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Management of patients with acute infections in resource-limited settings: towards new host biomarker-based tools to improve triage and risk stratification

Núria Balanza Lombardia

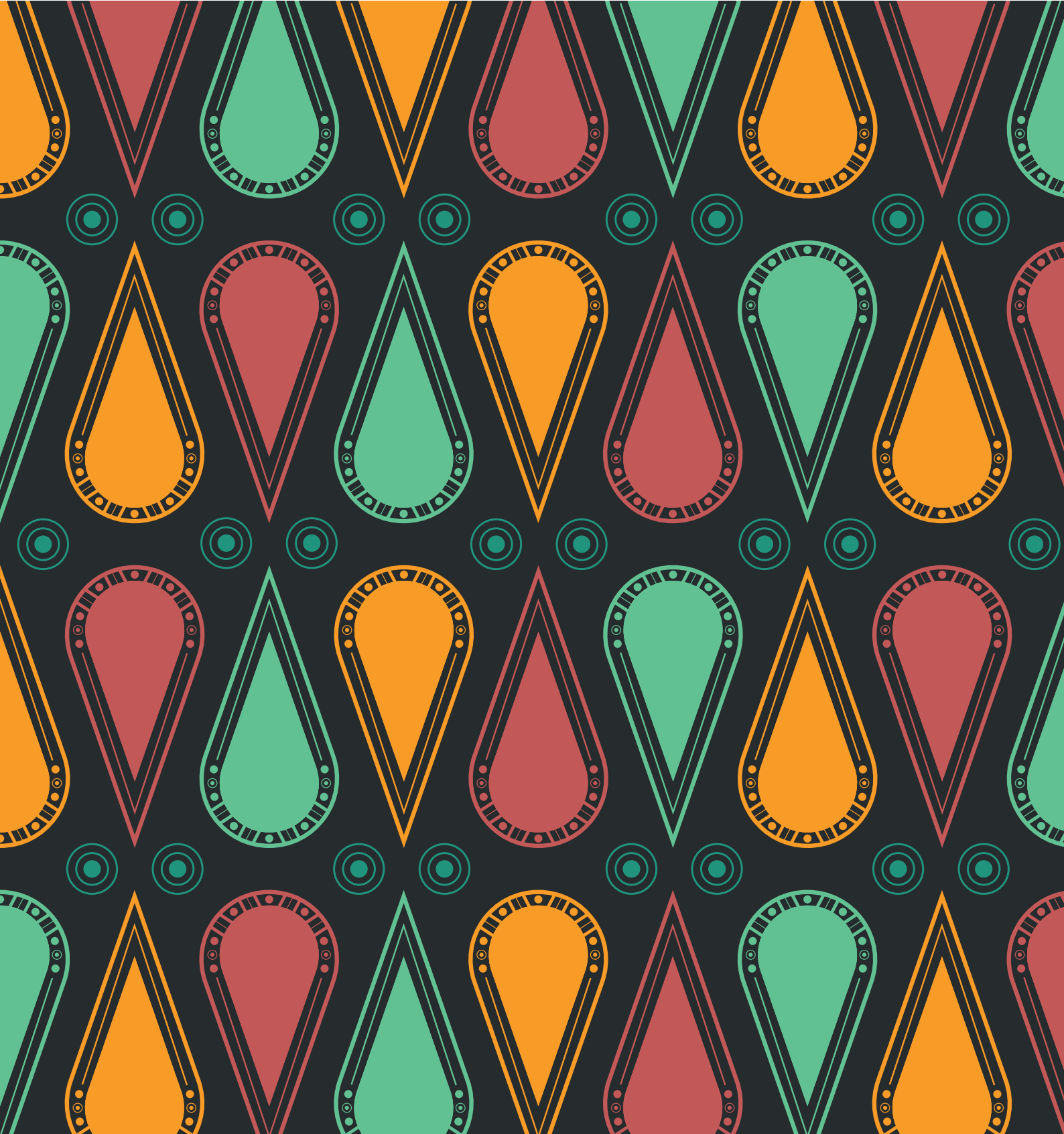
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Management of patients with acute infections in resource-limited settings: towards new host biomarker-based tools to improve triage and risk stratification

Núria Balanza Lombardia · Doctoral Thesis



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Management of patients with acute infections in resource-limited settings: towards new host biomarker-based tools to improve triage and risk stratification

*El maneig dels pacients amb infeccions agudes en entorns amb recursos limitats:
cap a noves eines basades en biomarcadors de l'hoste per a millorar el triatge
i l'estratificació del risc*

Doctoral thesis dissertation presented by **Núria Balanza Lombardia** to apply
for the degree of doctor at the University of Barcelona

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Doctoral Program in Medicine and Translational Research – International Health track,
Faculty of Medicine and Health Sciences, University of Barcelona

December 2024

Written by Núria Balanza Lombardia.
Cover design and layout by Ainoa Piñol Tabernero.

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Als meus pares

ACKNOWLEDGEMENTS

This thesis would not have been possible without the contribution and support of many people, to whom I want to express my heartfelt gratitude.

Al meu director de tesi, Quique Bassat, per la confiança des del primer dia per portar a terme aquest projecte. Gràcies per donar-me l'oportunitat d'aprendre moltíssim treballant en tants estudis diferents. Estaré sempre agraïda d'haver pogut aprendre de la teva manera de fer ciència, el teu esperit col·laboratiu, i els teus consells i visió de futur.

Al meu equip d'ISGlobal. Treballar junts i aprendre de vosaltres ha estat de les millors coses que m'emporto d'aquest doctorat. Gràcies a la Bàrbara, a la Sara Ajanovic, al Rosauero, al Pere, a la Cristina, al David, a l'Andrea, a l'Edie, a la Maria, a la Sara Arias i a la Carole. Gràcies pel suport constant, tantes estones compartides, els aprenentatges en pediatria i en l'àmbit clínic, les hores i hores netejant bases de dades, les discussions científiques, els cafès i dinars a l'oficina, el meu primer viatge a Moçambic de la mà de la Sara Ajanovic, els viatges a Toronto amb la Bàrbara, l'ESPID a Lisboa i la resta de congressos, la recerca a l'Hospital Sant Joan de Déu durant la primera onada de la pandèmia, i un llarg etcètera d'experiències dins i fora de l'oficina.

To Kevin Kain. As we say, you are the biomarker guru we all follow. Thank you for your invaluable contributions to this thesis and for welcoming me into your lab for nearly half a year. My sincere gratitude also goes to Clara for her support at the beginning of this journey. I am also deeply grateful to everyone I worked with in Toronto, who made my time there so enjoyable. Kathleen, Andrea, Valerie, Julie, Caroline, and Hridesh – thank you. Analyzing hundreds of blood samples and learning so much about biomarkers would not have been possible without you.

Aos colegas em Manhiça que tive o prazer de conhecer durante a minha breve estadia lá. Agradeço também a Sádía, Idalmiss, Bento e a todos os pesquisadores do CISM que conheci recentemente em Mopeia, com quem aprendi muito sobre os desafios da prática clínica em Moçambique e que tornaram essa experiência inesquecível.

To all co-authors of the studies of this thesis. Many thanks to the FIEBRE Consortium, ROSI study group, RAPID study group, and contributors to the Manhiça Health and Demographic Surveillance System. En especial, gràcies al Dan, per la paciència en ajudar-me a resoldre tots els meus dubtes d'estadística. I a la Caterina i el Llorenç, pels consells en el maneig i l'anàlisi de les bases de vigilància de morbiditat i demografia de Manhiça.

Al meu tutor de pràctiques docents, el doctor Andreu Prat. Gràcies pel suport i l'orientació durant aquests anys. Fer classes als alumnes de medicina de la Universitat de Barcelona ha estat una experiència molt positiva, de la qual he gaudit molt.

A Ainoa, gracias por el cariño y la dedicación en el diseño de la portada de esta tesis y en su maquetación.

Many thanks to everyone at ISGlobal with whom I have worked or shared moments. A la Carlota i tot l'equip d'Immunologia de la Malària, que durant unes pràctiques de la carrera al 2016 van fer que m'agradés tant ISGlobal que hi vaig voler tornar. To Jeroen and Jeff, who trusted me to come back as a fieldworker and research assistant. To everyone at Roselló 171 during my time there, thank you for the shared lunches and the nice memories. A Camila, por haber sido de las primeras amigas que hice en ISGlobal y por las risas desde entonces. Y a Paula, por abrirme las puertas de tu pisito y haber superado juntas los altibajos de los últimos meses.

Als amics i amigues fora d'ISGlobal que m'han acompanyat en aquest camí. A l'Anna, la Sarai, la Laura, l'Eva i la Míriam, per fer-nos costat i seguir vivint aventures juntes. Anna i Laura, tan llunyà que semblava el final del doctorat i ja ho tenim o estem molt a prop! Que bonic haver pogut compartir amb vosaltres aquesta experiència en paral·lel, amb les seves parts bones i els seus reptes, ja sigui en malalties cardiovasculars, infeccioses o càncer. A Cristina y Maria, por estar desde siempre y poder contar con vosotras en todo momento. Als amics del Masnou, per tot el que hem viscut durant aquests anys. Many thanks also to MJ, Dina, and Valeria, who made me feel at home in Toronto despite being thousands of kilometres away.

Als meus pares, per l'amor i el suport incondicional. Per animar-me a fer allò que em faci feliç, tot i que a vegades no sigui l'opció més fàcil. I per portar-me a Gàmbia l'estiu del 2011. Crec que tots els passos que he anat fent fins a aquesta tesi van començar, d'alguna manera, en aquell viatge. A la resta de la família, per l'acompanyament i els ànims en aquesta etapa. En especial, a mi abuela Carmen, por acogerme en la casa del pueblo durante la fase final de redacción de esta tesis y ayudarme a recargar energías a base de empanada y roscón.

E, finalmente, a todos os participantes dos estudos e às suas famílias. Sem a sua generosidade, esta tese não teria sido possível. O meu mais sincero agradecimento.

Massive thanks to all! Gràcies! ¡Gracias! Obrigada! Kanimambo!

FUNDING

The author of this thesis obtained an FPU (*“Formación de Profesorado Universitario”*) predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260) that supported the work developed in this thesis. She also obtained a mobility scholarship for PhD international research stays (*“Ajut per a estades de recerca a l’estranger per a fomentar la menció de Doctor Internacional del Programa de Doctorat de Medicina i Recerca Translacional”*) from University of Barcelona. ISGlobal receives support from the grant CEX2023-0001290-S funded by MCIN/AEI/10.13039/501100011033, and support from the Generalitat de Catalunya through the CERCA Program. Other funding sources of the studies included in this thesis are specified in detail in each article. The funders had no role in study design; data collection, analysis, or interpretation; writing of the reports; or decision to submit the articles for publication.

