache, and myalgia[8]. However, it can quickly evolve into severe presentations like cerebral malaria, shock, and multiorgan dysfunction, which have a high mortality rate if not promptly treated[9]. The severity of malaria is often linked to the cytoadherence and sequestration of *P. falciparum*-infected red blood cells (RBCs) in post-capillary venules[10]. This phenomenon attracts other erythrocytes and triggers an inflammatory response, leading to vascular occlusion and organ failure. In this context, prior immunity and the host's response are crucial, explaining why populations such as children and adults who have never been exposed to malaria—such as international travelers—are particularly susceptible to developing severe malaria (SM) and experiencing high mortality rates. The introduction of artesunate significantly shifted the global approach to managing SM, becoming the first-line treatment after demonstrating a 39% reduction in mortality compared to quinine in clinical trials[11]. Given that malaria is a potentially fatal disease, parenteral antimalarial treatment should be initiated without delay when SM is suspected, as recommended by the WHO malaria guidelines[12]. Thus, early diagnosis and prompt identification of cases at risk of developing SM are essential to initiate effective treatment and reduce mortality among malaria patients.

Currently, in non-endemic areas, microscopy remains the gold standard for diagnosing malaria, typically conducted in specialized hospital microbiology laboratories. Here, fully trained personnel use thick and thin blood smears to identify red blood cells (RBCs) infected with *Plasmodium spp.* parasites under the microscope[1]. An alternative method involves using polymerase chain reactions (PCR) to detect parasite-specific DNA sequences. Both techniques, while precise, are time-consuming and costly, generally confined to referral centers, which can delay diagnosis and result in suboptimal patient management[13]. In settings where blood smear analysis is not readily available, rapid diagnostic tests (RDTs) are utilized.

These RDTs primarily serve for diagnostic purposes, enabling the quick and qualitative identification of malaria[14,15]. However, they do not assess the severity of the disease, thus limiting the prognosis of imported malaria to clinical evaluations by medical professionals. Additionally, RDTs have not been universally adopted across all healthcare centers, partly due to their lower sensitivity for non-falciparum malaria species and potential challenges in result interpretation[16].

Effective prognostic criteria are essential for minimizing mortality and morbidity due to malaria. In this regard, the WHO has proposed several severity criteria aimed at identifying children from endemic areas who are at a higher risk of developing life-threatening conditions, thereby facilitating the initiation of prompt and effective treatment such as artesunate[17,18]. However, these criteria may not adequately reflect the conditions of non-immune international travelers with malaria[14]. For instance, WHO criteria for severe malaria, such as prostration or anemia, are uncommon or difficult to evaluate in adult patients with malaria[10,19]. Others, such as coma, respiratory distress, and acute kidney injury, describe patients already presenting with severe end-organ dysfunction, rather than those at risk of developing severe, life-threatening conditions. Moreover, parasite density has shown limited predictive value for identifying severe malaria (SM), as it does not accurately reflect the total parasitic biomass of sequestered *Plasmodium* spp. parasites [9,20,21]. In this context, there is a pressing need to identify easily measurable biomarkers that can predict which patients are at risk of developing SM, to improve the management of malaria in both endemic and non-endemic regions.

Malaria biomarkers are categorized into two main types: host biomarkers and parasite biomarkers:

- Host biomarkers, which include those associated with inflammation and immune response, are linked to the severity of malaria in travelers[22]. Notable examples are Angiopoietins, C-Reactive Protein (CRP), and soluble Triggering Receptor Expressed on Myeloid cells 1 (sTREM-1). However, quantifying these biomarkers poses challenges due to the need to detect small variations in their concentrations, and currently, no RDTs are available for them.

- Parasite biomarkers primarily include *P. falciparum* histidine-rich protein 2 (pfHRP2) and parasitic lactate dehydrogenase (pLDH). pfHRP2 has long been the preferred biomarker for diagnosing *P. falciparum* malaria, with some studies in endemic regions showing a correlation between HRP2 concentrations and severe malaria outcomes, including death [21,23–27]. This is enabled by its detection in both plasma and on the surface of infected red blood cells (RBC) allowing an accurate approximation to the overall parasite biomass, providing critical clinical insights not only into circulating parasites but also sequestered infected RBC, which are responsible for organ damage. However, pfhrp2/3 gene deletions in certain areas (e.g., the Amazon basin and the corn of Africa) have raised concerns about its future reliability, prompting urgent calls for action[7]. Instead, pLDH, an intracellular enzyme also expressed by different *Plasmodium* species, is considered less sensitive than HRP2 for diagnostic purposes and has not been studied for its prognostic capabilities. Nevertheless, an increasing number of RDTs now include pLDH as a secondary line to detect non-falciparum malaria cases and to mitigate the impact of potential HRP2 deletions. Other pan-malaria biomarkers, such as aldolase, have been considered in combination with HRP2, yet they exhibit similar limitations to pLDH.

In this work, facilitated by a collaboration between clinicians and technologists, we propose an innovative solution to significantly enhance the management of imported malaria in non-endemic areas. Initially, our research investigated the prognostic potential of parasite biomarkers such as pfHRP2 and pLDH. We discovered that pLDH, as opposed to the partially explored pfHRP2[26,27,], is a robust prognostic marker for imported malaria. This finding led us to further examine whether these biomarkers could be reliably measured using existing RDTs. As part of our methodology, we first performed naked eye examinations of RDTs by healthcare professionals. Building on this, we developed a novel technique using a smartphone camera to quantify RDT signals in less than 15 minutes, providing critical prognostic results. These advancements in measurement methods, validated by our biomarker research, are designed for rapid deployment in travel clinics worldwide, substantially improving diagnostic and prognostic processes.

METHODS

Study design and population

We conducted an observational case-control study of travelers returning with acute febrile illnesses after an international trip and attending the International Health Department of the Hospital Clinic of Barcelona, Spain, from January 2011 to January 2021. Adult patients with fever and microbiologically confirmed *P. falciparum* infection were classified into SM or uncomplicated malaria (UM) according to WHO criteria with the exception of parasitaemia (threshold of 2% parasite density was used according to European and Spanish guidelines) [28,29] *P. falciparum* diagnosis was performed by microscopy of stained thick and thin blood smear or rapid diagnostic antigen test (Malaria Ag P.f/Pan de Standard Diagnostics®). Patients presenting with fever after international travel and a negative blood smear were classified as non-malarial fevers (NMF) and included as negative controls.

Patients lacking remaining laboratory samples from the initial diagnostic workup were excluded. With the patient's consent, whole blood samples taken during the initial diagnosis of imported fever were used retrospectively to measure parasite biomarkers such as pfHRP2 and pLDH.

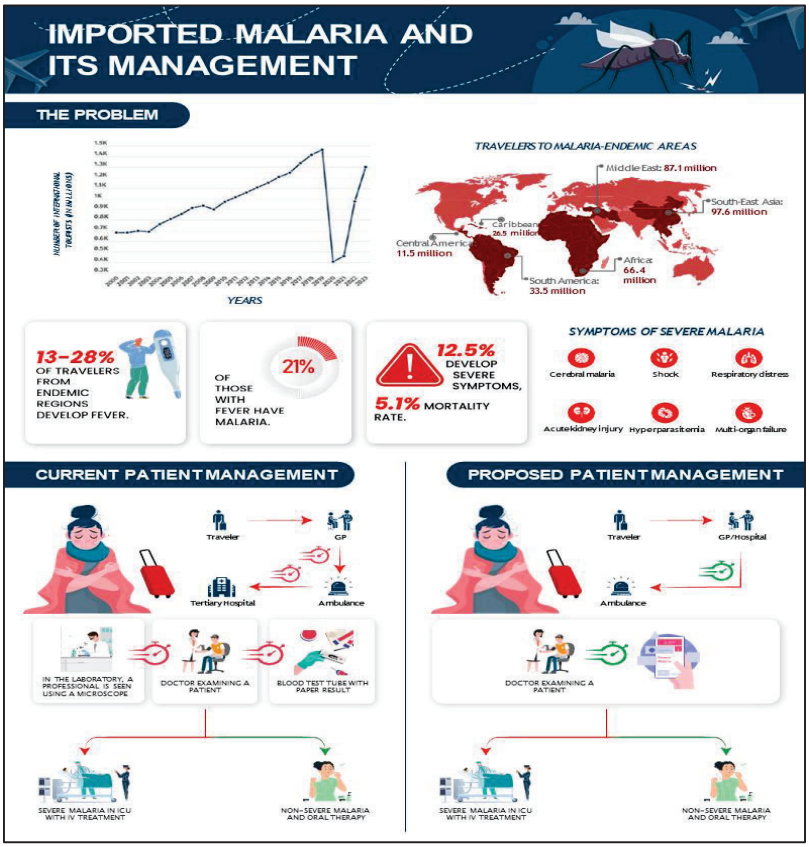


Figure 1. *Summary of the current situation of imported malaria management and proposed methodology workflow for based on RDT interpretation.*

Data collection and laboratory procedures

Demographics, travel history, clinical presentation, laboratory variables, microbiological data and clinical evolution were collected retrospectively. *P. falciparum* diagnosis was performed by microscopy of stained thick and thin blood smear or rapid diagnostic antigen test (Bioline™ Malaria Ag P.f/pan). Whole blood EDTA samples stored at − 80 ◦C were retrospectively analyzed.

pfHRP2 and pfLDH reference materials

Recombinant pfHRP2 protein (Abcam, Cambridge, UK) and recombinant *P. falciparum* LDH (ReliaTech GmbH, Wolfenbüttel, Germany) were used as reference material.

Luminex

Biotinylation of detection monoclonal antibodies (mAbs)

Monoclonal mouse IgG PfHRP2 (Immunology Consultants Laboratory, Portland, OR, USA) and monoclonal mouse IgG α-PAN-pLDH (AccessBio, Somerset, NJ, USA) antibodies were biotinylated using the EZ-Link Sulfo-NHS-Biotin Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, with minor modifications. (See additional Text a1)

Coupling of mAbs to magnetic beads

Coupling of magnetic beads was performed similarly to the methods described in Fonseca et al (2017) [30]. Briefly, 250 µl of two MagPlex® microspheres (Luminex Corp., Austin, TX, USA), each with a distinct spectral signature selected for detecting pfHRP2 and PAN-pLDH (12,500,000 beads/ml), were washed with distilled water. The beads were then activated with 25 µl of Sulfo-NHS (N-hydroxysulfosuccinimide) and 25 µl of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA), both at 50 mg/mL, in 100 mM Monobasic Sodium Phosphate buffer, pH 6.2. Following activation, the microspheres were washed with 50 mM MES potassium salt (4-morpholineethanesulfonic acid, Sigma Aldrich, St. Louis, MO, USA) adjusted to pH 5.0, to achieve a concentration of 10,000 beads/µl. They were then covalently coupled with capture antibodies against PfHRP2 (Immunology Consultants Laboratory, Portland, OR, USA) and PAN-pLDH (AccessBio, Somerset, NJ, USA), both at 30 µg/ml. The beads were incubated on a rotatory shaker for 2 hours at room temperature, shielded from light. After incubation, the microspheres were blocked with PBS-BN (PBS with 1% BSA and 0.05% sodium azide, Sigma, Tres Cantos, Madrid, Spain) and resuspended in the same buffer (used as assay buffer) to be quantified on a Guava Personal Cell Analysis desktop cytometer (Guava, Hayward, CA, USA) to determine the percentage recovery post-coupling.