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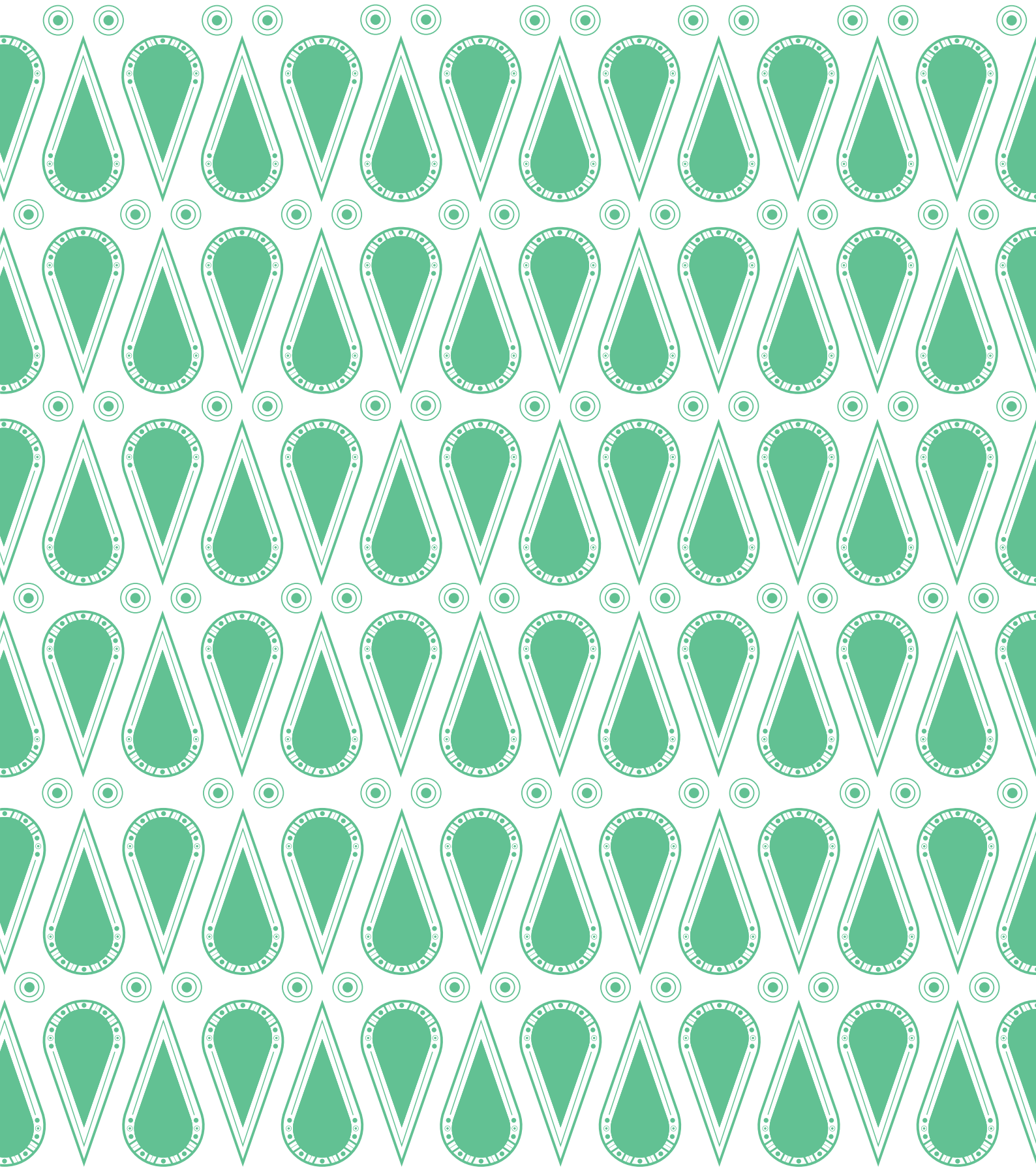
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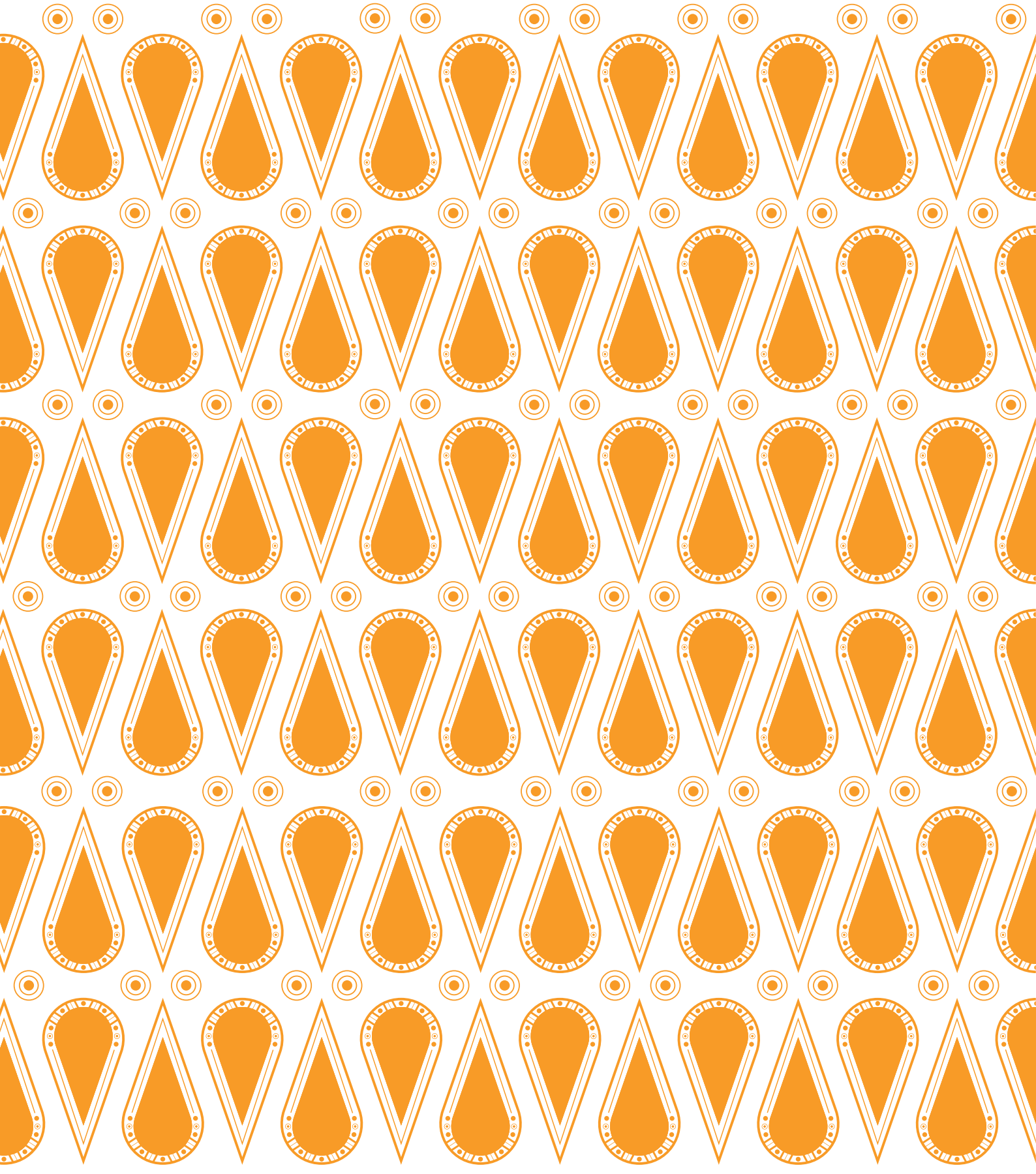
1. ABBREVIATIONS AND ACRONYMS



1. ABBREVIATIONS AND ACRONYMS

- **ACROBAT-newborns:** Appraising the Critical Role of prognostic Biomarkers in the Assessment and Triage of sick African newborns
- **AECID:** Spanish Agency for International Development Cooperation ("*Agencia Española de Cooperación Internacional para el Desarrollo*")
- **AIDS:** Acquired immune deficiency syndrome
- **AKI:** Acute kidney injury
- **Angpt-2:** Angiopoietin-2
- **AUROC:** Area under the receiver operating characteristic curve
- **CHI3L1:** Chitinase-3-like protein-1. It is also known as YKL-40
- **CI:** Confidence interval
- **CISM:** Manhica Health Research Centre ("*Centro de Investigação em Saúde de Manhica*")
- **COVID-19:** Coronavirus disease 2019
- **CRP:** C-reactive protein
- **CSF:** Cerebrospinal fluid
- **DALY:** Disability-adjusted life year
- **DAMP:** Damage-associated molecular pattern
- **EChLiBRiST:** Enhancing Children's Lives with Biomarkers for Risk Stratification and Triage
- **ED-PEWS:** Emergency Department Pediatric Early Warning Score
- **ELISA:** Enzyme-linked immunosorbent assay
- **EMA:** European Medicines Agency
- **FDA:** US Food and Drug Administration
- **FIEBRE:** Febrile Illness Evaluation in a Broad Range of Endemicities
- **GDP:** Gross domestic product
- **GFAP:** Glial fibrillary acidic protein
- **HDSS:** Health and Demographic Surveillance System
- **HIC:** High-income country
- **HIV:** Human immunodeficiency virus
- **I-FABP:** Intestinal fatty acid-binding protein
- **IL-6:** Interleukin-6
- **IL-8:** Interleukin-8
- **IMCI:** Integrated Management of Childhood Illness
- **IPD:** Individual participant data
- **IQR:** Interquartile range
- **IRR:** Incidence rate ratio
- **ISGlobal:** Barcelona Institute for Global Health
- **LBP:** Lipopolysaccharide binding protein
- **LMIC:** Low- and middle-income country
- **LODS:** Lambarené Organ Dysfunction Score
- **LqSOFA:** Liverpool quick Sequential Organ Failure Assessment
- **MEWS:** Modified Early Warning Score
- **NfL:** Neurofilament light chain
- **NGAL:** Neutrophil gelatinase-associated lipocalin
- **NSE:** Neuron-specific enolase
- **OR:** Odds ratio
- **PACK:** Practical Approach to Care Kit
- **PAMP:** Pathogen-associated molecular pattern
- **PCT:** Procalcitonin
- **POC:** Point-of-care
- **PRR:** Pattern recognition receptor
- **qSOFA:** Quick Sequential (Sepsis-Related) Organ Failure Assessment
- **RAPID:** Diagnostic biomarkers for respiratory infection and malaria
- **RDT:** Rapid diagnostic test
- **RISC-Malawi:** Respiratory Index of Severity in Children-Malawi
- **ROSI:** Rosiglitazone Adjunctive Therapy for Severe Malaria in Children
- **RSV:** Respiratory syncytial virus
- **S100 β :** Astrocyte calcium-binding protein β
- **sCD14:** Soluble complement of differentiation 14
- **SDG:** Sustainable Development Goal
- **sFit-1:** Soluble fms-like tyrosine kinase-1. It is also known as soluble vascular endothelial growth factor receptor-1 or sVEGFR-1
- **SPOT-SEPSIS:** Prediction of Disease Severity in Young Children Presenting With Acute Febrile Illness in Resource-limited Settings
- **sTNFR1:** Soluble tumor necrosis factor receptor-1
- **sTREM-1:** Soluble triggering receptor expressed on myeloid cells-1
- **suPAR:** Soluble urokinase-type plasminogen activator receptor
- **TFF3:** Trefoil factor 3
- **UCH-L1:** Ubiquitin C-terminal hydrolase L1
- **UI:** Uncertainty interval
- **UNICEF:** United Nations Children's Fund
- **UVA:** Universal Vital Assessment
- **WHO:** World Health Organization
- **ZO-1:** Zonula occludens-1

2. LIST OF ARTICLES



2. LIST OF ARTICLES

This is a thesis in compendium of publications format.

The thesis consists of two general objectives and four articles:

ARTICLE 1

Paediatric healthcare in Manhiça district through a gender lens: a retrospective analysis of 17 years of morbidity and demographic surveillance data

Núria Balanza, Aura Hunguana, Sara Ajanovic, Rosauero Varo, Justina Bramugy, Teodimiro Matsena, Tacilta Nhampossa, Dan Ouchi, Arsénio Nhacolo, Jéssica Dalsuco, Antonio Siteo, Llorenç Quintó, Sozinho Acácio, Ariel Nhacolo, Maria Maixenchs, Khátia Munguambe, Inácio Mandomando, Pedro Aide, Francisco Saúte, Caterina Guinovart, Charfudin Sacoor, Quique Bassat

Journal of Global Health. 2025;15:04010

Journal Impact Factor 2023: 4.5, Q1 (Public, Environmental & Occupational Health)

Published – *Research article*

ARTICLE 2

Prognostic accuracy of biomarkers of immune and endothelial activation in Mozambican children hospitalized with pneumonia

Núria Balanza, Clara Erice, Michelle Ngai, Chloe R. McDonald, Andrea M. Weckman, Julie Wright, Melissa Richard-Greenblatt, Rosauero Varo, Elisa López-Varela, Antonio Siteo, Pio Vitorino, Justina Bramugy, Miguel Lanaspá, Sozinho Acácio, Lola Madrid, Bàrbara Baro, Kevin C. Kain[#], Quique Bassat[#]

[#]Co-senior authors

PLOS Global Public Health. 2023;3(2):e0001553

Journal Impact Factor & Quartile not available yet

Published – *Research article*

ARTICLE 3

Soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) to risk-stratify paediatric and adult patients with febrile illness in southern Mozambique

Núria Balanza, Bàrbara Baro, Sara Ajanovic, Zumilda Boca, Justina Bramugy, Anelsio Cossa, Elizabeth JA Fitchett, Heidi Hopkins, Suzanne H. Keddie, Sham Lal, David C. W. Mabey, MD, Tegwen Marlais, Hridayesh Mishra, Campos Mucasse, Marta Valente, Andrea M. Weckman, Julie K. Wright, Shunmay Yeung, Kathleen Zhong, Kevin C. Kain[#], Quique Bassat[#]

[#]Co-senior authors

Communications Medicine

Journal Impact Factor 2023: 5.4, Q1 (Medicine, Research & Experimental)

Under review – *Research article*

ARTICLE 4

Neurofilament light chain as a biomarker of neuronal damage in children with malaria

Núria Balanza^{*}, Caroline K. Francis^{*}, Valerie M. Crowley, Andrea M. Weckman, Kathleen Zhong, Bàrbara Baro, Rosauero Varo, Quique Bassat[#], Kevin C. Kain[#], Rosiglitazone Adjunctive Therapy for Severe Malaria (ROSI) Study Group

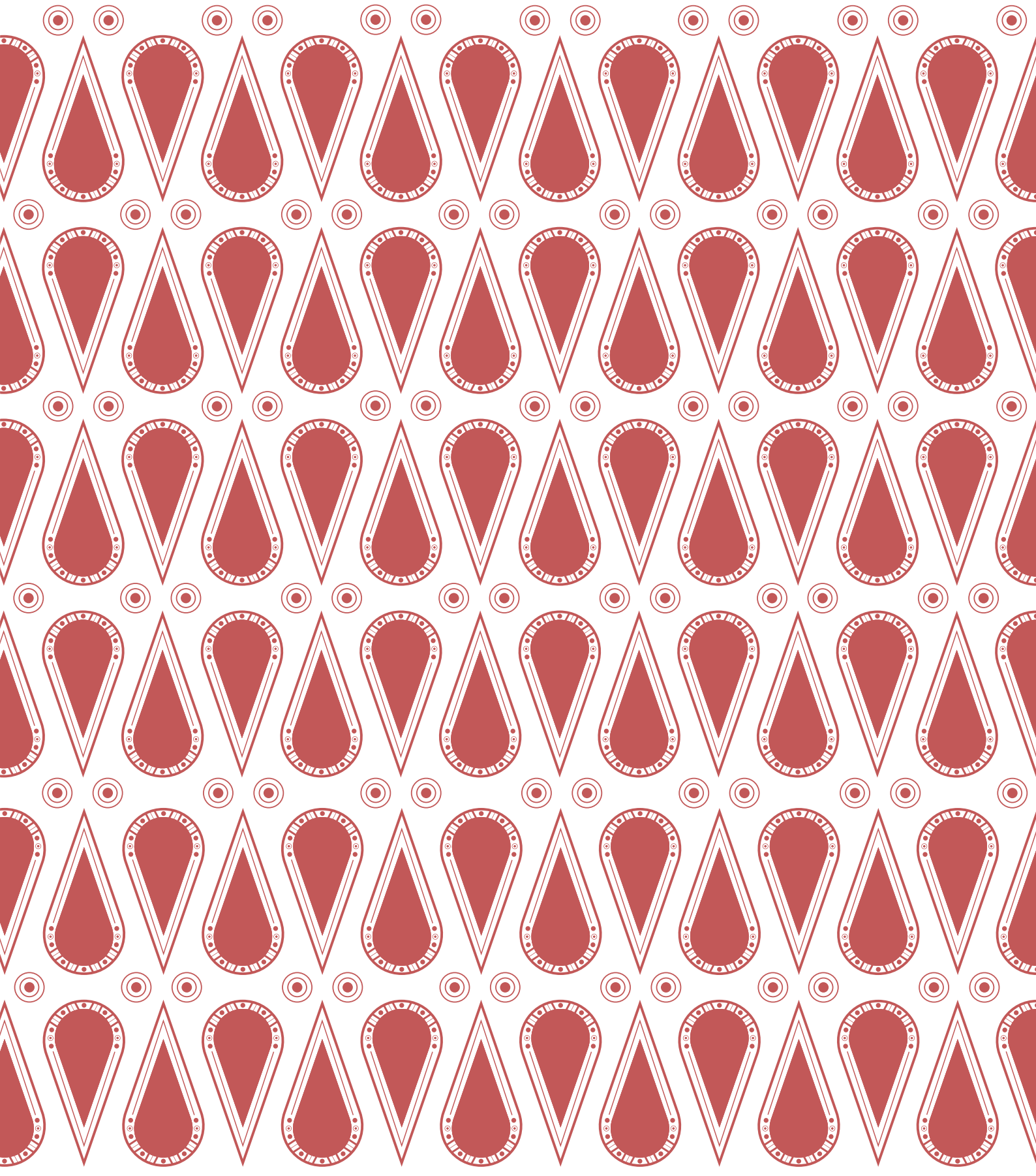
^{*}Co-first authors, [#]Co-senior authors

Journal of Infectious Diseases, 2024;229(1):183-188

Journal Impact Factor 2023: 5.0, Q1 (Infectious Diseases)

Published – *Brief report*

3. SUMMARY



3. SUMMARY

3.1. SUMMARY IN ENGLISH

Title: Management of patients with acute infections in resource-limited settings: towards new host biomarker-based tools to improve triage and risk stratification.

Introduction: Acute infections are a leading reason for seeking healthcare globally. However, the triage and risk stratification of patients with such infections remain challenging across different healthcare levels. In resource-limited settings, this is exacerbated by scarce clinicians, medical supplies, and equipment. Current strategies have inherent limitations and rely heavily on the subjective judgment of healthcare workers. Delays in the recognition and appropriate management of patients at risk of poor outcomes contribute to preventable mortality and morbidity, while unnecessary hospitalizations and overtreatment lead to misallocation of healthcare resources and promote antimicrobial resistance. Furthermore, overreliance on subjective criteria can result in inequities in clinical evaluation and decision-making, including gender-based disparities. New, simple, accurate, objective, and inexpensive tools for triage and risk stratification are needed.

Hypothesis: The overarching hypothesis of this thesis is that measuring host biomarkers in patients with acute infections would improve current patient triage and risk stratification practices in resource-limited settings and trigger more equitable management decisions, with a positive impact on clinical outcomes. Specific hypotheses are: i) Boys and girls in Manhica district, southern Mozambique, differ in access to and provision of healthcare, and ii) In pediatric and adult patients with suspected acute infections in Mozambique, measuring host biomarkers can help identify individuals at risk of adverse outcomes or with specific organ injury.

Objectives: This thesis presents two general objectives: i) To describe pediatric healthcare in Manhica district through a gender lens, and ii) To evaluate whether measuring host biomarkers can help identify individuals at risk of adverse outcomes or with specific organ injury in different clinical scenarios involving pediatric and adult patients with suspected acute infections in Mozambique.

Methods: This thesis comprises four studies. The first study addresses the first general objective, while the subsequent studies focus on the second general objective. The first study is a retrospective analysis of data from the Manhica Health and Demographic Surveillance System, including visits to outpatient clinics and hospitalizations from 2004 to 2020 in Manhica district by children aged <15 years. Different indicators reflecting access to and provision of healthcare were compared between boys and girls. The second and third studies are ancillary to two prospective observational studies conducted in Mozambique. The second study included children

aged <10 years hospitalized with clinical pneumonia and healthy community controls. The third study included children aged ≥ 2 months and adults with all-cause febrile illness presenting to hospital. In both, we retrospectively quantified plasma levels of different biomarkers of immune and endothelial activation at clinical presentation, and evaluated their prognostic performance for mortality and other adverse outcomes. The fourth study addresses a secondary objective of a randomized clinical trial in Mozambican children aged 1 to 12 years with malaria. We compared the dynamics of plasma neurofilament light chain (NfL) up to 14 days according to disease severity and the presence of neurological manifestations.

Main results: The first study included 1,139,962 pediatric visits to outpatient clinics and 41,278 hospitalizations. Overall, we found no major differences between boys and girls in most indicators of healthcare access and provision. However, girls were less frequently referred or admitted to hospital (OR=0.82 [95% CI: 0.79-0.86] and OR=0.85 [95% CI: 0.84-0.87], respectively) and were also less likely to present with severe manifestations. The second study included 472 pediatric pneumonia cases and 80 controls. Levels of all studied biomarkers were higher in cases compared to controls. Interleukin-8 (IL-8), soluble fms-like tyrosine kinase-1 (sFlt-1), and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) were the best predictors of in-hospital mortality, with AUROCs of 0.88 (95% CI: 0.78-0.97), 0.83 (95% CI: 0.73-0.94) and 0.82 (95% CI: 0.74-0.91), respectively, as well as the best predictors of 28-day and 90-day mortality. The third study included 1,955 pediatric and adult febrile patients. Levels of all studied biomarkers were higher in inpatients compared to outpatients. sTREM-1 was the best predictor of 28-day mortality with an AUROC of 0.82 (95% CI: 0.78-0.86). Its prognostic performance was consistent when stratifying by age and sex, but lower in HIV-positive patients. sTREM-1 levels were also associated with longer hospitalization and seeking further care in outpatients. In the second and third studies, best biomarkers outperformed C-reactive protein (CRP), procalcitonin (PCT), and lactate in predicting mortality, and their addition improved the AUROC of different clinical severity scores for mortality. The fourth study included 167 children with malaria. NfL levels were similar in all patients at hospital admission, but levels increased over time and this increment was significantly higher in cases with neurological manifestations.

Conclusions: Host biomarkers are a promising tool to improve current triage and risk stratification practices for patients with acute infections in resource-limited settings. Among these, sTREM-1 stands out as a robust predictor of mortality, while NfL could be useful for identifying brain injury. These biomarkers can also help reduce subjectivity in the provision of care. In the Manhica district, no major differences between boys and girls were observed in most of the studied indicators, but further research is needed. Overall, the findings of this thesis contribute towards the development of new host biomarker-based tools, whose clinical utility and impact should be evaluated in future interventional studies.

3.2. SUMMARY IN SPANISH

Título: El manejo de los pacientes con infecciones agudas en entornos con recursos limitados: hacia nuevas herramientas basadas en biomarcadores del huésped para mejorar el triaje y la estratificación del riesgo.

Introducción: Las infecciones agudas son una de las principales causas de consulta sanitaria en todo el mundo. Sin embargo, el triaje y la estratificación del riesgo en pacientes con dichas infecciones siguen siendo un reto en todos los niveles asistenciales. En entornos con recursos limitados, esta situación se ve agravada por la escasez de personal clínico, suministros médicos y equipamiento. Las estrategias actuales tienen limitaciones inherentes y dependen en gran medida de juicios subjetivos del personal sanitario. Los retrasos en el reconocimiento y manejo adecuado de los pacientes con riesgo de evolución desfavorable contribuyen a una mortalidad y morbilidad evitables, mientras que las hospitalizaciones innecesarias y el sobretratamiento se traducen en una asignación ineficiente de los recursos sanitarios y favorecen la resistencia a los antimicrobianos. Además, depender de criterios subjetivos puede generar inequidades en la evaluación y las decisiones clínicas, incluidas disparidades basadas en el género. Se necesitan nuevas herramientas de triaje y estratificación del riesgo que sean sencillas, precisas, objetivas y económicas.

Hipótesis: La hipótesis de esta tesis es que medir biomarcadores del huésped en pacientes con infecciones agudas mejoraría las prácticas actuales de triaje y estratificación del riesgo en entornos con recursos limitados y resultaría en un manejo clínico más equitativo, con un impacto positivo en los resultados clínicos. Las hipótesis específicas son: i) Los niños y las niñas del distrito de Manhica, en el sur de Mozambique, difieren en el acceso y prestación de atención sanitaria, y ii) En pacientes pediátricos y adultos con sospecha de infecciones agudas en Mozambique, medir biomarcadores del huésped puede ayudar a identificar a aquellos en riesgo de resultados adversos o con daño orgánico específico.

Objetivos: Esta tesis presenta dos objetivos generales: i) Describir la atención sanitaria pediátrica en el distrito de Manhica desde una perspectiva de género, y ii) Evaluar si medir biomarcadores del huésped, en pacientes pediátricos y adultos con sospecha de infecciones agudas en Mozambique, en diferentes escenarios clínicos, puede ayudar a identificar a aquellos en riesgo de resultados adversos o con daño orgánico específico.

Métodos: Esta tesis consta de cuatro estudios. El primero aborda el primer objetivo general, mientras que el resto se centran en el segundo objetivo general. El primer estudio es un análisis retrospectivo de datos del Sistema de Vigilancia Demográfica y Sanitaria de Manhica e incluye visitas a centros ambulatorios y hospitalizaciones de niños <15 años entre 2004 y 2020 en el distrito de Manhica. Se compararon diferentes indicadores que reflejan el acceso y prestación de atención sanitaria entre niños y niñas. El segundo y tercer estudio son ancillares a dos estudios observacionales prospectivos en Mozambique. El segundo incluyó a niños <10 años hospitalizados con neumonía clínica y

controles sanos de la comunidad. El tercero incluyó a niños ≥ 2 meses y adultos con fiebre que acudieron al hospital. En ambos se cuantificaron retrospectivamente los niveles plasmáticos de diferentes biomarcadores de activación inmunitaria y endotelial a la llegada al hospital, y se evaluó su valor pronóstico para mortalidad y otros resultados adversos. El cuarto estudio aborda un objetivo secundario de un ensayo clínico aleatorizado en niños mozambiqueños de 1 a 12 años con malaria. Se compararon las dinámicas de los neurofilamentos de cadena ligera (NfL) en plasma hasta los 14 días según la gravedad y la presencia de manifestaciones neurológicas.

Resultados principales: El primer estudio incluyó 1.139.962 visitas y 41.278 hospitalizaciones pediátricas. No se encontraron grandes diferencias entre niños y niñas en la mayoría de indicadores de acceso y prestación de atención sanitaria. Sin embargo, las niñas fueron menos referidas o ingresadas en el hospital (OR=0,82 [IC 95%: 0,79-0,86] y OR=0,85 [IC 95%: 0,84-0,87], respectivamente) y también presentaron menos manifestaciones graves a la llegada. El segundo estudio incluyó 472 casos pediátricos de neumonía y 80 controles. Los niveles de todos los biomarcadores estudiados fueron superiores en los casos respecto a los controles. La interleucina-8 (IL-8), la tirosina quinasa-1 soluble similar a fms (sFlt-1) y la forma soluble del receptor activador expresado en células mieloides-1 (sTREM-1) fueron los mejores biomarcadores para predecir mortalidad intrahospitalaria, con unas AUROCs de 0,88 (IC 95%: 0,78-0,97), 0,83 (IC 95%: 0,73-0,94) y 0,82 (IC 95%: 0,74-0,91), respectivamente, así como los mejores para predecir mortalidad a 28 y 90 días. El tercer estudio incluyó a 1.955 pacientes pediátricos y adultos febriles. Los niveles de todos los biomarcadores estudiados fueron superiores en los pacientes que hospitalizaron. sTREM-1 fue el mejor predictor de mortalidad a 28 días, con una AUROC de 0,82 (IC 95%: 0,78-0,86). Su valor pronóstico fue consistente al estratificar por edad y sexo, pero inferior en los pacientes VIH positivos. Los niveles de sTREM-1 también se asociaron con una hospitalización más prolongada y con buscar atención sanitaria adicional tras no ser hospitalizado. En el segundo y tercer estudio, los mejores biomarcadores superaron a la proteína C reactiva (PCR, o CRP en inglés), la procalcitonina (PCT) y el lactato para predecir mortalidad, y su combinación con escalas clínicas de gravedad mejoró la AUROC de éstas para mortalidad. El cuarto estudio incluyó a 167 niños con malaria. Los niveles de NfL fueron similares en todos los pacientes al ingreso, pero aumentaron con el tiempo y este incremento fue significativamente mayor en los que presentaron manifestaciones neurológicas.