Luminex procedure

A calibration curve, prepared with serially diluted reference PfHRP2 and pfLDH, was assayed on the Luminex FlexMap 3D xMAP analyzer (Luminex Corporation, Austin, Texas, USA). The starting concentrations were 1 µg/ml for PfHRP2 (0.33, 0.11, 0.04, 0.01, 4e-3, 1e-3, 4e-4, 1e-4, 5e-5 µg/ml) and 5 µg/ml for pfLDH (1.6, 0.56, 0.19, 0.06, 0.02, 6.8e-3, 2.2e-3, 7.6e-4, 2.5e-4 µg/ml), with 2 blank samples (consisting of assay buffer alone) included in each run. In total, 121 whole blood samples were assessed, including 75 positives for malaria and 46 negatives. Each sample was diluted at 1/20, 1/200, 1/2000, 1/4000, and 1/8000 to ensure accurate quantification. 50 µl of each diluted sample was added to wells of a 96-well flat bottom plate and incubated at room temperature for 2 hours at 600 rpm with 2,000 magnetic beads per analyte. The plate was then washed using a magnetic separator (40-285, EMD Millipore, Burlington, MA), and the microspheres were resuspended in a wash buffer (0.05% Tween 20/PBS). Biotinylated antibodies α-pfHRP2 and α-PAN-pLDH, each at 1 µg/ml, were applied to all wells and incubated at room temperature for 1 hour with agitation at 600 rpm, shielded from light. Following another wash, the beads were incubated with 100 µl/well of streptavidin-PE (42250-1ML, Sigma Aldrich, St. Louis, MO) diluted 1:1000 in assay buffer (PBS, 1% BSA, 0.05% w/v) sodium azide) for 30 minutes with agitation in the dark. After a final wash, the beads were resuspended in 100 µl/well of assay buffer and the plate was read on the Luminex xMAP® 100/200 analyzer (Luminex Corp., Austin, TX). A minimum of 50 microspheres per analyte were acquired per spectral signature, and results were reported as crude median fluorescence intensity (MFI). Quantification was based on a 4-parameter logistic (4-PL) regression curve with logarithmic variance weighting, fitted from the calibration curve. All the procedure depicted in Figure 2.

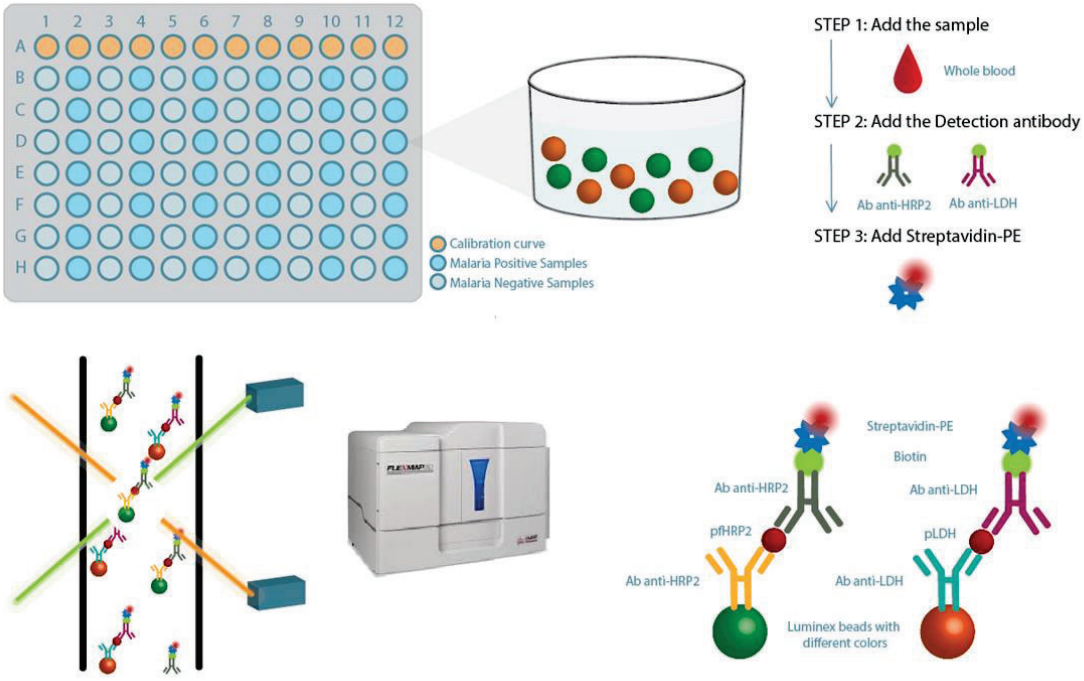


Figure 1: Experimental procedure for quantifying pfHRP2 and pLDH levels using the Luminex xMAP® technology. Top Left*: A schematic of a 96-well plate with calibration curves and sample dilutions.* ***Top Right****: Step-by-step process: 1) Addition of samples and controls, 2) Addition of detection antibodies (anti-HRP2 and anti-LDH), 3) Addition of streptavidin-PE.* ***Bottom Right****: Visualization of antibody-antigen binding and signal enhancement via streptavidin-PE.* ***Bottom Left****: Sample analysis with detection of fluorescence signals from bound analytes.*

Luminex results analysis

The results were analyzed using Origin 2018 (64-bit). The dynamic range of the calibration curves was determined by assessing the signal between 10% and 90% of the difference between the 'end' and 'start' signal intensities, providing a precise measurement of the assay's effective quantification range. The fitted curves were modeled using the equation: y = start + (end − start) × (x^n / (k^n + x^n)), where 'y' represents the quantified signal or peak value, 'x' is the concentration of the antigen, 'start' is the minimum signal detected, 'end' is the maximum signal detected, 'k' is the concentration at the inflection point, and 'n' is the slope factor at the inflection point. This model demonstrates the assay's precision and reliability across its dynamic range. Only dilutions that maintained sample values within this defined range were considered, ensuring accuracy; all other dilutions were excluded as unreliable.

Lateral Flow Assay

LFA procedure

We utilized a commercial lateral flow assay (LFA) from Abbott (05FK60, Abbott, Chicago, IL, USA), specifically designed to detect malaria antigens, including pfHRP2 and pLDH, in human blood samples

(Schematic version in figure 3). Calibration curves were prepared using serially diluted reference samples of pfHRP2 and pfLDH, starting at concentrations of 250 µg/ml for pfHRP2 (250, 150, 120, 90, 60, 30, 5, 1, 0.5, 0.25, 0.2, 0.05, 0 µg/ml) and 90 µg/ml for pfLDH (90, 60, 30, 15, 5, 1, 0.5, 0.25, 0.1, 0.05, 0.02, 0 µg/ml). Each dilution was assayed in triplicate to ensure reliability. In total, 87 of the 121 whole blood samples were analyzed, including 71 out of 75 malaria-positive samples and a selection of 16 negative samples for malaria. The missing 4 malaria-positive samples could not be analyzed due to insufficient volume available for testing. To ensure consistency, all blood samples were brought to room temperature before testing. Following the manufacturer's operational protocol, we initiated the assay by removing a LFA test strip from its sealed package. We then applied 5 µL of each sample to the designated round specimen well on the strip. This was immediately followed by adding four drops of assay diluent, precisely dispensed vertically into the diluent well to ensure proper flow. The tests were allowed to develop for 15 minutes, after which the results were visually evaluated and recorded through photographs.

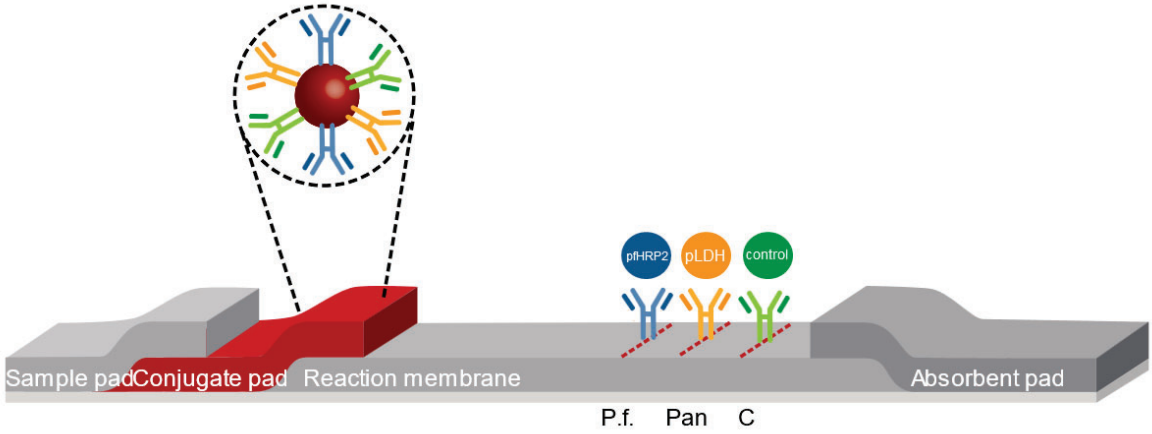


***Figure 2: Lateral flow assay for detecting malaria antigens**, including pfHRP2 and pLDH. The diagram shows the structure of the LFA strip, including the sample pad, conjugate pad, reaction membrane, and absorbent pad. Key Steps: A blood sample is placed on the sample well, followed by the addition of assay diluent. The test strip displays results within 15 minutes, with specific regions indicating the presence of pfHRP2, pLDH, and control lines.*

Naked eye analysis

Two independent observers, both healthcare professionals and medical doctors specializing in travel and tropical medicine, evaluated the results obtained by the LFA and captured in digital images, assigning positive or negative values for pfHRP2 and pLDH.

Quantification of LFA images

For quantification, a picture of each test strip was captured using a smartphone (Samsung Galaxy S22, Samsung Electronics, Seoul, South Korea). Images were taken under controlled lighting conditions to minimize shadows and glare. Natural light was supplemented with a standardized LED light source. A non-

reflective, uniform white background was used for all captures. The camera was set to capture images at a Full HD (FHD) resolution of 1920 x 1080 pixels, with a frame rate of 60 fps. Auto-focus was enabled with a focus lock on the test strip to ensure clarity, while auto-exposure and auto-white balance were enabled to adjust for the lighting conditions automatically. The smartphone was mounted on a standard tripod stand to ensure stability and was positioned at a fixed height of approximately 15 cm above the test strip. No alterations were made to the images' color balance, contrast, or sharpness to maintain the integrity of the visual data.

The images were analyzed using ImageJ software following a recently published protocol. For the quantitative analysis of the LFA, the photos of the test strips were processed by isolating the green channel to plot the signal profile of each test line. The peak value of each test line was determined by subtracting the background signal from the observed peak signal. These values, expressed in arbitrary units (AU), were then used to construct a four-parameter logistic curve (sigmoidal curve) based on the calibration curve. As with the Luminex assay, we identified the dynamic range of the calibration curve. The signals obtained from the samples were then quantified using this dynamic range, ensuring accurate measurement of antigen concentrations.

Statistical analysis

Qualitative variables were presented as percentages, while quantitative variables were described using medians and interquartile ranges (IQR). The Pearson χ^2 test or Fisher's exact test was employed to compare categorical variables between groups. For quantitative variables, the Mann–Whitney U test or Kruskal–Wallis test was used. The concentrations of all biomarkers were compared between malaria and NMF, as well as between SM and UM groups.

We evaluated the diagnostic accuracy and predictive ability of pfHRP2 and pLDH, measured by both Luminex and LFA, by estimating the area under the ROC curve (AUC-ROC), sensitivity, specificity, positive predictive value, and negative predictive value. The Kappa index was used to assess the agreement between LFA results observed by two independent observers and those measured by ImageJ software. Finally, correlation coefficients were calculated to assess the agreement between pfHRP2 and pLDH concentrations measured by Luminex and those obtained by LFA.

Ethics

The study was approved by the Institutional Review Board and the Ethics Committee of the Hospital Clinic of Barcelona (HCB/2019/0839), and written informed consent was obtained from all study participants. The study was designed in compliance with Good Clinical Practice and following the Declaration of Helsinki. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for reporting case-control studies was followed (Supplementary Table a.0).

RESULTS

Clinical assessment

Overall, 121 participants were included in the study: 75 patients with malaria and 46 participants with NMF (Figure 4). Globally, 44 (36.4%) were female and the median age was 36 years. Supplementary tables a1.1 and a1.2 shows patients' baseline characteristics. Previous malaria episodes, trips to Africa, and visits to friends and relatives were more common in participants with malaria. No patient with malaria took antimalarial chemoprophylaxis.

Among malaria patients, 25 were UM and 50 were classified as SM. The most common severity criteria were hyperparasitemia >2% (n=43, 86%), jaundice (n=20, 40%), prostration (n=10, 20%), shock (n=10, 20%), acidosis (n=8, 16%), and acute kidney injury (n=8, 16%) (Supplementary Table a2). Following local guidelines, 90% of participants with SM were admitted to an intensive care unit (ICU).

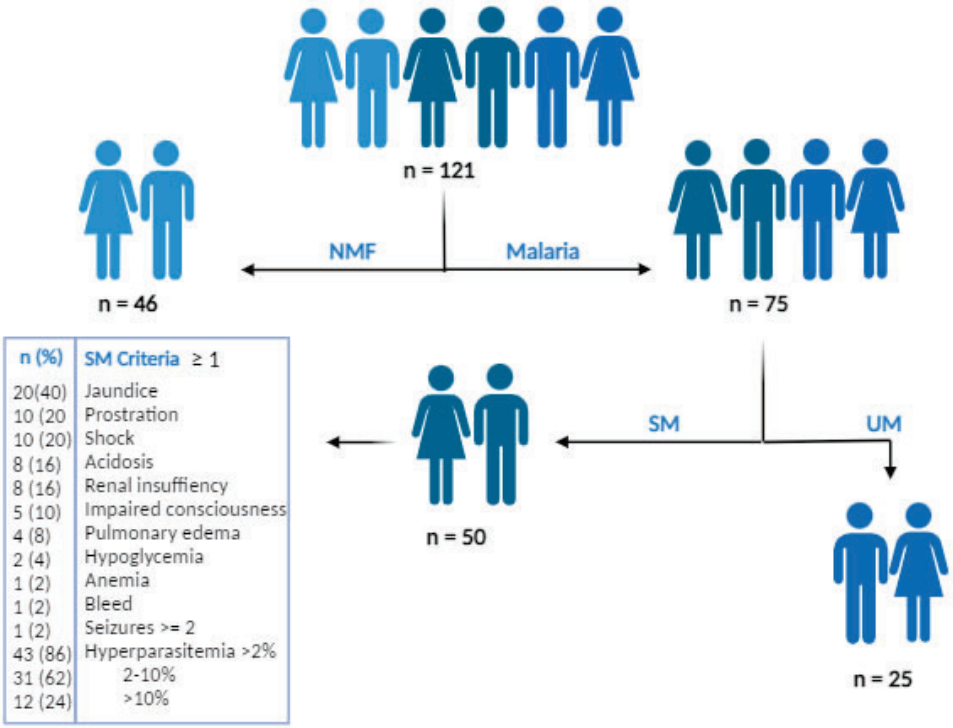


Figure 3: Patient classification based on malaria status and severity. *A total of 121 patients were assessed and classified in Non-Malaria Febrile (NMF) conditions, or malaria infected. Among the malaria patients they were categorized between Uncomplicated Malaria (UM) or Severe Malaria (SM), meeting at least one severe malaria criteria listed on the table. The table details the specific severe malaria criteria and the frequency of each condition among the SM patients.*

Luminex results

HRP2 Luminex results

The dynamic range obtained from the calibration curves ranges from 49.19 ng/ml to 493.76 µg/ml.

Diagnostic results for pfHRP2 (Luminex assay): Among the 121 individuals tested, median pfHRP2 concentration was 1047.1 ng/ml (IQR: 0 to 17171.5 ng/ml), showing significant differences between malaria patients and participants with NMF ($p<0.001$). Patients diagnosed with malaria (n=75) exhibited a median pfHRP2 concentration of 8537.4 ng/ml (IQR: 1621.16 - 41109.3 ng/ml), while in participants with NMF (n=46) pfHRP2 levels were consistently undetectable (Table 1.A). In our cohort, pfHRP2 measured by Luminex showed 100% sensitivity, 100% specificity, 100% PPV and 100% NPV for the diagnosis of malaria (AUC-ROC 1) (Table 3.A).

Prognostic results for pfHRP2 (Luminex assay)**:** Median pfHRP2 concentrations were significantly higher in participants with SM compared to participants with UM (24471.2ng/ml vs. 1081.4 ng/ml, $p<0.001$) (Table 1.B). pfHRP2 concentration determined by Luminex showed an AUC-ROC of 0.86 for predicting SM. pfHRP2 showed 82.0% sensitivity (95%CI 71.4-92.6), 84.0% specificity (95%CI: 69.6-98.4), 91.1% PPV (95%CI: 82.7-99.4), and 70.0% NPV (95%CI: 53.6-86.4) to identify SM patients (Table 3.A).

LDH Luminex results

The dynamic range obtained from the calibration curves ranges from 28.82 ng/ml to 185.03 µg/ml.

Diagnostic results for pLDH (Luminex assay): Among the 121 individuals tested, median pLDH level was reported at 61.6 ng/ml (IQR: 0 - 791.7 ng/ml). pLDH concentrations differed significantly between participants with NMF and malaria patients ($p<0.001$). Particularly, pLDH resulted undetectable in individuals with NMF (n=46), while in patients with malaria (n=75), the median pLDH concentration resulted 505.5 ng/ml (IQR: 84.1 - 1314.6 ng/ml) (Table 1.A). pLDH concentration measured by Luminex showed an AUC-ROC of 0.93 (95%CI: 0.89-0.97) for diagnosing patients with imported malaria. In terms of diagnostic performance, pLDH sensitivity was 85.3% (95%CI: 77.3-93.3), specificity was 100% (95%CI: 100-100), PPV was 100% (95%CI: 100-100) and NPV was 80.7% (CI 70.4-90.9) (Table 3.A).

Prognostic results for pLDH (Luminex assay): Regarding the prognostic evaluation of pLDH in malaria patients, pLDH concentrations resulted significantly higher in patients with SM compared to patients with UM ($p<0.001$). Patients with UM (n=25) displayed median pLDH concentrations of 0ng/ml (IQR 0 - 187.7 ng/ml). In contrast, participants with SM (n=50) exhibited pLDH concentrations of 381.5 ng/ml (IQR: 200.0 - 543.4 ng/ml) (Table 1.B). Determination of pLDH by Luminex showed an AUC-ROC of 0.88 (95%CI: 0.80-0.96) to identify SM cases. The sensitivity of pLDH as a prognostic marker was found to be 84.0% (95%CI: 73.8-94.2), while the specificity was 88.0% (95%CI: 75.3-100). The PPV was estimated at 93.3% (95%CI: 86.0-100), and the NPV stood at 73.3% (95%CI: 57.5-89.2) (Table 3.A).

Prognostic value of the combination of HRP2 and LDH

A logistic regression model showed that the combination of pfHRP2 and pLDH measured by Luminex showed an AUC-ROC of 0.88 (95%CI 0.80-0.96) to predict SM, which did not significantly differ from the predictive capacity of pLDH alone. Sensitivity and specificity of the combination of pfHRP2 and pLDH measured by Luminex to predict SM were 88.0% (95%CI: 75.3-100%) and 86.0% (95%CI: 76.4-95.6%),

respectively. Predictably, the diagnostic value was 1, as no negative patients exhibited detectable levels of these biomarkers, indicating a perfect discriminatory ability in this sample (Table 3.A).

Table 1: Parasite biomarker concentration in Luminex (ng/mL)				
A. Diagnostic	**Total (n=121)**	**NMF (n=46)**	**Malaria (n=75)**	**p-value**
pfHRP2 (IQR)	1047.1 (0-17171.5)	0 (0-0)	8537.4 (1621.16-41109.3)	< 0.001
pLDH (IQR)	61.6 (0-791.7)	0 (0-0)	505.5 (84.1-1314.6)	< 0.001
B. Prognostic	**Malaria (n=75)**	**UM group (n=25)**	**SM group (n=50)**	**p-value**
pfHRP2 (IQR)	8537.4 (1621.2-41109.3)	1081.4 (435.8-3048.1)	24471.2 (7022.0-64682.5)	< 0.001
pLDH	219.8 (0-441.4)	0 (0-187.7)	381.5 (200.0-543.4)	< 0.001

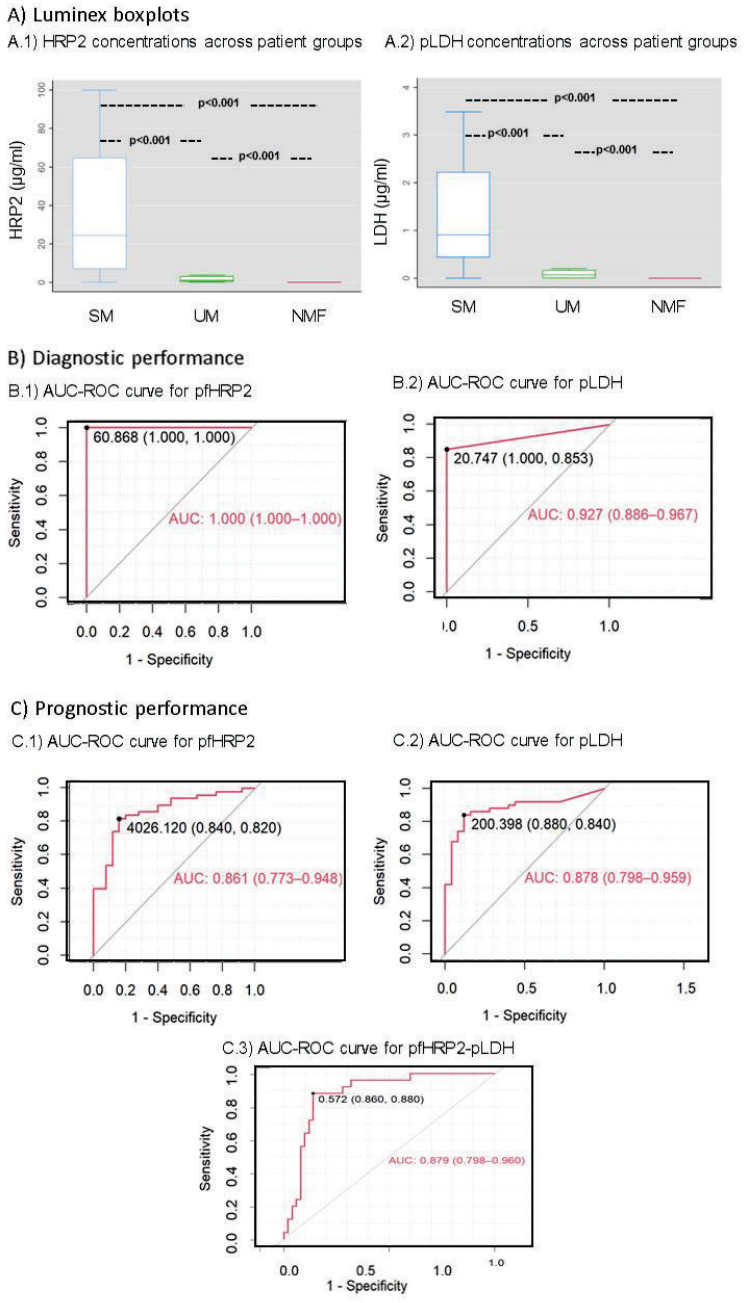


Figure 4: Performance Metrics of Biomarkers in Diagnosing and Predicting Malaria Severity measured by Luminex. A) Luminex boxplots. A.1) pfHRP2 Concentrations across patient groups: *Distribution of pfHRP2 concentrations (µg/ml) among different patient groups: SM, UM, NMF. The boxplot indicates higher median pfHRP2 levels in SM compared to UM and NMF groups ($p < 0.001$).* ***A.2.) pLDH concentrations across patient groups:*** *Distribution of pLDH concentrations (µg/ml) among different patient groups: SM, UM, NMF. The boxplot shows elevated pLDH levels in the SM group compared to UM and NMF ($p < 0.001$).* ***B) Diagnostic performance. B.1) AUC-ROC Curve for pfHRP2:*** *As a diagnostic marker shows an Area Under the Curve (AUC) of 1.000 (1.000 - 1.000), indicating perfect diagnostic performance.*