Conclusiones: Los biomarcadores del huésped son una herramienta prometedora para mejorar las prácticas actuales de triaje y estratificación de riesgo en pacientes con infecciones agudas en entornos con recursos limitados. Entre ellos, sTREM-1 destaca como un predictor robusto de mortalidad, mientras que NfL podría ser útil para identificar daño cerebral. Estos biomarcadores también pueden ayudar a reducir la subjetividad en la prestación de atención sanitaria. En el distrito de Manhica, no se observaron grandes diferencias entre niños y niñas en la mayoría de los indicadores estudiados, pero es necesario seguir investigando. Los hallazgos de esta tesis contribuyen al desarrollo de nuevas herramientas basadas en biomarcadores del huésped, cuya utilidad clínica e impacto deberán evaluarse en futuros estudios de intervención.

3.3. SUMMARY IN CATALAN

Títol: El maneig dels pacients amb infeccions agudes en entorns amb recursos limitats: cap a noves eines basades en biomarcadors de l'hoste per a millorar el triatge i l'estratificació del risc.

Introducció: Les infeccions agudes són una de les principals causes de consulta sanitària arreu del món. Tanmateix, el triatge i l'estratificació del risc en pacients amb aquestes infeccions continuen sent un repte en tots els nivells assistencials. En entorns amb recursos limitats, aquesta situació es veu agreujada per l'escassetat de personal clínic, subministraments mèdics i equipament. Les estratègies actuals tenen limitacions inherents i depenen en gran manera de judicis subjectius del personal sanitari. Els retards en el reconeixement i maneig adequat dels pacients amb risc d'evolució desfavorable contribueixen a una mortalitat i morbiditat evitables, mentre que les hospitalitzacions innecessàries i el sobretractament es tradueixen en una assignació ineficient dels recursos sanitaris i afavoreixen la resistència als antimicrobians. A més, dependre de criteris subjectius pot generar inequitats en l'avaluació i les decisions clíniques, incloses disparitats basades en el gènere. Es necessiten noves eines de triatge i estratificació del risc que siguin senzilles, precises, objectives i econòmiques.

Hipòtesi: La hipòtesi d'aquesta tesi és que mesurar biomarcadors de l'hoste en pacients amb infeccions agudes milloraria les pràctiques actuals de triatge i estratificació del risc en entorns amb recursos limitats i resultaria en un maneig clínic més equitatiu, amb un impacte positiu en els resultats clínics. Les hipòtesis específiques són: i) Els nens i les nenes del districte de Manhíça, en el sud de Moçambic, difereixen en l'accés i prestació d'atenció sanitària, i ii) En pacients pediàtrics i adults amb sospita d'infeccions agudes a Moçambic, mesurar biomarcadors de l'hoste pot ajudar a identificar a aquells en risc de resultats adversos o amb dany orgànic específic.

Objectius: Aquesta tesi presenta dos objectius generals: i) Descriure l'atenció sanitària pediàtrica en el districte de Manhíça des d'una perspectiva de gènere, i ii) Avaluar si mesurar biomarcadors de l'hoste, en pacients pediàtrics i adults amb sospita d'infeccions agudes a Moçambic, en diferents escenaris clínics, pot ajudar a identificar a aquells en risc de resultats adversos o amb dany orgànic específic.

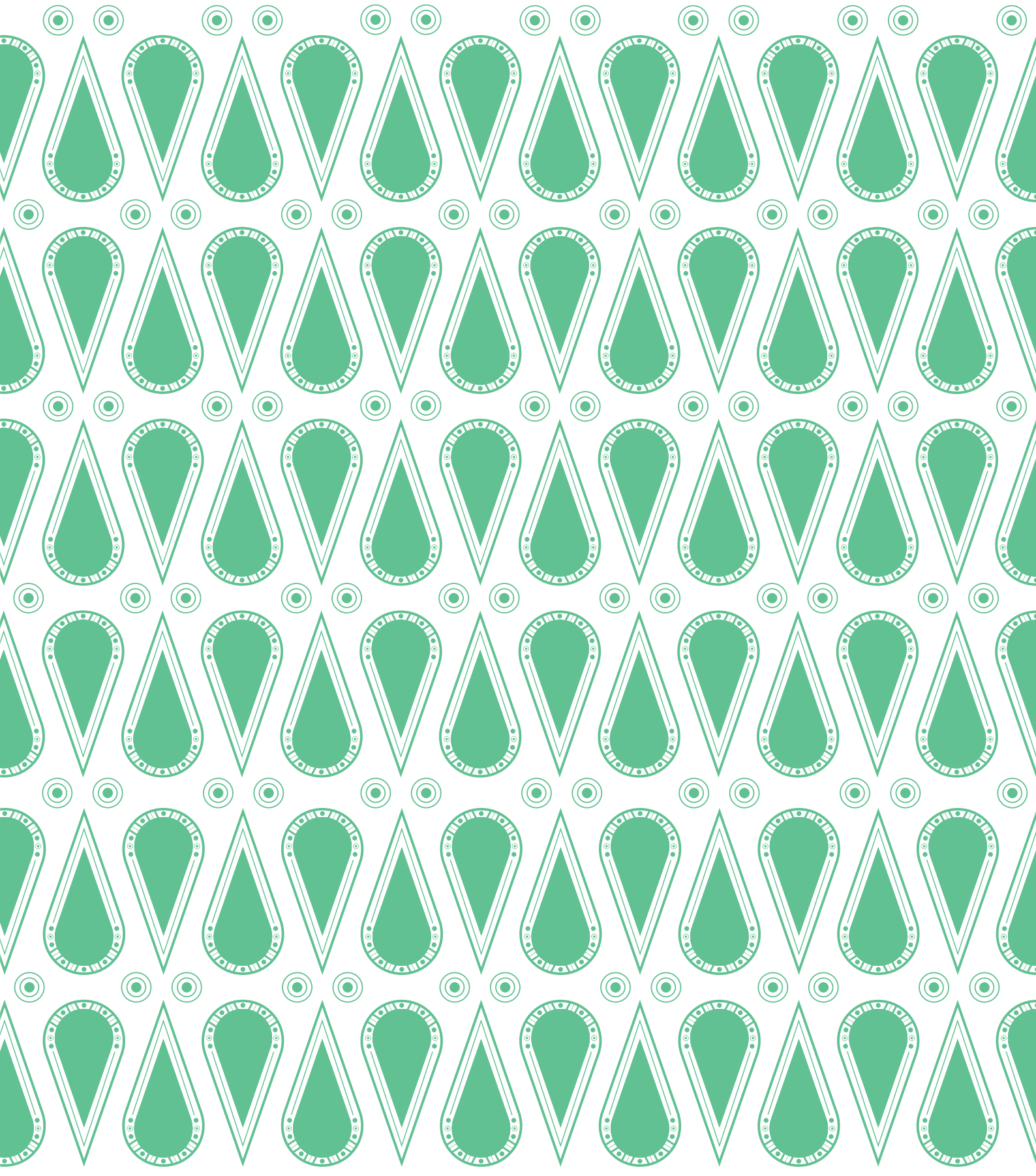
Mètodes: Aquesta tesi consta de quatre estudis. El primer aborda el primer objectiu general, mentre que la resta se centren en el segon objectiu general. El primer estudi és una anàlisi retrospectiva de dades del Sistema de Vigilància Demogràfica i Sanitària de Manhíça i inclou visites a centres ambulatoris i hospitalitzacions de nens <15 anys entre 2004 i 2020 en el districte de Manhíça. Es van comparar diferents indicadors que reflecteixen l'accés i prestació d'atenció sanitària entre nens i nenes. El segon i tercer estudi són ancil·lars a dos estudis observacionals prospectius a Moçambic. El segon va incloure nens <10 anys hospitalitzats amb pneumònia clínica i controls sans de la comunitat. El tercer va incloure nens ≥2 mesos i adults amb febre que van acudir a l'hospital. En tots dos es van quantificar retrospectivament els nivells plasmàtics de diferents biomarcadors

d'activació immunitària i endotelial a l'arribada a l'hospital, i es va avaluar el seu valor pronòstic per a mortalitat i altres resultats adversos. El quart estudi aborda un objectiu secundari d'un assaig clínic aleatoritzat en nens moçambiquesos d'1 a 12 anys amb malària. Es van comparar les dinàmiques dels neurofilaments de cadena lleugera (NfL) en plasma fins als 14 dies segons la gravetat i la presència de manifestacions neurològiques.

Resultats principals: El primer estudi va incloure 1.139.962 visites i 41.278 hospitalitzacions pediàtriques. No es van trobar grans diferències entre nens i nenes en la majoria d'indicadors d'accés i prestació d'atenció sanitària. No obstant això, les nenes van ser menys referides o ingressades a l'hospital (OR=0,82 [IC 95%: 0,79-0,86] i OR=0,85 [IC 95%: 0,84-0,87], respectivament) i també van presentar menys manifestacions greus a l'arribada. El segon estudi va incloure 472 casos pediàtrics de pneumònia i 80 controls. Els nivells de tots els biomarcadors estudiats van ser superiors en els casos respecte als controls. La interleucina-8 (IL-8), la tirosina quinasa-1 soluble similar a fms (sFlt-1) i la forma soluble del receptor activador expressat en cèl·lules mieloides-1 (sTREM-1) van ser els millors biomarcadors per predir mortalitat intrahospitalària, amb unes AUROCs de 0,88 (IC 95%: 0,78-0,97), 0,83 (IC 95%: 0,73-0,94) i 0,82 (IC 95%: 0,74-0,91), respectivament, així com els millors per predir mortalitat a 28 i 90 dies. El tercer estudi va incloure 1.955 pacients pediàtrics i adults febrils. Els nivells de tots els biomarcadors estudiats van ser superiors en els pacients que van hospitalitzar. sTREM-1 va ser el millor predictor de mortalitat a 28 dies, amb una AUROC de 0,82 (IC 95%: 0,78-0,86). El seu valor pronòstic va ser consistent en estratificar per edat i sexe, però inferior en els pacients VIH positius. Els nivells de sTREM-1 també es van associar amb una hospitalització més prolongada i amb buscar atenció sanitària addicional després de no ser hospitalitzat. En el segon i tercer estudi, els millors biomarcadors van superar a la proteïna C reactiva (PCR, o CRP en anglès), la procalcitonina (PCT) i el lactat per predir mortalitat, i la seva combinació amb escales clíniques de gravetat va millorar la AUROC d'aquestes per a mortalitat. El quart estudi va incloure 167 nens amb malària. Els nivells de NfL van ser similars en tots els pacients a l'ingrés, però van augmentar amb el temps i aquest increment va ser significativament major en els quals van presentar manifestacions neurològiques.

Conclusions: Els biomarcadors de l'hoste són una eina prometedora per a millorar les pràctiques actuals de triatge i estratificació de risc en pacients amb infeccions agudes en entorns amb recursos limitats. Entre ells, sTREM-1 destaca com un predictor robust de mortalitat, mentre que NfL podria ser útil per a identificar dany cerebral. Aquests biomarcadors també poden ajudar a reduir la subjectivitat en la prestació d'atenció sanitària. En el districte de Manhiça, no es van observar grans diferències entre nens i nenes en la majoria dels indicadors estudiats, però és necessari continuar investigant. Les troballes d'aquesta tesi contribueixen al desenvolupament de noves eines basades en biomarcadors de l'hoste, la utilitat clínica i impacte de les quals hauran d'avaluar-se en futurs estudis d'intervenció.

4. INTRODUCTION



4. INTRODUCTION

4.1. INFECTIOUS DISEASES

4.1.1. Global burden of infectious diseases

Infectious diseases remain, still in 2024, a significant cause of morbidity and mortality globally. Despite a general epidemiological transition towards an increased burden of non-communicable diseases, lower respiratory infections, diarrheal diseases, human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), tuberculosis, and malaria are current leading causes of illness, disability, and mortality [1,2]. Globally in 2019, over a quarter (27.7%) of the total 2,540 million (95% uncertainty interval [UI]: 2,290-2,810) disability-adjusted life years (DALYs) were associated with 85 different pathogens [3]. Bacterial infections were associated with 415 million DALYs (58.9%), viral infections with 178 million DALYs (25.3%), parasitic infections with 72 million DALYs (10.2%), and fungal infections with 18.5 million DALYs (2.6%) [3].

Low- and middle-income countries (LMICs) are disproportionately affected by infectious diseases, with sub-Saharan Africa bearing the highest burden. The factors driving this disparity are multifaceted, including weak health systems, insufficient vaccination coverage, poor sanitation and water access, environmental and climate factors, and a high prevalence of malnutrition. Children younger than five years are also particularly impacted, with 65.5% of their DALYs associated with infections (79.3% in sub-Saharan Africa) compared to 19.1% of DALYs in individuals aged five years and older (45.2% in sub-Saharan Africa) (**Figure 1**) [3].

4.1.2. Sepsis – a major global health concern

Sepsis is a syndromic response to infection and the final common pathway to death from most infectious diseases [4]. According to the Sepsis-3 definition, sepsis is currently defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [5]. The concept and the understanding of sepsis have evolved over time [6,7]. Now it encompasses bacterial, fungal, viral, and parasitic pathogens; requires only a suspected infection acknowledging that many cases lack definitive confirmation; focuses on a non-homeostatic host response as the main source of morbidity and mortality; and entails evaluation of organ dysfunction through clinical scores [5]. The subset of sepsis patients with particularly profound circulatory, cellular, and metabolic abnormalities is defined as having septic shock [5]. Recently, the specific criteria for pediatric sepsis and septic shock were updated to be aligned with those in current use for adults [8,9].