***B.2) AUC-ROC Curve for pLDH**: As a diagnostic marker, with an AUC of 0.927 (0.860 - 0.987), demonstrating high diagnostic accuracy. **C) Prognostic performance. C.1) AUC-ROC Curve for pfHRP2:** The prognostic performance of pfHRP2, with an AUC of 0.881 (0.773 - 0.948), indicates good predictive capability. **C.2) AUC-ROC Curve for pLDH**: The prognostic performance of pLDH, with an AUC of 0.878 (0.789 - 0.958), suggests a suggests a strong prognostic ability. (Calibration curves are provided in the SI) **C.3) AUC-ROC Curve for Combined HRP2 and LDH**: The prognostic performance of the combination of pfHRP2 and pLDH, with an AUC-ROC of 0.879 (95% CI: 0.798-0.96) suggests a suggests a strong prognostic ability with no significantly improved performance compared to the individual biomarkers.*

LFA results picture quantification

HRP2 Picture quantification

The dynamic range obtained from the calibration curves ranges from 325.02 ng/ml to 4445.77 ng/ml.
Diagnostic results for pfHRP2 (LFA assay): Regarding the diagnostic evaluation of pfHRP2 for malaria in LFA 87 cases were evaluated: 71 malaria patients and 16 NMF. Consistently with Luminex results, NMF cases had undetectable pfHRP2 levels, while those participants with imported malaria had a median pfHRP2 concentration of 1577.1 ng/ml (IQR: 1092.8-2351.2 ng/ml) ($p < 0.001$). The diagnostic performance of pfHRP2 detection via LFA showed an AUC-ROC of 1 (95% CI 1.00-1.00), with 100% sensitivity, specificity, PPV, and NPV.
Prognostic results for pfHRP2 (LFA assay): pfHRP2 concentrations in LFA were determined in 25 UM and 46 SM, showing 1577.1 ng/ml (IQR: 1165.5-2351.2 ng/ml) and 1568.3 ng/ml (IQR: 1040.9-2298.4 ng/ml) concentrations, respectively, with no significant differences between the two groups (p=0.847). The AUC-ROC of LFA pfHRP2 concentrations to detect SM cases was 0.51 (95%CI: 0.37-0.66), with a sensitivity of 17.4% (95%CI: 6.4-28.3), specificity of 96.0% (95%CI: 88.3-100), PPV of 88.9% (95%CI: 68.4-100), and a NPV of 38.7% (95%CI: 26.6-50.8).
Comparison with Luminex results for pfHRP2: The analysis examined the relationship between pfHRP2 levels quantified using the Luminex platform and the pfHRP2 lateral flow assay (LFA). The calculated correlation coefficient between these two measurement methodologies is -0.0973, indicating a negligible negative correlation. (lineal regression: R2 =0.0094). Although this result is somewhat surprising, it is important to consider the different capabilities of the two techniques. The Luminex platform uses a lengthy, multi-step protocol that maximizes sensitivity and specificity, providing precise quantification of pfHRP2 levels. In contrast, the LFA is designed for rapid and straightforward detection, which might not capture the full range of pfHRP2 concentrations as accurately. Moreover, the antibodies employed in the two techniques are likely different, with varying binding affinities and kinetics that can significantly affect the quantification of results. These differences in assay design, antibody characteristics, and detection capabilities underscore the importance of selecting the appropriate method based on the specific requirements for sensitivity and quantification in malaria diagnosis and monitoring.

LDH Picture quantification

The dynamic range obtained from the calibration curves ranges from 134.61 ng/ml to 1887.24 ng/ml.
Diagnostic results for pLDH (LFA assay): Regarding pLDH evaluation by LFA, participants with NMF (n=16) had undetectable pLDH levels (0 ng/ml), whereas patients with malaria (n=71) had a median pLDH level

of 219.8 ng/ml (IQR: 0-441.4 ng/ml) (p < 0.001). The diagnostic performance showed an AUC-ROC of 0.82 (95% CI: 0.77-0.88), a sensitivity of 64.8% (95% CI: 53.7-75.9), specificity of 100% (95% CI: 100-100), a PPV of 100% (95% CI: 100.0-100.0), and a NPV of 39.0% (95% CI: 24.1-53.9).

Prognostic results for pLDH (LFA assay): For the prognostic assessment of pLDH by LFA, the median pLDH concentrations resulted significantly higher in participants with SM (n=46) compared to participants with UM (n=25) (p<0.001). Particularly, median pLDH concentration determined by LFA were estimated in 0ng/ml (IQR: 0 - 187.7 ng/ml), whereas median pLDH concentration in the SM group was 381.5 ng/ml (IQR: 200.0-543.4 ng/ml). The AUC-ROC of pLDH determined by LFA was 0.85 (95% CI: 0.76-0.93), allowing estimating a sensitivity of 73.9% (95%CI: 61.2-86.6), specificity of 88.0% (95%CI: 75.2-100), PPV of 91.8% (95% CI: 83.1-100), and NPV of 64.7% (95% CI: 48.6-80.7) to identify imported SM cases.

Comparison with Luminex results for pLDH: The comparison between pLDH levels measured by Luminex and the pLDH LFA yielded a correlation coefficient of 0.6175.

Prognostic value of the combination of HRP2 and LDH

A logistic regression model showed that the combination of pfHRP2 and pLDH measured by LFA and captured by ImageJ showed an AUC-ROC of 0.87 (95%CI 0.78-0.95) to predict SM, and did not significantly differed from the prognostic capacity of pLDH alone. Sensitivity and specificity of the combination of pfHRP2 and pLDH to predict SM were 88.0% (95%CI: 75.3-100%) and 80.4% (CI: 69.0-91.9%), respectively. Predictably, the diagnostic value was 1, as no negative patients exhibited detectable levels of these biomarkers, indicating a perfect discriminatory ability in this sample (Table 3.B).

Table 2: Parasite biomarker concentration in LFA (ng/ml)

A. Diagnostic	**Total (n=87)**	**NMF (n=16)**	**Malaria (n=71)**	**p-value**
pfHRP2 (IQR)	1331.2 (648.6-2262.2)	0 (0-0)	1577.1 (1092.8-2351.2)	< 0.001
pLDH (IQR)	177.6 (0-422.5)	0 (0-0)	219.8 (0-441.4)	< 0.001
B. Prognostic	**Malaria (n=71)**	**UM group (n=25)**	**SM group (n=46)**	**p-value**
pfHRP2 (IQR)	1577.1 (1092.8-2351.2)	1577.1 (1165.5-2351.2)	1568.3 (1040.9-2298.4)	0.847
pLDH (IQR)	219.8 (0-441.4)	0 (0-187.7)	381.5 (200.0-543.4)	< 0.001

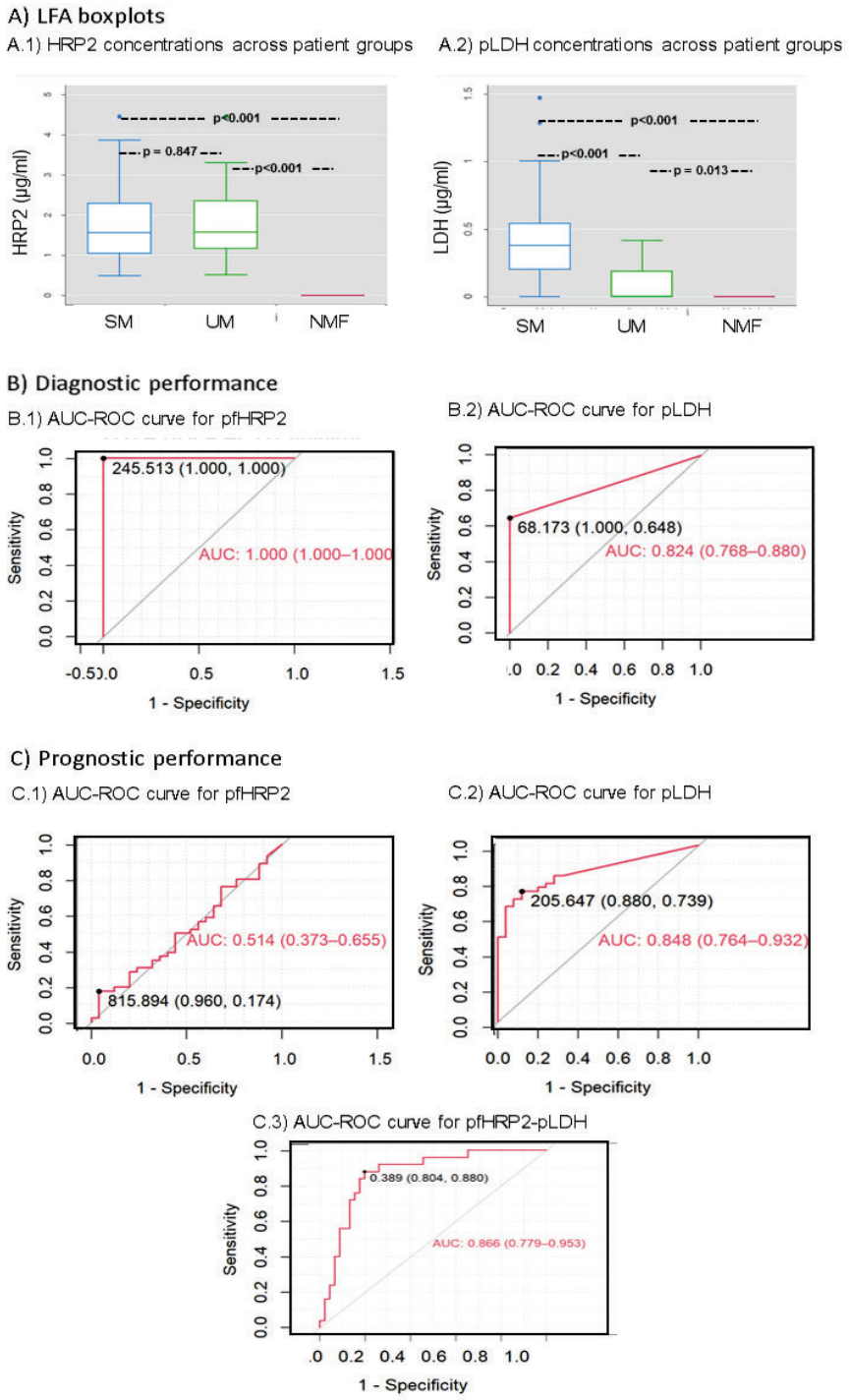


Figure 5: Performance Metrics of Biomarkers in Diagnosing and Predicting Malaria Severity measured by LFA. A) LFA boxplots. A.1) pfHRP2 Concentrations across patient groups: *Distribution of pfHRP2 concentrations (µg/ml) among different patient groups: SM, UM, NMF. The boxplot indicates similar median pfHRP2 levels in the SM and UM groups (p = 0.847) , both of which are higher compared to the NMF group (p < 0.001).* ***A.2.) pLDH concentrations across patient groups:*** *Distribution of pLDH*

concentrations (µg/ml) among different patient groups: SM, UM, NMF. The boxplot shows elevated pLDH levels in the SM group compared to the UM and NMF groups (p < 0.001), but there is no significant difference between UM and NMF (p = 0.013). ***B) Diagnostic performance. B.1) AUC-ROC Curve for pfHRP2:*** *As a diagnostic marker shows an Area Under the Curve (AUC) of 1.000 (1.000 - 1.000), indicating perfect diagnostic performance.* ***B.2) AUC-ROC Curve for pLDH****: As a diagnostic marker, with an AUC of 0.924 (0.746 - 0.994), demonstrating high diagnostic accuracy.* ***C) Prognostic performance. C.1) AUC-ROC Curve for pfHRP2:*** *The prognostic performance of pfHRP2, with an AUC of 0.516 (0.373 - 0.655), indicating moderate predictive capability.* ***C.2) AUC-ROC Curve for pLDH****: The prognostic performance of pLDH, with an AUC of 0.818 (0.746 - 0.892), suggests a suggests a strong prognostic ability. (Calibration curves are provided in the SI)* ***C.3) AUC-ROC Curve for Combined HRP2 and LDH****: The prognostic performance of the combination of pfHRP2 and pLDH, with an AUC-ROC of 0.866 (95% CI: 0.779-0.953) suggests a suggests a strong prognostic ability with no significantly improved performance compared to the individual biomarkers.*

Table 3: Performance of HRP2, pLDH and HRP2/pLDH combination for the diagnosis and prognosis* of imported P. falciparum malaria.

		Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)	Cut-off value	AUC-ROC % (95%CI)
A. Luminex							
HRP2	**Diagnostic**	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	60.868	1 (M) (1.00-1.00)
	Prognostic	82.0 (71.4-92.6)	84.0 (69.6-98.4)	91.1 (82.7-99.4)	70.0 (53.6-86.4)	4026.12	0.86 (SM) (0.77-0.93)
LDH	**Diagnostic**	85.3 (77.3-93.3)	100 (100-100)	100 (100-100)	80.7 (70.4-90.9)	20.747	0.93 (M) (0.89-0.97)
	Prognostic	84.0 (73.8-94.2)	88.0 (75.3-100)	93.3 (86.0-100)	73.3 (57.5-89.2)	200.398	0.88 (SM) (0.80-0.96)
LDH/HRP2	**Diagnostic**	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	0.5	1 (M) (1.00-1.00)
	Prognostic	88.0 (75.3-100)	86.0 (76.4-95.6)	75.9 (60.3-91.4)	93.5 (86.3-100)	0.572	0.88 (SM) (0.80-0.96)
B. LFA							
HRP2	**Diagnostic**	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	245.513	1 (M) (1.00-1.00)
	Prognostic	17.4 (6.4-28.3)	96.0 (88.3-100)	88.9 (68.4-100)	38.7 (26.6-50.8)	815.894	0.51 (SM) (0.37-0.66)
LDH	**Diagnostic**	64.8 (53.7-75.9)	100 (100-100)	100 (100-100)	39.0 (24.1-53.9)	68.173	0.82 (M) (0.77-0.88)
	Prognostic	73.9 (61.2-86.6)	88.0 (75.2-100)	91.8 (83.1-100)	64.7 (48.6-80.7)	205.647	0.85 (SM) (0.76-0.93)

LDH/HRP2	**Diagnostic**	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	0.5	1 (M) (1.00-1.00)
	Prognostic	88.0 (75.3-100)	80.4 (69.0-91.9)	71.0 (55.0-86.9)	92.5 (84.3-100)	0.389	0.87 (SM) (0.78-0.95)

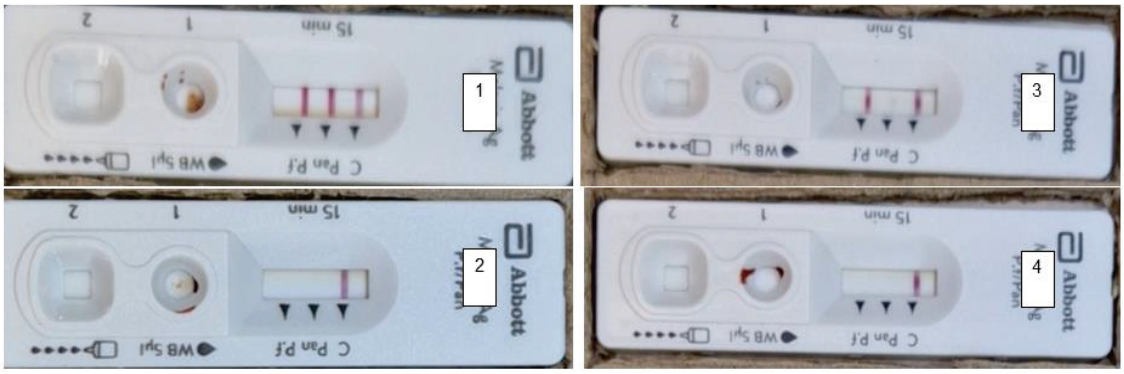
*Prognosis of imported malaria was defined as the identification of severe malaria (SM) cases based on WHO criteria except for hyperparasitaemia (threshold of 2% according to European and Spanish guidelines)

LFA results naked eye

Two independent observers evaluated the results obtained by LFA and captured in digital images, assigning positive or negative values for pfHRP2 and pLDH. A total of 87 tests were evaluated, 71 malaria patients and 16 NMF, with a percentage agreement of 100% for pfHRP2 between the observers and the analysis with ImageJ software. For pLDH, the percentages of agreement were 81.6% for one observer and 85.1% for the other compared to the analysis with ImageJ software. All disagreements were cases detected by the software but not by the observers.

The comparison of results obtained by the two observers and those interpreted by ImageJ software showed good agreement for pfHRP2 (kappa 1, 95%CI: 1-1) but not for pLDH (kappa 0.62, 95%CI: 0.46-0.79, and 0.69, 95%CI: 0.54-0.85, for each observer, respectively).

Naked eye



Malaria		+ (1)	+ (2)	+ (3)	- (4)
Obs1	P.f	+	-	+	-
	Pan	+	-	+	-
Obs2	P.f	+	-	+	-
	Pan	+	-	-	-

Figure 6: Results of the LFA for detecting malaria. ***Top****: Photographic images of the LFA test strips under different conditions. Images 1-3 represent positive patients (with image 2 being a false negative), while image 4 shows a negative patient.* ***Bottom****: Observer 1 accurately detected both pfHRP2 and pLDH in the positive cases. Observer 2 also correctly identified pfHRP2 and pLDH, but showed slight discrepancies in the detection of pLDH in test 3.*

DISCUSSION

Besides children and pregnant women in malaria-endemic areas, international travelers are recognized as the third population most at risk of developing SM. As mentioned in the introduction, the current clinical practice for managing imported malaria in non-endemic areas faces two general problems: firstly, it heavily relies on WHO guidelines designed specifically for children from endemic areas, thereby not accurately reflecting the clinical conditions of adults previously unexposed to malaria and consequently failing to accurately identify severe cases; secondly, the diagnosis and prognosis of malaria depend on referral hospitals and lengthy laboratory assays. In response to these challenges, this work proposes the implementation of the parasite biomarker pLDH as a prognostic biomarker, with its applicability extending directly to the bedside of patients in healthcare facilities of any complexity, ranging from outpatient clinics to emergency departments and ICUs, using a smartphone to read commercially available RDTs.

Our Luminex data showed that while pfHRP2 remains the best diagnostic biomarker with a sensitivity and specificity of 100%. pLDH also provided good diagnostic performance with a sensitivity of 85% and specificity of 100%. As prognostic biomarkers, the two proteins had similar performance, with HRP2 showing 82% sensitivity and 84% specificity in distinguishing between SM and UM (AUC-ROC 0.86); pLDH had slightly better values of 84% sensitivity and 88% specificity (AUC-ROC 0.88, not presenting statistical differences). Furthermore, we also show that their combination does not significantly enhance the prognostic ability, with a result of 88% sensitivity and 86% specificity (AUC-ROC 0.88). Although pfHRP2 has already been proposed as a valuable prognostic biomarker in endemic regions, where elevated levels have been linked to severe outcomes such as cerebral malaria and mortality, its role in imported malaria cases remains limited, and the absence of established cut-offs hinders its broader clinical application. Moreover, the growing prevalence of *P. falciparum* strains with hrp2/3 gene deletions and the persistence of pfHRP2 after treatment poses a significant threat to its diagnostic and prognostic effectiveness and its implementation as a tool for treatment response monitoring[23,31,32]. This study represents the first effort to evaluate pLDH as a potential severity biomarker, overcoming some of the limitations associated with pfHRP2.

While our implementation of pLDH effectively resolves the issue of accurately identifying severe imported malaria, thanks to the capabilities of tertiary hospitals in high-income countries to implement laboratory-based immunoassays such as Luminex, it does not overcome the second challenge. These assays, though precise for guiding patient management and identifying high-risk patients, require dedicated personnel and specialized facilities to execute such complex and lengthy protocols. This poses a significant limitation, as their extended duration—from several hours to days, depending on the availability of trained microbiologists—is incompatible with scenarios that demand immediate clinical decisions, such as the management of imported malaria patients. On the other hand, new technologies such as the use of aptamers or electrochemical sensors are being evaluated to quantify pLDH. However, all of these have been developed with diagnostic purposes in mind in endemic settings, and currently, no commercial tests are available for their use[33–35].

To further address the need for timely and practical testing methods, we explored the use of a common RDT for malaria that tests for both pfHRP2 and pLDH. By following the kit instructions, medical doctors at our clinic were able to visually identify malaria cases with remarkable accuracy, but failed to detect between 15% and 18% of positive pLDH strains. This method of using RDTs not only provided rapid results but also allowed us to bypass the lengthy assays typically associated with traditional laboratory tests. However, this qualitative assessment still heavily relies on the reader's experience. Although two studies suggested a possible association of malaria RDT results with parasitemia, naked eye evaluation of malaria LFA do not allow to discriminate between severe and UM cases[36,37]. Moreover, the ability to distinguish between strong and weak test lines significantly depends on the reader's familiarity with such tests. More experienced readers, who have seen many tests, can better interpret these results, a skill particularly critical in settings where malaria is not endemic.

To eliminate user bias from the LFA readings, we analyzed the signal intensities using a smartphone and the open-source software ImageJ. This approach ensured an extremely low cost, which could be close to zero assuming each clinic, at least in high-resource settings, has access to a smartphone. Analyzing the results, while we confirmed the diagnostic ability of HRP2 with both sensitivity and specificity of 100%, the performance of pLDH was lower with a sensitivity of 64.8% and specificity of 96%. We also found that in terms of prognostic ability, pLDH surpassed pfHRP2; specifically, pfHRP2 had a sensitivity of 17% and specificity of 96% (AUC-ROC 0.51), while pLDH had a sensitivity of 73.9% and specificity of 88% (AUC-ROC 0.85). Their combination did not significantly increase the prognostic performance of the biomarkers, providing a sensitivity of 88% and a specificity of 80.4% (AUC-ROC 0.87). This indicates that by reading the pLDH alone and in combination with pfHRP2, we could achieve a sensitivity and specificity of 88%. Employing an already commercially available RDT for a cost of approximately 20 dollars, a smartphone, which is ubiquitous nowadays, and open-source software, it is possible to identify SM patients with a sensitivity and specificity of 88% in approximately 15 minutes. This is invaluable information for clinicians who are assessing patients with a potentially deadly disease to identify the best use of the healthcare resources available.