Sepsis is recognized as a serious worldwide health threat, characterized by high incidence and mortality, as well as severe morbidities and *sequelae* among survivors [4,10]. Despite the scarcity of high-quality epidemiological data on the burden of sepsis [11], a global study estimated 48.9 million (95% UI: 38.9-62.9) sepsis cases and 11.0 million (95% UI: 10.1-12.0) sepsis-related deaths in 2017 [12]. This represents around one in every five (19.7%) of all annual deaths globally. The largest contributors to sepsis incidence and mortality for all age groups were diarrheal diseases and lower respiratory infections, respectively [12]. Sepsis especially affects vulnerable populations such as neonates, young children, pregnant and recently pregnant women, older adults, individuals with chronic medical conditions, and those immunocompromised [4]. In 2017, 41.5% of all estimated sepsis cases worldwide and 26.4% of sepsis-related deaths occurred in children younger than five years [12]. Furthermore, the burden of sepsis varies significantly across regions, with an inverse relationship between a country's income level and sepsis incidence and mortality. As a result, much of the burden of sepsis is concentrated in LMICs, particularly in sub-Saharan Africa (**Figure 2**) [12].

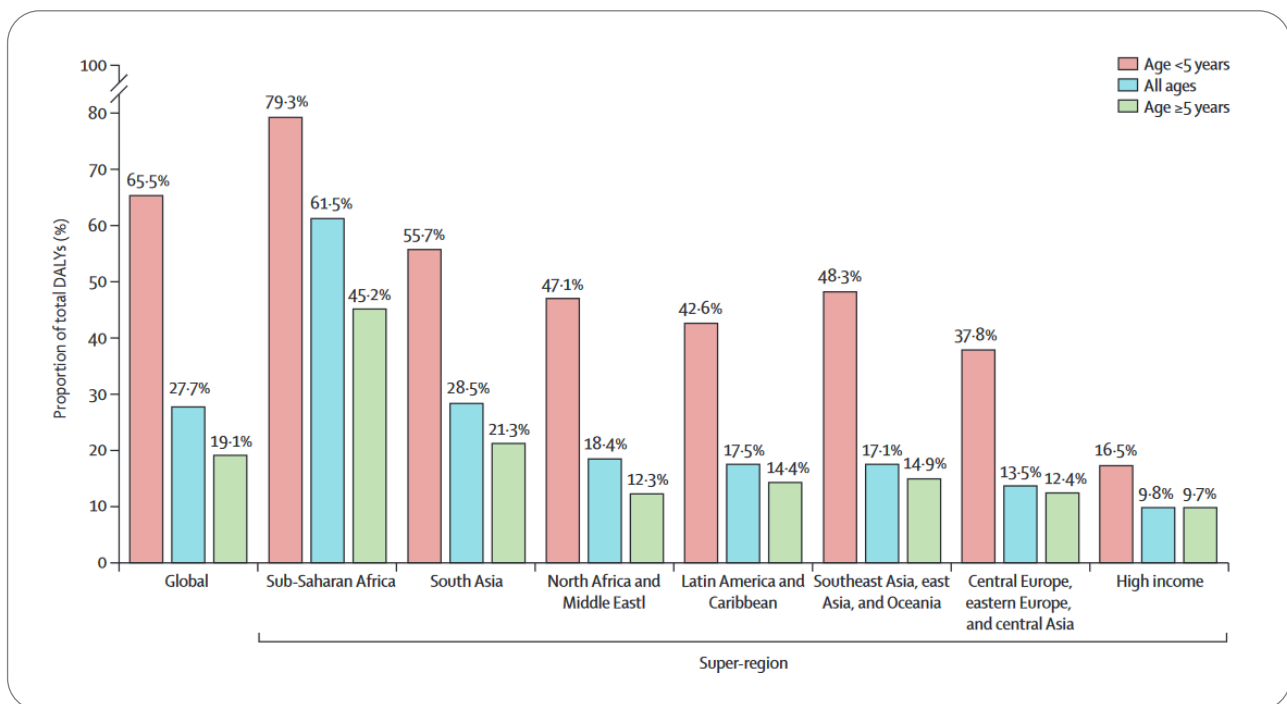


Figure 1: Proportion of total global and regional DALYs associated with 85 pathogens in 2019. Abbreviations: DALYs=disability-adjusted life-years. Reproduced from reference [3].

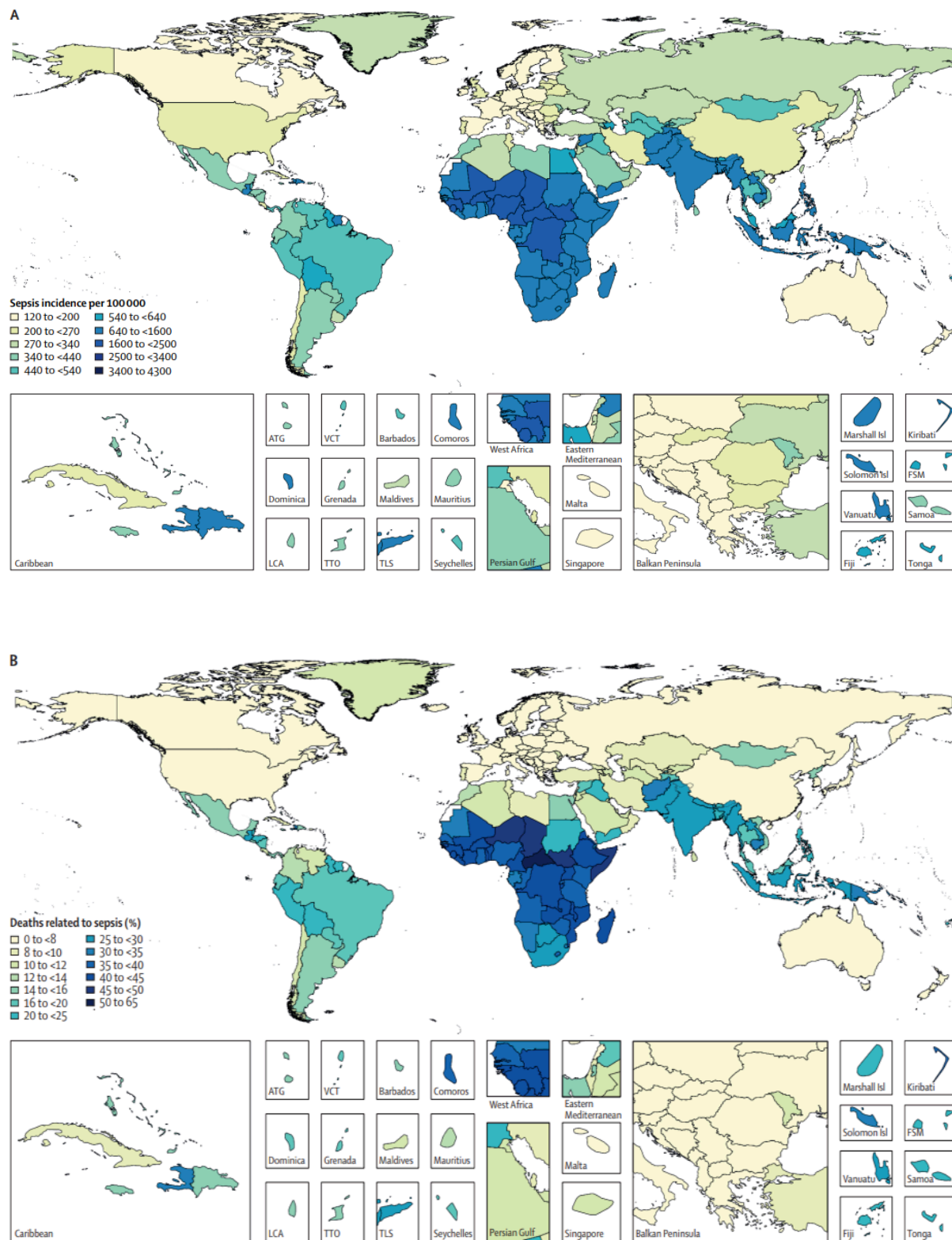


Figure 2: Global estimates on the burden of sepsis in 2017. A) Age-standardized sepsis incidence per 100,000 population for both sexes in 2017. B) Percentage of all deaths related to sepsis, age-standardized for both sexes in 2017. Abbreviations: ATG=Antigua and Barbuda, FSM=Federated States of Micronesia, LCA=Saint Lucia, TLS=Timor-Leste, TTO=Trinidad and Tobago, VCT=Saint Vincent and the Grenadines. Reproduced from reference [12].

4.1.3. Infections affecting the central nervous system

Beyond sepsis, infections affecting the central nervous system are also of particular interest, because of their frequent association with sepsis, the diagnostic challenges they may pose due to overlapping manifestations, their high associated mortality, and the fact that they often lead to serious long-term complications among survivors. Infections such as meningitis, encephalitis, and cerebral malaria entail significant brain dysfunction and injury and often result in long-term neurological deficits and cognitive impairment [13–17]. They cause a significant global disease burden, with sub-Saharan Africa and Asia being the most affected regions. In 2019, meningitis alone accounted for an estimated 2.51 million (95% UI: 2.11–2.99) incident cases and 236,000 (95% UI: 204,000–277,000) deaths globally [18]. In the same year, there were an estimated 1.44 million (95% UI: 1.28–1.61) incident cases and 89,900 deaths (95% UI: 76,530–122,870) due to encephalitis. Malaria remains a global health concern, with approximately 249 million clinical cases in 2022, of which 1–2% progressed to severe forms, including cerebral malaria (the most severe neurological manifestation of the disease) [19]. In addition, sepsis itself can also lead to brain damage due to neuroinflammation and disruption of brain homeostasis, with clinical manifestations ranging from mild delirium to coma and neurological *sequelae* in survivors [20,21].

4.2. MANAGEMENT OF ACUTE INFECTIONS

4.2.1. Challenges in the triage and risk stratification of acute infections

Acute infections are a common reason for individuals to seek healthcare. Most are characterized by their cardinal sign, fever, which is a primary motivation for health consultation globally [22]. In addition to assessing the cause of a patient's illness, healthcare workers must evaluate the urgency of the patient's condition and determine whether they are at risk of severe or fatal progression. At the community or primary care level, most individuals with a suspected acute infection will have an uncomplicated and self-limited course and can be managed conservatively with minimal supportive interventions to increase comfort, alleviate symptoms, and prevent dehydration [23]. Only a small proportion of cases involve life-threatening infections and may progress to sepsis. These require prompt and aggressive treatment to prevent adverse outcomes, including hospitalization or referral to higher-level care for advanced treatment, intensive care, or resuscitative techniques [24]. Healthcare providers must rely on a combination of clinical judgment, patient medical history, and basic available tests to distinguish between non-severe and severe infections. However, early in the course of illness, it is often challenging to identify individuals at risk of severe and fatal infections among the majority who are not, since initial presentations of severe infections

may be subtle and non-specific [24,25]. Furthermore, among those patients considered to have severe conditions and admitted to hospital, it is not straightforward to determine how to prioritize available healthcare resources. It can be difficult to identify which inpatients would benefit most from increased monitoring, high-cost or adjunctive therapies, admission to high-dependency units, or transfer to a higher-level health facility. Additionally, it is also challenging to determine when patients can be safely discharged from the hospital and identify those who would benefit most from closer follow-up after discharge (**Figure 3**) [26].

Delays in the recognition and adequate treatment of patients with severe infections can result in increased mortality and long-term *sequelae* in survivors. On the other hand, unnecessary or prolonged admissions, dispensable referrals to higher-level facilities, and overtreatment entail misallocation of healthcare resources, overloading of healthcare facilities, contribution to the development of antimicrobial resistance, and even harm to patients due to nosocomial infections and exposure to unnecessary investigations and treatments [27–30].

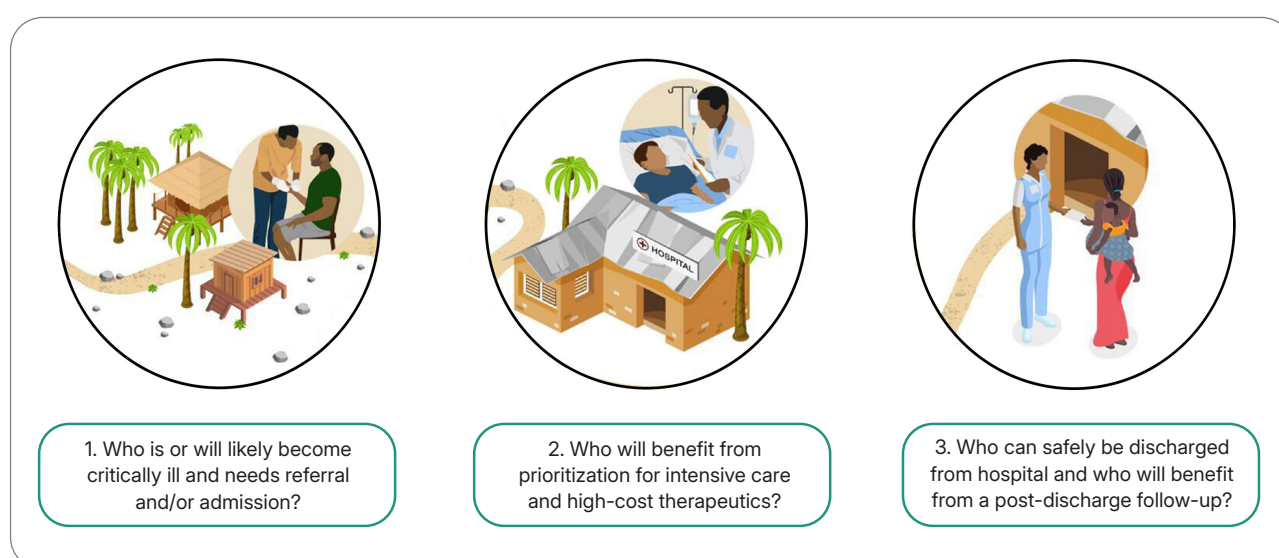


Figure 3. Key questions on disease severity and prognosis that guide clinical decision-making. Adapted from reference [26] by the author.