CONCLUSIONS

In conclusion, pLDH presents itself as a viable prognostic biomarker for malaria, with the potential to be widely implemented across various healthcare settings using smartphones to analyze commercially available rapid diagnostic tests (RDTs). While pfHRP2 remains the most reliable diagnostic biomarker, pLDH has also demonstrated strong diagnostic capabilities. When evaluated as prognostic biomarkers, both pfHRP2 and pLDH exhibited comparable performance, with pLDH showing a slight edge in sensitivity and specificity. The integration of smartphones and open-source software for RDT analysis offers a promising approach to minimize user bias, delivering a cost-effective and timely method for both diagnosing malaria and predicting its severity. This innovation could significantly enhance the accessibility and accuracy of malaria management, particularly in resource-limited settings.

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Supplementary information

Table a.0. STROBE checklist		
	Nº	**Localization in the text**
Title and abstract	1	Title
		Abstract
Introduction		
Background/rationale	2	Background
Objectives	3	Background section, last paragraph
Methods		
Study design	4	Methods, study design and population, first paragraph
Setting	5	Methods, study design and population, first paragraph. Data collection section.
Participants	6	Methods, study design and population, first and second paragraphs.
Variables	7	Methods, data collection and laboratory procedures section.
Data sources / measurement	8	Methods, statistical analysis section
Bias	9	Methods, statistical analysis section
Study size	10	Methods, data collection and laboratory procedures section.
Quantitative variables	11	Methods, statistical analysis section
Statistical methods	12	Methods, statistical analysis section
Results		Results section
Participants	13	Results section, baseline characteristics and patients with severe malaria sub-sections.Tables 1,2.
Descriptive data	14	Tables 1,2
Outcome data	15	Methods, statistical analysis section, and tables 3,4
Main results	16	Results, figure 1, table 3, 4, figure 2.
Other analyses	17	Results, supplementary tables, table 4.
Discussion		

Key results	18	Discussion, first paragraph and conclusion section
Limitations	19	Discussion, third paragraph
Interpretation	20	Discussion, last paragraph
Generalisability	21	Discuss the generalizability (external validity) of the study results

Table a1.1- Patients' baseline characteristics.	**All groups (n=121)**	**NMF (n=46)**	**Malaria (n=75)**	**p-value**
Female, n(%)	44 (36.4)	21 (45.7)	23 (30.7)	0.096
Age, Md (IQR)	37 (28-46)	30 (26-40)	39 (32-49)	0.011
Previous malaria episodes, n (%)	18 (14.9)	1 (2.2)	17 (22.8)	0.008
Previous medical condition				
HTA, n (%)	10 (8.3)	2 (4.4)	8 (10.7)	0.220
Obesity, n (%)	7 (5.8)	0 (0.0)	7 (9.33)	0.033
Diabetes, n (%)	3 (2.5)	0 (0.0)	3 (4.0)	0.170
Immunosuppression, n (%)	6 (5.0)	1 (2.2)	5 (6.7)	0.269
Travel destination: WHO regions				
Africa, n (%)	89 (73.6)	15 (32.6)	74 (98.7)	< 0.001
Travel reason				< 0.001
Tourism and work, n (%)	64 (53.8)	36 (80.0)	28 (37.3)	-
VFR, n (%)	31 (26.1)	3 (6.7)	28 (37.3)	-
Cooperation and expatriate, n (%)	18 (15.1)	5 (11.1)	13 (17.3)	-
Migration, n (%)	6 (5.0)	1 (2.2)	5 (6.67)	-
Treatment				
Profilaxis, n (5)	0 (0.0)	0 (0.0)	0 (0.0)	-
Treatment before malaria, n (%)	17 (14.0)	0 (0.0)	17 (22.7)	0.330

UCI				
UCI, n (%)	46 (38.0)	0 (0.0)	46 (61.3)	< 0.001
Length stay UCI, Md (IQR)	3 (2-5)	0 (0-0)	3 (2-5)	< 0.001

Table a1.2- Patients' baseline characteristics. Malaria.	**Malaria (n=75)**	**UM (n=25)**	**SM (n=50)**	**p-value***
Female, n(%)	23 (30.7)	7 (28.0)	16 (32.0)	0.723
Age, Md (IQR)	39 (32-49)	38 (30-46)	41 (32-49)	0.475
Previous malaria episodes, n (%)	17 (22.8)	7 (28.0)	10 (20.0)	0.624
Previous medical condition				
HTA, n (%)	8 (10.7)	2 (8.0)	6 (12.0)	0.597
Obesity, n (%)	7 (9.33)	2 (8.0)	5 (10.0)	0.779
Diabetes, n (%)	3 (4.0)	0 (0.0)	3 (6.0)	0.211
Immunosuppression, n (%)	5 (6.7)	2 (8.0)	3 (6.0)	0.743
Travel destination: WHO regions				
Africa, n (%)	74 (98.7)	25 (100)	49 (98.0)	0.477
Travel reason				0.423
Tourism and work, n (%)	28 (37.3)	6 (24.0)	22 (44.9)	-
VFR, n (%)	28 (37.3)	13 (52.0)	15 (30.6)	-
Cooperation and expatriate, n (%)	13 (17.3)	5 (20.0)	8 (16.3)	-
Migration, n (%)	5 (6.67)	1 (4.0)	4 (8.2)	-
Treatment				
Profilaxis, n (5)	0 (0.0)	0 (0.0)	0 (0.0)	-

Treatment before malaria, n (%)	17 (22.7)	4 (16.0)	13 (26.0)	0.330
UCI				
UCI, n (%)	46 (61.3)	1 (4.0)	45 (90.0)	< 0.001
Length stay UCI, Md (IQR)	3 (2-5)	3 (3-3)	3(2-5)	0.773

Table a2.- Severity criteria	
Jaundice, n (%)	20 (40.0)
Prostration, n (%)	10 820.0)
Shock, n (%)	10 (20.0)
Acidosis, n (%)	8 (16.0)
Renal insufficiency, n (%)	8 (16.0)
Glasgow, n (%)	5 (10.0)
Pulmonary edema, n (%)	4 (8.0)
Hypoglycemia, n (%)	2 (4.0)
Anemia, n (%)	1 (2.0)
Bleed, n (%)	1 (2.0)
Seizure, n (%)	1 (2.0)
Hyperparasitemia >2%, n (%)	43 (86.0)
Hyperparasitemia 2-10, n (%)	31 (62.0)
Hyperparasitemia >10%, n (%)	12 (24.0)

Text a1. Biotinylation of detection mAbs. Biotinylation of monoclonal mouse IgG PfHRP2 (ImmunologyConsultantsLaboratory, Portland, OR, USA) and α-PAN-pLDH (AccessBio, Somerset, NJ, USA) antibodies was performed following manufacturer instructions with minor modifications. Briefly, we prepared the sulfo-NHS-LC-Biotin 50-fold molar excess instead of 20-fold molar excess and when mixing the antibody and the diluted biotin, incubate the reaction for 1.5 hours on ice and 30 minutes at room temperature instead of 2 hours on ice. After biotinylation, the antibody concentration in the collected flow-through was measured by spectrophotometry (Epoch Microplate Spectrophotometer, BioTek). The antibody solution was adjusted to the desired concentration, aliquoted and stored at 4 °C.

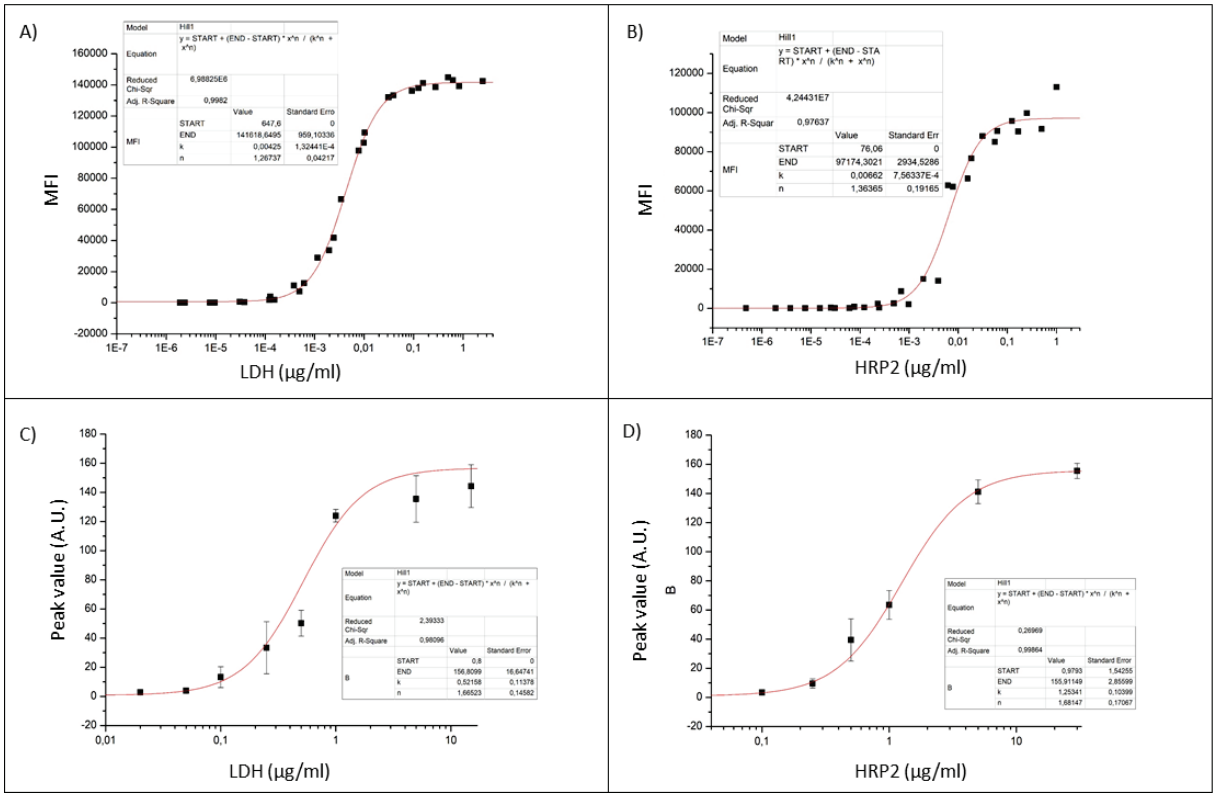

Figure 6: Calibration curves. A) Luminex pfLDH B) Luminex pfHRP2 C) LFA pfLDH D) LFA pfHRP2

VIII.DISCUSSION

For a large portion of the global population, malaria remains one of the deadliest infectious diseases, partly due to the lack of healthcare resources for adequate patient management. It is noteworthy that many of the regions that have eliminated malaria are those with high healthcare resources. However, since it is an aggressive and relatively uncommon disease compared to other non-imported infections, there is also room for improving the initial management of malaria patients in non-endemic regions. This includes early diagnosis and precise handling of the entire spectrum of severity that this disease entails.

This thesis, composed of four articles, explores various aspects of malaria management in non-endemic regions. Each article addresses a specific facet of the improvement areas, referred to as challenges, collectively contributing to a broader and deeper understanding of the approach to managing a patient with imported malaria. Furthermore, these challenges have been addressed by attempting to step away from the perspective of a high-resource (both human and technical) tertiary center such as the Hospital Clinic of Barcelona and exploring solutions that can be applicable to regional hospitals and even primary care centers.

The first challenge focuses on trying to shorten the diagnostic time for malaria in a patient who presents with a fever (or other compatible symptoms) after staying in a malaria-endemic area. Despite the fact that most malaria-endemic countries are geared towards malaria control and eradication[14], imported malaria cases barely have changed in the past, and during pandemics a higher risk for severe malaria was described due to delay in diagnosis[73]. Also, COVID-19's irruption has changed the diagnostic probabilities of acute fever, and clinicians should be again prepared to evaluate febrile patients after international travel. It is difficult to influence a person's behavior to achieve early

contact with the healthcare system, but once the patient enters the healthcare circuit, it is essential for clinicians to understand malaria diagnosis as an emergency, and therefore identification of patients at high risk of having malaria becomes mandatory. However, a Spanish retrospective study of 297 patients with imported malaria showed that malaria was misdiagnosed in 46% of patients at first medical visit(pre-print)[121], with those patients being at higher risk of SM. Another concerning aspect is that microbiological tests are only available in referral centers. In this context, algorithms or score systems are effective ways to incorporate predictive variables into clinical practice, therefore reassuring the clinician to put malaria on top of their differential diagnosis list. Although such systems have been proposed for malaria in endemic areas or for arbovirus infections in non-endemic regions[77,122], there is currently a lack of scores specifically tailored for travel-related malaria.

The **article 1** investigates the role of ML to detect patients at high risk of having malaria. As a result of this diagnostic challenge, we created MALrisk, a clinical decision support system to promptly suspect malaria in patients with imported fever. With only 6 variables that are easy to obtain through medical history (travel destination, chemoprophylaxis intake, presence of rash and respiratory symptoms) and basic laboratory results (platelet count and presence of hyperbilirubinemia), that can be obtained even at almost any primary care centers, MALrisk achieved 100% sensitivity and 72% specificity to identify malaria cases. In the evaluated population, all malaria cases, regardless malaria species or severity were correctly classified by MALrisk.

ML involves an interesting step forward in terms of considering clinical variables of predictive value. ML contributes to the development of these scores by providing a comparable level of accuracy while offering adaptability. This adaptability stems from the model's capacity to be trained with diverse populations, enabling it to handle large sets of variables and demographics, while maintaining an easy-to-use approach for the MALrisk application by a clinician: a digital application that asks 5 “yes or no" questions and needs a number to be registered for the platelet value.

Referring to MALrisk's accuracy, when specific information that could define a disease (symptoms, signs on physical examination or basic laboratory test) is lacking after evaluating a patient, reassurance in the decision-making process is crucial to ensure an appropriate patient triage. The mentioned reassurance is difficult to achieve if there is a lack of precise resources, not to mention the time constraints that clinicians face in emergency departments or primary health centers. All these factors could abruptly stop the patient's diagnostic process or could misinterpret the triage work up, potentially leading to the patient being sent home with a fatal illness or forcing the clinician to transfer the patient without a clear diagnosis (undifferentiated fever). A decision support system could be the key to enabling a physician to make decisions they might otherwise be unable to make. This means that since MALrisk identifies patients with high risk of having malaria, the result should encourage clinicians to start an urgent package of measures, which may involve transferring patients to a referral center urgently under a strong alarm (malaria suspicion), or to initiating empirical antimalarial treatment while waiting for confirmatory results, depending on the setting. Albeit the use of empirical antibiotic therapy is widely implemented, the prescription of empirical antimalarials is not widespread, despite the demonstrated safety of antimalarials over time[123]. Therefore, MALrisk could also support clinicians into starting urgent antimalarial treatment, if diagnostic tests are unavailable within a few hours or if the patient's clinical severity requires it.

On the other hand, the model misclassified 28% of non-malarial febrile patients as suspected malaria cases. This implies that, in less than one-third of patients, the model recommends urgent testing and assesses the need to start an empirical treatment. To understand this outcome is important to point out that for MALrisk, platelet value and "coming from an African country" were the features that contributed most to the model, and this is the reason why in all the incorrectly classified cases, at least one of these two features were present. Some might argue that misclassifying 28% of patients is an unacceptable figure from the standpoint of diagnostic accuracy. However, from a triage point of view, we believe that emphasizing the need to quickly rule out malaria in febrile

travelers returning from Africa or presenting with thrombocytopenia, is still a solid and necessary message in clinical practice. In other words, if we really want to identify all malaria cases out of febrile patients and if we truly want to shorten diagnostic times, we should be prepared to accept a certain rate of negative tests as a part of the initial diagnosis work up of the patient. Additionally, based on current guidelines, all febrile patients returning from malaria endemic areas should be tested for malaria, which means that MALrisk could avoid 72% of these tests or referrals.

While this study serves as a proof of concept for imported malaria early diagnosis, it opens several avenues for future research. Expanding studies to include external validation in independent, large populations will be crucial for assessing the performance of MALrisk across diverse groups. This additional research could refine the model, potentially altering the importance of certain features like travel history to Africa and platelet values. Successfully implementing these steps could enhance the model's performance and facilitate its integration into computer software or digital applications. Additionally, exploring the feasibility of these advancements could lead to significant practical impacts across various healthcare settings. The advantage of ML lies in its adaptability, allowing it to be implemented as a smartphone application (even in the most remote locations smartphones are present). Furthermore, ML and specifically MALrisk can be integrated into health care system software, enabling alerts or notifications based on the analyzed variables. Although this solution is more costly and complex, it could even detect those patients in whom the physician has not even thought about or considered the possibility of malaria, and therefore, could potentially eliminate the factor of 'human error' linked to misdiagnosis, as demonstrated in the previously mentioned studies.[121]

To summarize, the MALrisk is a promising tool to promptly identify suspected malaria cases in patients with imported fever in all clinical settings, allowing the initiation of empiric antimalarials and reinforcing the need for urgent transfer. This resource could be easily scalable to a digital application and could help clinicians in the decision-making process of the patient.

Early diagnosis of malaria is the first essential step in managing the patient. However, once this step has been achieved, the clinical question guiding the following objectives and articles of this thesis has been how to identify those cases with poor prognosis to guide the initial management of malaria patients. Initial decisions will likely determine the rest of the patient's clinical course. Are all severe malaria cases of the same severity? Are the actual clinical guidelines accurate at predicting patient's prognosis? To address these questions, in **article 2** we thoroughly characterized a cohort of 506 malaria cases attended at Hospital Clínic of Barcelona, providing detailed information about the patients treated for malaria and their outcomes. This approach allowed identifying high-risk subgroups for life-threatening conditions, leading to the use of VSM and LSM classification systems to assess the risk for developing these conditions. Therefore, a patient was classified as having VSM in the presence of at least one of the following criteria: parasitemia >10 %, pulmonary edema, impaired consciousness, seizures, renal failure, metabolic acidosis or hyperlactatemia, shock or hypoglycemia. In patients with SM and no criteria for VSM, LSM was defined by: 2–10 % parasitemia, hyperbilirubinemia, prostration, anemia or minor bleeding. In our cohort, 35 % of patients had a SM episode based on classical WHO criteria for non-endemic areas, however no patient belonging to the LSM group developed a life-threatening condition. That means that almost 60 % of cases labeled initially as severe did not present severe organ damage. By contrary, 50 % of patients with VSM developed life-threatening conditions. Therefore, these results suggest that a nuanced selection of SM cases would enable the accurate management of patients with imported malaria and optimize healthcare resources by restricting ICU care to those patients with VSM.

The spectrum of malaria severity exhibits different distinguishing features in children and adults, and our cohort is a good reflect of that: children more frequently present with anemia, acidosis, and convulsions, whereas in adults, renal failure and pulmonary involvement are more prevalent characteristics of SM[39,124–126] , as were in our cohort**.** On the other hand, while evaluating individual criteria, some criteria merit comments. Starting with the prostration criteria, prostration is an easily detectable and

definable criterion in children, but it is subjective and imprecise for adults. Even if it was a criterion described in our cohort, it obviously does not resemble the definition used for children at all, and it leaves the interpretation of prostration in the hands of the treating physician. Is the patient's self-perceived prostration? Or perhaps it is the subjective perception of severity that the physician has when evaluating the patient? Could it be that symptoms similar to those of encephalopathy, a prelude to cerebral malaria, are being categorized as prostration? What is clear is that current guidelines do not provide an exact definition of what prostration means in adults, making it a criterion that is neither reproducible nor assessable. Despite that, what is indisputable is that the presentation with the highest morbidity and mortality, regardless of age, is cerebral malaria. In our cohort, all patients died from cerebral malaria, which is primarily defined as a Glasgow Coma Scale score of <11 points[127] . This punctuation defines a patient that has already a stablished coma and will probably need intubation. Therefore, this criterion does not allow early detection of neurological deterioration in malaria-infected patients. To try to solve this, some guidelines include any neurological impairment as a severity criterion[59,87]. Another parameter that triggers debate as a defining factor of severity is parasitemia. It is historically described the increase in mortality with the increase of parasite load[43,128]. However, this is only an indirect measure of the real pathogenesis in target organs (whose failure is the origin of morbidity and mortality). In our cohort, parasitemia between 2 and 10 %, jaundice and prostration were the most common criteria, accounting for 56 %, 37 % and 17 % of patients, all belonging to LSM group. On the other hand, guidelines in non-endemic areas do not include the assumption of "uncomplicated hyperparasitemia" [127], a clinical scenario which represented the 36% of patients with SM in our cohort but did not threatening conditions.

Thereupon, to summarize the specific proposed recommendations for individual severity criteria redefinition, we would suggest, first, eliminating prostration, and second, widening the neurologic criterion. Our third recommendation focuses on the fact that patients with uncomplicated hyperparasitemia could be safely treated with

artesunate outside the ICU without expecting complications; therefore, nuanced definitions and recommendations on parasitemia should be added into guidelines. Finally, we would suggest dividing SM patients into VSM and LSM groups to guide the need for admission to ICU. Currently, VSM and LSM classifications are only described in French guidelines, and they have not been adopted by other non-endemic recommendations. Although this study is based on these concepts, it does not completely share the same criteria. French guidelines set the parasitemia threshold for LSM at 4% (in contrast to our range of 2–10%) and included isolated seizures and moderate renal failure as criteria for LSM. Despite the slight differences in the specific criteria defining VSM and LSM among the studies or guidelines, our study fully supports the idea of dividing the severity group into two sub-groups of severity.

On the other hand, large cohorts in non-endemic areas describe risk factors (different from those collected by the WHO) associated with mortality [31,75,76]. Easy to obtain variables such as age and reason for travelling could be considered when classifying patients, as well as the duration of fever, reflected in some guidelines[88] , albeit in our cohort symptoms' duration has not been found as a risk factor. Another special population at risk are pregnant women; in our cohort 50 % had severe malaria, and the only patient with UM who needed ICU intervention was pregnant. Additionally, in our cohort, high LDH, severe thrombocytopenia and elevated CRP were consistently risk factors for developing life-threatening conditions. Based on what was previously mentioned, special populations and laboratory values should be also considered in future guidelines.

Looking ahead, it is crucial to enhance the prognostic value of certain severity criteria by studying larger and more diverse cohorts to capture their infrequent occurrences more accurately. Additionally, prospective studies involving various populations, including children, are crucial to determine if the results can be extrapolated across different demographics. Moreover, increasing the statistical power of studies by including larger sample sizes could help establish the significance of classical risk factors, such as the duration of travel or pregnancy, which currently lack statistical significance. All these

steps could finally define the spectrum of severity subgroups and could help deciding the variables and thresholds needed to include in future classifications, although our approach has been stated throughout the text.

As stated at the beginning, guidelines are necessary to have a common language among health care providers and to standardize processes. The **article 2** has paved the way for reflecting on the revision of the content of the guidelines we currently have, but it is in the **article 3 and 4** where the key to definitively tipping the balance between severe and non-severe groups might be found. Being able to rely on objective values that directly reflect the pathophysiological cascade occurring in the patient is undoubtedly the precision medicine we seek today, given that the language of numbers is more compelling and exhaustive than the compendium of signs and symptoms.