4.2.2. Additional challenges in resource-limited settings

In resource-limited contexts in LMICs, where the burden of infectious diseases is highest, the triage and risk stratification of acute infections are further complicated by the chronic shortage of qualified medical personnel and the limited laboratory facilities, radiology services, and special equipment for other investigations. Stockouts and supply chain issues of tests or reagents, essential medicines, and devices typically complicate patient prioritization and management decisions [31]. Moreover, other common challenges in these settings include:

- **Overcrowded facilities:** During seasonal peaks of transmission (e.g., those caused by malaria, respiratory syncytial virus (RSV) bronchiolitis, influenza, etc.) or outbreaks (e.g., those caused by meningitis, cholera, dengue fever, measles, etc.) the healthcare facilities are vulnerable to overcrowding, the workload of healthcare workers increases, and triage and risk stratification become even more challenging [26]. Scarce resources are stretched further and resource prioritization becomes more complex. The coronavirus disease 2019 (COVID-19) pandemic serves as an exemplary illustration of a similar issue on a global scale, affecting high-income countries (HICs) as well.
- **Difficult access to the healthcare system:** Particularly in the most rural and remote areas, making appropriate decisions about who to discharge home and who to refer or admit is even more critical. Geographic obstacles, climatic conditions, sociocultural dynamics, and political considerations can hinder access to healthcare facilities [26]. As an example, in Mozambique, it is estimated that 67% of the population lives more than 60 minutes away from a health facility on foot [32]. If a patient is lucky enough to make its way to the healthcare system, that contact may become a unique opportunity to ascertain clinical severity and not miss recognition of a life-threatening infection. The decision to discharge patients following hospitalization is equally important. In many LMICs, mortality during the first weeks following hospital discharge is often as high as during hospitalization, with most deaths occurring at home rather than through readmissions, a reality that is well documented for children but also reported in adults [33,34].

4.2.3. Case management strategies for resource-limited settings

To assist healthcare providers with these daily challenges in resource-limited settings, simple clinical decision-support algorithms and scores have been developed. The overarching aim is to systematize clinical practice, considering the limited available resources, and to improve the processes of triage, risk stratification, diagnosis, and treatment of common diseases (**Figures 4 & 5**).

In the mid-1990s, the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) developed the Integrated Management of Childhood Illness (IMCI) strategy [35]. This comprises a set of clinical algorithms aimed at improving the quality of disease management of children in primary healthcare settings, using a syndromic approach that focuses on the most common causes of childhood illness including pneumonia, diarrhea, malaria, measles, and malnutrition. Since then, similar guidelines have been developed by WHO/UNICEF with an integrated management approach for different levels of healthcare and patient groups, which are updated periodically to reflect new evidence and practices (**Figure 6**) [36–39]. Their implementation helps healthcare providers in the identification and treatment of the most common conditions, systematizes the assessment of danger signs to establish priority and disease severity, strengthens the counseling of patients or caretakers, and encourages health promotion and disease prevention measures.



Figure 4: Triage at the outpatient department of the Manhica District Hospital, Manhica, Mozambique. Photo taken by David Torres Fernández.

Since their appearance, these guidelines have been implemented in many resource-limited settings [40]. The different proposed clinical algorithms have shown variable results, but evidence suggests that they have generally contributed to improving the quality of healthcare and reducing morbidity and mortality [41–44]. Nevertheless, their overreliance on clinical signs and symptoms is problematic. Many different infections overlap in symptomatology, especially in children, and individual signs and symptoms have poor diagnostic accuracy [45]. Moreover, the accurate identification of patients at risk of severe and fatal infections with these algorithms is also challenging. Different studies highlight that the recognition of the proposed danger signs is subjective, resulting in significant interobserver variability in patient severity categorization [46,47]. Especially low-skilled healthcare workers may have a low competency in reliably recognizing severe disease traits. In addition, these danger signs also lack sensitivity and specificity for adverse outcomes, which can lead to both under- and over-admission and treatment, and some only appear late in the disease course [22,48]. Finally, continuous training and supervision are needed to maintain an optimal adherence to these guidelines [28,49–52]. Other clinical decision support tools have been developed, usually expanding the scope of the WHO/UNICEF-proposed guidelines, but they suffer from similar shortcomings. An example used on a wide scale for adult healthcare consultations is the Practical Approach to Care Kit (PACK) program, initially developed in South Africa and now being expanded to cover all age groups and community care [53].



Figure 5: Patients in the outpatient department of the Manhiça District Hospital, Manhiça, Mozambique. Photo taken by Andalu Vila San Juan.

In parallel, different scoring systems have been developed or adapted for patients in resource-limited settings, either for general use or when specific infectious diseases are suspected. These scores rank the severity or risk of adverse outcomes according to the presence or absence of a combination of different signs, symptoms, laboratory results, and other patient characteristics. Thus, they could be used for triage and risk stratification strategies and be combined with response algorithms. For instance, the Lambarené Organ Dysfunction Score (LODS) is a 0-3 score that combines presence of coma, prostration, and deep breathing, which was derived to predict fatal malaria cases in children and can serve as a tool to help recognize those in need of referral or increased care [54]. Another score for adults is the Universal Vital Assessment (UVA), derived with data from admitted patients to African hospitals and aimed to better identify adults at risk of in-hospital death. The UVA score gives a 0-12 punctuation based on temperature, heart rate, respiratory rate, systolic blood pressure, oxygen saturation, consciousness, and HIV serostatus [55]. Like these two examples, several other scores exist, including scores aimed at identifying patients at risk of post-discharge death [56–59]. However, the performance of scores is highly dependent on the ability of healthcare workers to identify subjective signs on which they are based, as well as the availability of all data required for score calculation for a patient. Many existing scores require variables that are not feasible to collect in resource-limited settings [60]. Additionally, several suffer from methodological issues, are typically not well validated in different settings from where they were derived, have not been appropriately evaluated for clinical utility, and are not widely implemented in routine clinical practice in resource-limited environments for children [46,61–65] or adults [47,66–68].

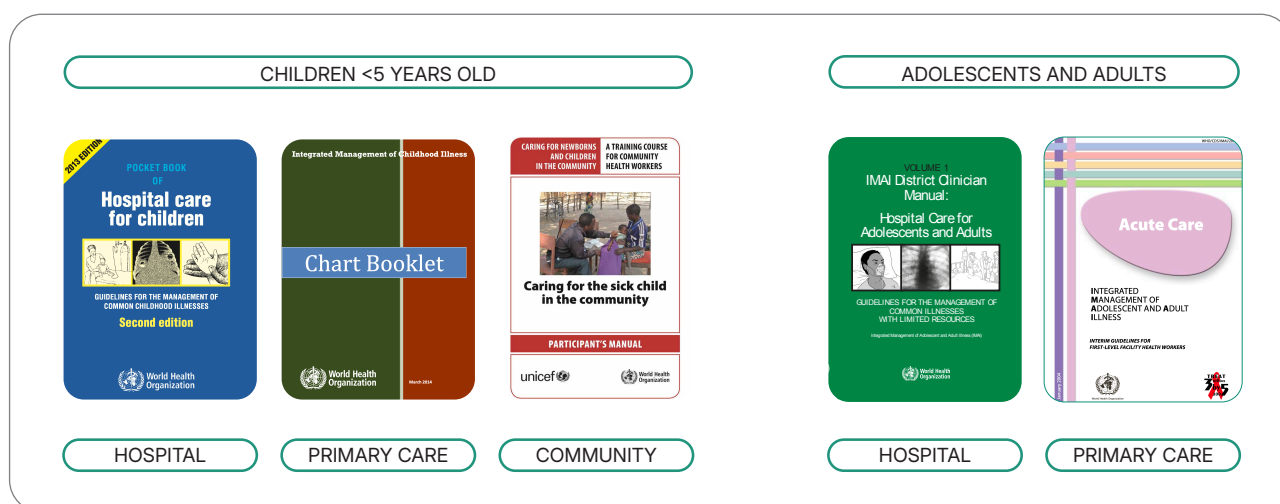


Figure 6: Guidelines for the clinical management of common illnesses in resource-limited settings. Five WHO/UNICEF-proposed guidelines targeting different age groups and levels of the healthcare system. Covers of the guidelines reproduced from references [35-39].

Tools for identifying and quantifying brain injury and *sequelae* include neurologic and cognitive tests, neuroimaging techniques, and electroencephalograms. However, these methods have drawbacks, as they cannot detect subtle or early-stage brain injuries, require specialized interpretation, and are resource-intensive. Their availability and use in clinical practice is very restricted in resource-limited settings [69]. Consequently, this hinders efforts to manage appropriately patients with infections affecting the central nervous system and to provide rehabilitation strategies to those at risk of persistent neurocognitive dysfunction.

4.2.4. Focusing on pathogens rather than patients

Efforts in the management of suspected infections have traditionally focused, beyond clinical evaluation, on identifying the underlying cause and establishing whether an infection is viral, bacterial, fungal, or parasitic in origin, to trigger specific antimicrobial treatment if necessary. In resource-limited settings, given the paucity of therapeutic resources available, this focus on the pathogen has been epitomized by a need to identify bacterial and malaria infections, as they are common and potentially lethal infections and likely to benefit from specific treatments (i.e. antibiotics or antimalarials) [70]. In recent times, this approach also aims to address the emerging global antimicrobial resistance crisis and reduce unnecessary antimicrobial use.

Malaria rapid diagnostic tests (RDTs) have simplified, and in some way also democratized, malaria diagnosis in endemic areas. However, identifying bacterial infections amenable to antibiotic treatment is challenging. Microbiology and radiology investigations require specialized services and infrastructure, which are costly and scarcely available in resource-limited settings. Even when available, these services still have caveats, such as long turnaround times, possible inconclusive results, or low sensitivity as in the case of blood cultures. While rapid diagnostic tests for

pathogens other than malaria are also available, they vary in diagnostic accuracy, and assays with superior performance are needed [71]. In addition, for many localized infections, specimens are not accessible without invasive procedures that are not commonly performed, such as lumbar punctures [72].

All this is further challenged by the presence of mixed or multiple infections, as well as the complexities of differentiating asymptomatic microbial colonization from infection and disease. Studies aimed to identify causes of fever in children and adults in LMICs have reported the detection of multiple pathogens in a significant percentage of cases [73–76]. Other large studies investigating the etiologies of pneumonia and diarrhea as syndromes have also identified different pathogens in many patients. Moreover, they have reported presence or carriage of pathogens in control populations composed of individuals who were healthy or had non-infectious diseases, therefore decreasing the attributable fraction of those syndromes being caused specifically by those pathogens [77–80]. In a large cohort of febrile children in Europe, 75% of cases could not be conclusively diagnosed with either a bacterial or viral infection using local diagnostics, and this percentage showed little improvement even with the addition of extensive molecular pathogen detection approaches. In addition, most of these pediatric patients with a definitive or probable bacterial infection also had viruses identified in blood or throat swabs [81]. The use of minimally-invasive post-mortem methods to investigate cause of death also supports the extremely common co-existence of multiple pathogens during disease and a progressive abandonment of the idea that only single-pathogen infections lead to disease and death [82].

In the context of the global problem of antibiotic overuse, pathogen-based diagnostic approaches may not necessarily reduce antibiotic prescription and even increase their inappropriate use depending on the setting [81,83]. Furthermore, healthcare workers in LMICs can overprescribe antimicrobials because of their low confidence in the clinical evaluation or diagnostic test results. For example, when malaria RDTs were introduced, to improve malaria diagnosis and reduce unnecessary use of antimalarials in non-malarial cases, early studies reported varying levels of adherence to negative test results, which improved over time [84]. Besides, other studies have also suggested that the introduction of malaria RDTs may have led to an increase in antibiotic prescriptions for febrile syndromes when test results are negative [85].

Lastly, and most importantly, determining the presence or absence of a given pathogen does not indicate who will develop an uncomplicated, self-resolving infection versus a life-threatening one. While the presence of some specific pathogens can be associated with a worse prognosis, patients with the same infectious etiology can experience significantly different illness trajectories. For instance, a positive malaria RDT does not indicate the severity of the disease the patient may experience. Therefore, while assessing the cause of a patient's illness is important, using an etiology-focused approach to acute infections has notable limitations. A patient-focused approach should always be prioritized, as early recognition of severe infections is crucial for saving lives.

4.2.5. Subjectivity and healthcare inequities

At the lower levels of the healthcare system, especially in resource-limited settings with scarce tools and insufficient numbers of well-trained personnel, clinical evaluation and management often rely heavily on subjective factors. Healthcare workers must trust their ability to recognize and interpret key signs and indicators of severity and make decisions based on these. This process is influenced by healthcare workers' experiences, biases, and subconscious perceptions. Patients (or their caregivers in the case of young children) describe symptoms and medical history according to sociocultural influences and personal factors, adding another layer of subjectivity. Moreover, complex sociocultural dynamics can impact healthcare-seeking behaviors and refusal to accept recommendations by healthcare workers related to referral, hospitalization, or treatment. Leaving against medical advice is common in some settings, often influenced by cultural and financial factors [56]. Together, these factors can exacerbate healthcare disparities, ultimately impacting patient outcomes.