It is in this search for objective markers of severity that we carried out **article 3 and 4**. In **article 3**, all evaluated host biomarkers except sTREM-1 (i.e Ang-1, Ang-2, CRP and platelets), allowed early identification of SM cases according to WHO criteria for non-endemic regions. The combination of biomarkers from two different pathways, such as Ang-2 and CRP, showed the best diagnostic performance when comparing clinically severe cases, with 84.6% (95%CI 58.9–98.1) sensitivity and 77.4% (95%CI 65.9–87.7) specificity.

Available data on Ang-Tie axis biomarkers suggest that they may predict strong outcomes such as death or cerebral malaria in malaria infected children in endemic areas[95,106] . Moreover, a systematic review describes a correlation between Ang-1 and Ang-2 levels and malaria severity[107]. However, patients from non-endemic regions represent a different population as they are commonly adults with no prior or recent exposure to malaria. There is only one study in imported malaria that measures Ang-Tie axis biomarkers[108], describing higher levels of Ang-2 in severe cases but not providing a cut-off point. As Ang-1 levels decrease in pathological scenarios, in contrast to Ang-2, the latter might be easier to interpret and therefore a strong candidate for inclusion in rapid prognostic tests or routine laboratory workups. Among other studied biomarkers,

sTREM-1 diagnostic performance in our study was worse than Ang-2. Although it was able to identify the most severe patients after modifying the WHO classification, it failed to show significant differences when classical WHO criteria were applied. Due to divergent results with other works commented previously [113,114], prospective cohorts could be essential to cast light on discrepancies[97,115]. Another protein that illustrates inflammation is CRP, a well-known acute phase protein that has been shown to correlate with disease severity in imported malaria and as a monitoring tool[109–111]. Our results were consistent with other studies [109–111]. In contrast, in the mentioned prospective French study, CRP detected severe cases but failed to discriminate VSM from LSM cases. Due to the very low co-infection rate in our patients (5.5%), it is unlikely that host biomarkers were raised because of co-infection. Similarly, as previously mentioned, thrombocytopenia is a common finding in these patients and platelet count on admission in endemic regions correlates with disease severity and death[117,118]. In our study, platelets were lower among severe patients, whether it was according to WHO criteria or applying the modified classification. Notwithstanding, its performance as a single biomarker to identify clinically severe patients was poorer than other biomarkers. On top of that, our study shows that the combination of different pathway biomarkers, via ML modeling, adds value to already achieved results, and provides a simple tool (the nomogram) to estimate the individual risk of developing SM.

During the initial management of a patient, physicians should always ask themselves whether they are adequately controlling all factors that could impact the patient's progression. In the case of malaria, the suspicion of a bacterial co-infection always looms over the case. This is a transversal aspect of all the previous articles. The WHO recommends having a low threshold to initiate antibiotic therapy and specifically recommends administering it to children with SM in areas of high malaria transmission due to the high prevalence described of *Salmonella* spp. infections and the increased predisposition to bacterial translocation associated to malaria from capillary sequestration.[127,129]. As indicated by local protocol, broad use of antibiotics was prescribed in our cohort. However, the frequency of co-infections was only 5.5 % in our

cohort (article 2), with no differences between groups. This number is slightly lower than other cohorts in non-endemic areas[97,130], where 8% of community acquired co-infections were described in a literature review of SM patients managed in ICUs, and 24% of same type of infections were observed in a French the other hand, as stated previously, CRP was proposed as a severity biomarker. Therefore, although there is enough evidence to support a more restrictive use of antibiotics, unfortunately for "dynamic changes" policies in real life, high CRP has classically been a marker for bacterial infections[131]and although not an highly specific marker, it is widely used in Emergency Departments to decide on empiric antibiotic therapy initiation. Therefore, stating that CRP is a malaria severity biomarker could be insufficient to prevent clinicians from starting empiric antibiotic therapy. This same logic can also be applied to procalcitonin, which is classically elevated in patients with SM. [115,120]. Overall, despite not having the medical imagery in our favor, we believe that malaria could also fit into antibiotic stewardship programs. This means that empiric antibiotic therapy could be only started in case of a clinical suspicion of a concomitant infection or in VSM cases.

In this thesis, we have emphasized the measurement of biomarkers as a classification tool, and host biomarker proteins proved to be reliable tools. Hence, for the host-parasite interaction, we wanted to prove whether parasite biomarkers could also serve as prognostic tools. However, for this evaluation we also considered it crucial that this measurement capability could be rapid and accessible[132], allowing clinicians to make timely decisions (i.e artesunate's initiation and evaluate the need of an ICU) regardless of the healthcare facility complexity. As it has been explained, HRP2 and pLDH are the main targets in RDTs, but their detection it is only expressed in a qualitative way in LFA. By upgrading the lecture of the RDT we aimed to evaluate the quantitative performance of parasite biomarkers.

The idea of correlating qualitative variables (such as the HRP2 or pLDH band) with a quantitative one (parasitemia) and, consequently, with prognostic implications (severity) based on an RDT, has been previously described[100–102]. The most recent one is

a retrospective study of 273 cases of imported malaria in Spain, where the presence of only the HRP2 reactivity with the absence of pLDH co-reactivity showed a 100% negative predictive value for high parasitemia[102]. This study, along with the other mentioned two studies based on the same concept, confirmed a semi-quantitative way to exclude potential SM cases[100,101]. This approach to RDT is of great interest and lays the conceptual foundation for taking the next step, the **article 4**, where the prognostic capabilities of HRP2 and pLDH were evaluated.

Article 4 showed that HRP2 and pLDH had good performances as prognostic biomarkers, HRP2 showed 82% sensitivity and 84% specificity in distinguishing between SM and UM (AUC-ROC 0.86). While pLDH had slightly better values of 84% sensitivity and 88% specificity (AUC-ROC 0.88), these values did not reach a significant difference with HRP2. Furthermore, their combination does not significantly enhance the prognostic ability. Although HRP2 has already been proposed as a valuable prognostic biomarker in endemic regions, where elevated levels have been linked to severe outcomes such as cerebral malaria and mortality[92], its role in imported malaria cases remains limited[98], and the absence of established cut-offs hinders its broader clinical application. Moreover, the growing prevalence of *P. falciparum* strains with hrp2/3 gene deletions and the persistence of HRP2 after treatment poses a significant threat to its diagnostic and prognostic effectiveness and its implementation as a tool for treatment response monitoring[67,133,134]. This study represents the first effort to evaluate pLDH as a potential severity biomarker, overcoming some of the limitations associated with HRP2.

Although pLDH can be a reliable candidate, the need for Luminex as a laboratory immunoassay fails to achieve the goal of serving a rapid and accessible tool. The need for dedicated personnel and specialized facilities to execute such complex and lengthy protocols are a clear limitation to the biomarkers' implementation. The pursued rapid solution should at least reduce the hours of work required by an expert microbiologist to read a blood smear. On the other hand, being time such an important feature, one of the questions in this work could be why not attempt to establish the quantification of biomarkers through technologies that would allow a POC to directly provide numeric

values. The most well-known POC in the medical field is the glucometer, which is based on electrochemical sensors. In addition, there are more novel technologies, such as those based on aptamers, but their commercial use is not yet approved, and their development would be more costly and complex, though interesting for the future[135]. Therefore, to stick in the line of upgrading the use of a common RDT, and to eliminate user bias from the LFA readings, we analyzed the signal intensities using a smartphone and the open-source software ImageJ. This approach ensured an extremely low cost, which could be close to zero assuming each clinic, at least in high-resource settings, has access to a smartphone. In terms of prognostic ability, pLDH surpassed HRP2; specifically, HRP2 had a sensitivity of 17% and specificity of 96% (AUC-ROC 0.51), while LDH had a sensitivity of 73.9% and specificity of 88% (AUC-ROC 0.85). It is worth taking a moment to assess the difference in performance between Luminex and RDT. The Luminex technology is a lengthy (several hours), multi-step laboratory-based technique that requires several washing and incubation steps, and the use of an expensive reading machine for the quantification of results. This ensures maximum sensitivity and specificity when analyzing complex samples, such as those employed in this study. In contrast, RDTs require a two-step protocol (application of the sample and washing buffer) and only 15 minutes. Furthermore, the quantification is achieved using a common smartphone, not equipment costing over a hundred thousand euros. This inevitably leads to poorer analytical results compared to Luminex technology, which are commonly used by trained personnel in fully equipped clinical laboratories. In addition to the technical specifications of the two techniques, we should also consider that they most likely employ different biorecognition elements (i.e., antibodies) for the detection of the target. The use of different antibodies is a crucial aspect since, while they detect the same target, their affinity and specificity may differ.

All in all, employing an already commercially available RDT for a cost of approximately 20 dollars, a smartphone, which is ubiquitous nowadays, and open-source software, it is possible to identify SM patients with a sensitivity and specificity of 88% in approximately 15 minutes. This is invaluable information for clinicians who are assessing

patients with a potentially deadly disease to identify the best use of the healthcare resources available.

To conclude, throughout my thesis journey, I have gained valuable insights into the process of addressing clinical questions through research. Recognizing that significant changes often stem from policy interventions—such as primary prevention and education for both patients and physicians—I have deliberately chosen to focus on providing accessible tools and leveraging technology to advance and improve the management of imported malaria.

Given the extensive characterization of the disease over centuries of coexisting with the parasite, further knowledge enhancement must now come from integrating technological advancements into medical practice. This integration aims to refine our clinical practices while optimizing healthcare resources. The future lies in harnessing new technologies to improve our workflows, including the application of machine learning and fostering closer collaborations between laboratory research and clinical medicine. The synergy between these distinct yet essential perspectives is crucial for making a tangible impact on patient outcomes. Moving forward, the prospective validation of these proof-of-concept studies and the subsequent review of guidelines should be the next steps in enhancing the management of imported malaria.

IX.CONCLUSIONS

1. MALrisk is a promising tool to promptly identify suspected malaria cases in patients with imported fever in all clinical settings, allowing the initiation of empiric antimalarials and reinforcing the need for urgent transfer.
2. Although one third of our cohort met the criteria for severe malaria, only a small proportion of cases (7.3%) developed life-threatening conditions or death.
3. Current severity criteria definitions for non-endemic areas would benefit from a review, and a subdivision of the severity groups into very severe malaria and less severe malaria cases could help to optimize health care resources.
4. Patients with malaria exhibiting any of the following conditions—parasitemia >10%, pulmonary edema, altered consciousness, seizures, renal failure, metabolic acidosis or hyperlactatemia, shock, or hypoglycemia—should be classified as very severe malaria and managed in the Intensive Care Unit, as half of these cases may progress to life-threatening conditions
5. Patients reclassified as less severe malaria accounting for 60% of severe malaria cases, have a negligible risk of developing a life-threatening condition and thus could be managed out of an Intensive Care Unit.
6. In patients with imported malaria, the prevalence of co-infections was low. Empirical antibiotic therapy should be restricted to very severe malaria cases and cases with a suspicion of a concomitant infection.
7. Laboratory parameters such as platelets < 50 $10x9/L$, Lactate dehydrogenase > 500 U/L and C-Reactive Protein > 10mg/dL and individual factors such as pregnancy are associated with the development of life-threatening conditions and should be considered in the management of patients with imported malaria.
8. The combination of Angiopoietin-2 and C-Reactive Protein may be a reliable tool for the early identification of severe imported malaria.
9. Histidin-Rich-Protein-2 and plasmodium-Lactate dehydrogenase evaluated by Luminex have a reliable performance as prognostic biomarkers for severity in imported malaria.

10. Plasmodium-lactate dehydrogenase could be used as a prognostic tool for malaria, which can be applied in any healthcare settings using smartphones to read commercially available Rapid Diagnostic Tests.

11. Using a smartphone and open-source software to analyze Rapid Diagnostic Tests can help reduce user bias and could provide a cost-effective, timely method for diagnosing and predicting malaria severity.

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