Different axes of inequities in health and healthcare include gender, age, socioeconomic status, ethnicity, race, and disability. For instance, gender can influence healthcare access and provision, often resulting in women, girls, and gender minorities facing disadvantages [86,87]. Particularly in Asia, previous studies have reported that girls sought healthcare later in the disease course, were admitted to hospital less frequently, and had higher in-hospital mortality [88–90]. These differences were suggested to be at least partly explained by societal norms and family or cultural practices, rather than entirely by sex-related reasons. Consequently, integrating objective and quantitative indicators of disease severity and prognosis into the management of acute infections could reduce subjective bias in medical evaluations and decision-making, leading to more equitable management decisions regardless of patient background or circumstances.

4.2.6. Innovative tools

Various technological innovations have emerged to address shortcomings in patient management in resource-limited settings, including those related to acute infections [23]. Existing technologies and those in development are focused on four main areas:

- **Electronic clinical decision support algorithms:** These can provide simplicity and enhance adherence to guidelines, accommodate a more nuanced diagnosis considering patient factors (e.g., medical history, symptoms and signs, and diagnostic test results) and external factors (e.g., local disease prevalence), and facilitate timely updates as new evidence becomes available [31,91,92]. Different existing algorithms include digital adaptations of the WHO/UNICEF-proposed guidelines, algorithms that improve these guidelines in some

manner by incorporating additional clinical signs or test results, and those that incorporate real-time surveillance and machine learning approaches [93,94]. While some electronic algorithms have shown promising results on effectiveness, further research is needed and consideration of the existing digital infrastructure in each setting is important [31].

- **Devices for measuring vital signs:** Measurement of heart rate, respiratory rate, temperature, blood pressure, and oxygen saturation is important for assessing disease and severity. However, accurate measurement can be challenging due to multiple factors, including lack of appropriate tools, inadequate training of healthcare workers, and high workloads [95]. A growing variety of devices are expanding the capability and quality of measuring key vital signs. Especially in the field of pneumonia, advances have been made to develop automated respiratory rate counters and scale-up pulse oximeters [31].
- **Multiplex point-of-care (POC) diagnostic platforms:** With the goal to better understand the cause of infection, multiplex POC or near-POC diagnostic platforms have been proposed [96–98]. These are intended to simultaneously test for multiple pathogens with a single sample. As of October 2022, over 80 near-POC molecular diagnostic platforms existed, with at least 65 on the market and 19 in the pipeline. Most of these have high multiplexing potential, but still limited offering of tests [96]. However, platforms with very high multiplexing capacity may still be considered complex and expensive for LMICs and, as a result, remain unavailable for clinical care [96].
- **Host biomarkers:** A biomarker, also known as a biological marker, is a measurable indicator of a biological process, condition, or response to treatment. Host biomarkers are the subgroup of biomarkers that specifically originate from the host organism or patient. This contrasts with pathogen biomarkers, which originate from infectious agents. Host biomarkers include molecules such as proteins, metabolites, genetic material, or cellular components. Using host biomarkers could contribute to improving the management of acute infections in resource-limited settings, aiding in i) assessing disease severity, specific organ injury, and prognosis of infections [26,99], or ii) distinguishing etiologies, especially between bacterial and non-bacterial causes [100].

In the following section, host biomarkers are discussed in detail in the context of acute infections, focusing on their application to determine disease severity, organ injury, and prognosis. Considering all that has been previously discussed, it is crucial to develop tools to improve triage and risk stratification strategies for patients with acute infections in different clinical scenarios in resource-limited settings. New accurate, simple, rapid, objective, and inexpensive tools are needed, and host biomarkers present a possible solution in this regard.

4.3. HOST BIOMARKERS IN ACUTE INFECTIONS

4.3.1. Host response to infection

Upon infection, pathogens encounter the innate immune system of the host. Leukocytes and parenchymal cells, such as epithelial and endothelial cells, are involved in the early local immune response. These cells detect pathogens by recognizing pathogen-associated molecular patterns (PAMPs) through different cell-surface and intracellular pattern recognition receptors (PRRs). In most cases, the immune system efficiently mounts a protective and balanced response to infection, which results in pathogen elimination. This process involves a variety of pro-inflammatory reactions, including cytokine and chemokine release, phagocyte recruitment, and local activation of the complement and coagulation systems. The intensity and scope vary according to factors of both the pathogen (e.g., species, load, and virulence) and the host (e.g., age, comorbidities, genetics, and medication). Subsequently, there is a return to homeostasis through compensatory mechanisms aimed at restraining the initial inflammation and preventing collateral tissue damage [101].

However, during certain infections, the host response can become unbalanced and harmful, and progress towards sepsis (**Figure 7**). A dysregulated host response to infection can involve both sustained excessive inflammation and immune suppression [102,103]. Excessive inflammation is mediated through the release of pro-inflammatory mediators by multiple cell types, liberation of damage-associated molecular patterns (DAMPs) by necrotic cell death, and activation of the interconnected coagulation system, complement system, and vascular endothelium. This hyperinflammation can cause damage to healthy tissue, which results in the release of DAMPs that further activate PRRs and contribute to a vicious circle of cell injury and sustained hyperinflammation. On the other hand, immune suppression is characterized by effector cell apoptosis, T cell exhaustion, increase in immunosuppressive cells, the presence of leukocytes with dysfunctional features, reduced antigen presentation, and a diminished capacity to produce pro-inflammatory cytokines [101,104]. Ultimately, all these dysregulated responses can lead to recurrent and persistent infections, organ damage and dysfunction, and, in the most severe cases, patient death.

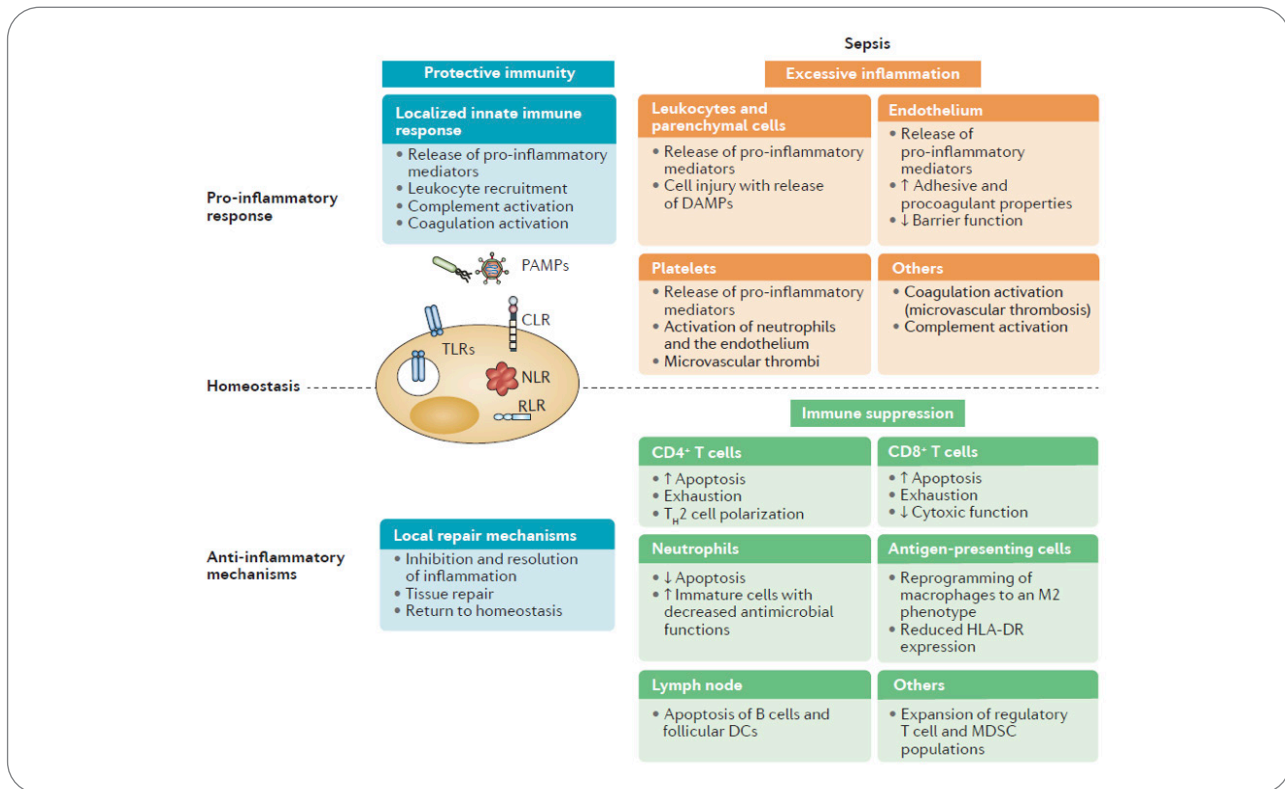


Figure 7. The host response to infection and during sepsis. Abbreviations: CLR=C-type lectin receptor, DAMP=damage-associated molecular pattern, DC=dendritic cell, HLA-DR= Human Leukocyte Antigen - DR isotype, MDSC=myeloid-derived suppressor cell, NLR=nucleotide-binding oligomerization domain-like receptor, PAMP=pathogen-associated molecular pattern, RLR=retinoic acid-inducible gene-like receptor, Th2=T helper 2, TLR=Toll-like receptor. Reproduced from reference [101].

Disruption of the integrity of the vascular endothelial barrier plays an important mechanistic role in the pathogenesis of infections (**Figure 8**) [101,104]. The endothelium coats the vascular system and separates the fluid phase of the blood compartment from the tissues. Comprising up to 60 trillion cells and covering an estimated surface area of 700 to 7000m², it is the largest interconnected organ in the human body [105,106]. Under homeostatic conditions, the vascular endothelium serves as a dynamic surface that regulates the flow of molecules between the bloodstream and tissues. It also has a role in coagulation balance, blood pressure regulation, angiogenesis, and innate immunity as it is equipped with PRRs to recognize PAMPs and DAMPs. Its barrier function is maintained by the cell cytoskeleton, the glycocalyx, intercellular adhesion molecules, and other supportive proteins [107]. In a localized infection, these structures become disrupted in response to leukocyte and platelet adhesion to the endothelial surface, the release of inflammatory mediators, and toxic oxidative and nitrosative intermediates [104]. The vascular endothelium shifts from quiescent to activated and becomes permeable, prothrombotic, and proinflammatory. This is crucial for leukocyte trafficking, pathogen elimination, and vascular remodeling and repair. Nevertheless, excessive inflammation augments these processes and can become detrimental if uncontrolled, persistent, or widespread, thereby contributing to vascular endothelial barrier dysfunction and tissue injury [107]. A loss of vascular integrity causes leakage of intravascular proteins and plasma into the extravascular space, tissue edema, impaired microvascular perfusion, and ultimately, patient shock [101].

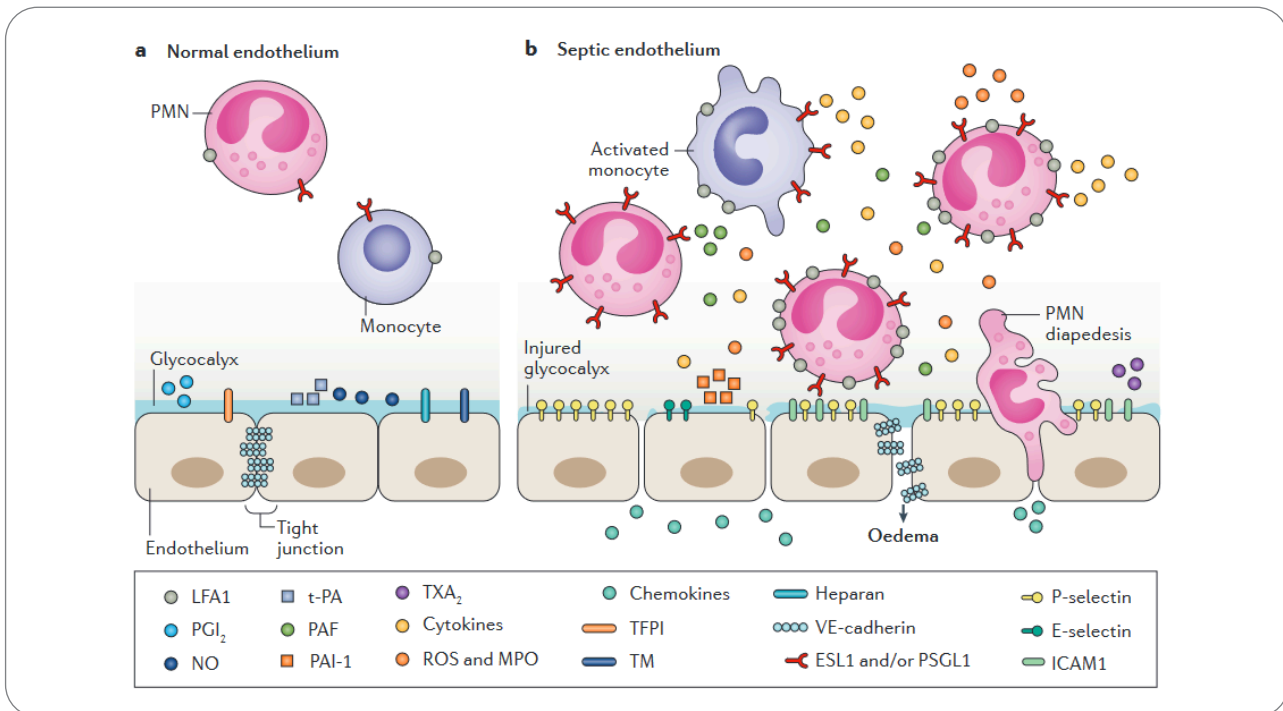


Figure 8. Changes in the vascular endothelium in response to inflammatory stimuli during sepsis. Abbreviations: ESL1=E-selectin ligand 1, ICAM1=intercellular adhesion molecule 1, LFA1=lymphocyte function-associated antigen 1, MPO=myeloperoxidase, NO=nitric oxide, PAF=platelet activating factor, PAI-1=plasminogen activator inhibitor 1, PGI₂=prostaglandin I₂, PMN=polymorphonuclear leukocyte, PSGL1=P-selectin ligand 1, ROS=reactive oxygen species, TFPI=tissue factor pathway inhibitor, TM=thrombomodulin, t-PA=tissue plasminogen activator, TXA₂=thromboxane A₂, VE=vascular endothelial. Reproduced from reference [104].

4.3.2. Classic host response biomarkers

Measuring different host biomarkers in human bodily fluids offers valuable insights into ongoing pathophysiological processes in infections. C-reactive protein (CRP) and procalcitonin (PCT) are two host biomarkers well studied and commonly used in high-resource settings in the context of infections. CRP is primarily synthesized in the liver in response to cytokines during acute inflammation. CRP plays a crucial role in the immune response by binding to bacteria and damaged cells, thereby activating the complement system to facilitate their clearance [108]. In contrast, PCT is a precursor protein primarily produced by thyroid C cells. PCT rapidly converts to calcitonin under normal conditions and regulates calcium levels, but during infections and systemic inflammation, levels rise significantly due to its production in extrathyroidal tissues [109]. CRP serves as a general marker of inflammatory conditions, whereas PCT is more specific to bacterial infections and can help distinguish them from other causes of inflammation.

CRP and PCT can assist clinicians in decision-making related to infectious diseases, including differential diagnosis, prognosis, disease monitoring, and guidance of antibiotic therapy. However, previous studies have reported diverse results regarding their clinical utility, depending on the population and specific application, and recommendations for their use differ across clinical guidelines [109–111]. In resource-limited settings, CRP and PCT measurement are rarely available, either through laboratory assays or POC tests. Additionally, there is limited evidence regarding

their clinical utility and relevant thresholds in populations in LMICs [112–115,23]. Studies conducted in LMICs have sometimes found that CRP and PCT performed worse than in studies conducted in HICs. This is suggested to be due to the impact of prevalent co-infections (e.g. HIV, malaria, and soil-transmitted helminths) and comorbidities (e.g. malnutrition and untreated diabetes), which can interfere with the expression of these biomarkers [31,116–119].

Lactate is another widely used host biomarker in the context of infections, with POC tests readily available [120]. Lactate was previously seen as a waste product of anaerobic metabolism that simply indicated tissue hypoperfusion, but nowadays it is also regarded as a metabolic fuel and a marker of cellular stress [121]. High levels indicate organ dysfunction and an increased risk of mortality, while changes in concentrations can offer insights into the response to treatment [122–124]. Measuring blood lactate is recommended in international guidelines for the management of sepsis and septic shock [125], and elevated lactate is part of the Sepsis-3 definition of septic shock [5]. However, similar to CRP and PCT, data on the clinical utility of lactate in populations in LMICs are limited, and it is not commonly used in these settings due to the lack of methods for its measurement [126–134].

4.3.3. Novel biomarkers of immune and endothelial activation

Current research indicates that several analytes from immune and endothelial activation pathways may have clinical utility in patients with acute infections, serving as indicators of disease severity and progression. In recent years, the number of published studies on this topic has increased exponentially. In a myriad of etiologies (including malaria, bacterial infections, COVID-19, dengue, and Ebola virus) and clinical syndromes (including febrile illness, meningitis, clinical pneumonia, and sepsis), levels of different biomarkers of immune and endothelial activation have been associated with disease severity traits, clinical deterioration, subsequent adverse outcomes, and mortality [135–138]. Some of these biomarkers have consistently demonstrated robust prognostic performance across different etiologies. This suggests that various pathogens share common pathways of host response and that some biomarkers can reflect pathogen-agnostic mechanisms of severe infection.

Proteins and small molecules are especially interesting candidates as they are currently the most straightforward type of biomarkers to incorporate into potential simple, fast, and inexpensive tests. Although a large body of literature exists, some protein biomarkers of particular significance that are studied in this thesis are angiopoietin-2 (Angpt-2), chitinase-3-like protein 1 (CHI3L1), interleukin-6 (IL-6), interleukin-8 (IL-8), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble tumor necrosis factor receptor-1 (sTNFR1), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), and soluble urokinase-type plasminogen activator receptor (suPAR). **Table 1** provides an overview of their biological function and clinical evidence supporting their potential clinical utility in acute infections. These novel candidates offer an alternative to classic biomarkers such as CRP, PCT, and lactate [139].

Table 1. Overview of the biological function and clinical evidence of promising biomarkers of immune and endothelial activation in infections

BIOMARKERS	BIOLOGICAL FUNCTION	CLINICAL EVIDENCE
Angpt-2	Angpt-1 and Angpt-2 bind the Tie-2 receptor on endothelial cells. Angpt-1 interaction with Tie-2 stabilizes the endothelium, while Angpt-2 acts as an antagonist and disrupts the endothelial barrier. During an infection, endothelial cells increase Angpt-2 secretion, leading to a higher Angpt-2:Angpt-1 ratio [140].	High levels of Angpt-2 have been consistently reported as indicative of disease severity and predictor of adverse outcomes in infections. Evidence is especially compelling for malaria and sepsis, where Angpt-2 levels have been associated with disease severity, organ injury, longer clinical recovery times, and mortality [136,140–142].
CHI3L1	CHI3L1 is a glycoprotein secreted by different cell types, including immune and endothelial cells. CHI3L1 overexpression has been observed in inflammatory conditions, including infections. It is involved in driving immune cell activation and differentiation, but it also plays a role in tissue regeneration and repair [143].	CHI3L1 has been studied in different infectious diseases including febrile illness, sepsis, and COVID-19. High levels of CHI3L1 have been associated with disease severity and risk of adverse outcomes and death [144,145].
IL-6	IL-6 is a pleiotropic cytokine, secreted by different cell types during infection, inflammation, or neoplastic disease. Among others, it promotes differentiation of B cells into antibody-producing cells, stimulates T cell balance, and induces production of acute-phase proteins [146].	Being one of the most studied biomarkers in the context of infections, high levels of IL-6 have been associated with severe infections including sepsis [138,146,147]. Its clinical use has increased in recent years, and it is now included in some guidelines for sepsis management and COVID-19 treatment [148].
IL-8	IL-8 is a pro-inflammatory chemokine produced by various cell types like macrophages and epithelial cells during infection. It primarily acts as a potent chemoattractant for neutrophils, but it also promotes neutrophil activation and angiogenesis [149].	High levels of IL-8 have been associated with increased disease severity, poor outcomes, and subsequent death in different infectious diseases, including pneumonia, malaria, and dengue [147,150,151].
sFlt-1	VEGF is a key regulator of normal angiogenesis, but it also plays a role in the disruption of the endothelium during inflammation. The effects of VEGF are mediated by two receptors: Flt-1 and Flk-1. Both the transmembrane and soluble forms of Flt-1 bind VEGF and prevent its binding to Flk-1, which is capable of transducing a much stronger signal [136].	High concentrations of sFlt-1 have been associated with disease severity, adverse outcomes, and mortality risk in cohorts of patients with suspected infection, sepsis, pneumonia, malaria, and dengue [136,141].
sTNFR1	TNFR1 is expressed across all human tissues and binds the proinflammatory cytokine TNF. sTNFR1 is the soluble form of TNFR1 and acts as a decoy receptor. sTNFR1 helps to modulate the inflammatory response and limit excessive TNF signaling [152].	In individuals with severe or fatal infectious diseases, levels of sTNFR1 are usually higher. This association is supported by evidence from cohorts of patients with sepsis, dengue, malaria, or pneumonia [153–156].
sTREM-1	TREM-1 is a cell-surface receptor expressed mostly by myeloid immune cells, which amplifies inflammation in infections. The soluble form sTREM-1 acts as a decoy receptor. sTREM-1 counteracts excessive inflammatory reactions, but high levels may reflect an underlying immune dysfunction [157,158].	High levels of sTREM-1 have been consistently associated with sepsis and risk of death in different populations of patients with infectious diseases [159–161]. sTREM-1 has also been associated with disease severity and other adverse outcomes during an infection, but evidence is less robust [162].
suPAR	suPAR is the soluble form of uPAR, a cell-surface receptor that binds uPA. The uPA/uPAR system has a central role in fibrinolysis and modulation of inflammatory and immune responses. Three forms of suPAR are produced and they are involved in the regulation of various cellular processes, uPA binding, and chemotaxis of immune cells [163].	High suPAR levels have been linked to disease progression, poor prognosis, and mortality risk in malaria, bacteremia, sepsis, meningitis, hemorrhagic fevers, and most recently COVID-19 [163–165]. A few studies in COVID-19 patients successfully used suPAR testing to risk-stratify patients and provide early targeted treatment with a drug intervention [166–168].

Abbreviations: Angpt-1=angiopoietin-2, Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein-1, Flk-1=fetal liver kinase-1, Flt-1=fms-like tyrosine kinase-1, IL-6=interleukin-6, IL-8=interleukin-8, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor-1, sTREM-1=soluble triggering receptor expressed on myeloid cells-1, suPAR=soluble urokinase-type plasminogen activator receptor, TNF=tumor necrosis factor, TNFR1=tumor necrosis factor receptor-1, TREM-1=triggering receptor expressed on myeloid cells-1, uPA=urokinase plasminogen activator, uPAR=urokinase plasminogen activator receptor, VEGF=vascular endothelial growth factor. Table created by the author.

Over the past five years, concurrent with the completion of this thesis, relevant findings from studies on novel host biomarkers of immune and endothelial activation conducted specifically in resource-limited settings have been published. **Table 2** provides a summary of studies that included large populations from LMICs (with sample sizes of over 400 individuals) and compared the prognostic performance of several host biomarkers in blood listed in **Table 1**.

- In young children:** The largest study included 2,502 hospitalized children with febrile illness in Uganda and compared levels of several biomarkers of immune and endothelial activation at clinical presentation. In this study cohort, sTREM-1 had the best prognostic performance for 7-day mortality, with an area under the receiver operating characteristic curve (AUROC) of around 0.90. However, sFlt-1, IL-8, Angpt-2, CHI3L1, and IL-6 also had high discrimination ability for 7-day mortality [169]. The same cohort restricted to the 805 children who fulfilled criteria for clinical pneumonia gave consistent results for 48-hour mortality [128]. In another large study involving 592 Ugandan children hospitalized with severe malaria, sTREM-1, sTNFR1, sFlt-1, and CHI3L1 levels at presentation were good predictors of in-hospital mortality [170]. In 900 children with clinical pneumonia who visited an outpatient clinic in a refugee camp on the Thailand-Myanmar border, levels of Angpt-2, IL-8, sFlt-1, PCT, IL-1ra, and IL-6 were strongly associated with the need of supplemental oxygen at the illness visit. Among these biomarkers, Angpt-2 demonstrated the best discrimination for this outcome (AUROC of 0.81, 95% confidence interval [CI]: 0.74-0.87) [162].
- In adults and older children:** Three large studies on prognostic biomarkers were conducted in adults in Thailand, Tanzania, and India. In 760 Thai adults hospitalized with suspected infection, IL-8, sTNFR-1, Angpt-2, and sTREM-1 concentrations at clinical presentation were strongly associated with 28-day mortality, with sTREM-1 having an AUROC of 0.81 (95% CI: 0.77-0.85) [171]. In 507 febrile adults attending outpatient facilities in Tanzania, sTREM-1, sTNFR1, IL-6, and sFlt-1 were good predictors of 28-day mortality. In this population, sTREM-1 had the best prognostic performance with an AUROC of 0.87 (95% CI: 0.81-0.92) [172]. Among 420 adults presenting with moderate COVID-19 to hospitals in India, host biomarkers had different prognostic utility depending on the severity of the outcome assessed. However, IL-1Ra, IL-6, IL-10, and IL-8 exhibited dose-response relationships with subsequent disease progression [173,174]. In addition, a multi-country study in Asia and Latin America, which included both adults and older children, found that increased levels of several biomarkers were associated with an increased risk of developing severe or moderate dengue. However, IL-Ra, SDC-1, and IL-8 were the most robust biomarkers [175].

Table 2. Large studies in low- and middle-income countries evaluating the prognostic performance of biomarkers of immune and endothelial activation

STUDY	STUDY DESIGN	COUNTRY	STUDY PERIOD	HEALTHCARE LEVEL	AGE GROUP	CLINICAL SYNDROME	INCLUSION & EXCLUSION CRITERIA	SAMPLE SIZE	PRIMARY OUTCOME	OUTCOME PREVALENCE	BIOMARKERS ANALYZED	BEST BIOMARKERS
Leigodowicz <i>et al.</i> (2021) [169]	Prospective cohort	Uganda	2012-2013	Hospital (Jinja Regional Hospital in Uganda)	2 months to 5 years	Febrile illness	Presenting to the emergency department with a history of fever in the past 48 h or an axillary temperature >37.5 °C, and admitted according to the treating physician's judgement.	2,502	7-day mortality	95/2,084 (3.8%)	Angpt-1, Angpt-2, CHI3L1, CRP, IL-6, IL-8, IP-10, PCT, sFit-1, sICAM-1, sTNFR1, sTREM-1, sVCAM-1	sTREM-1 had the best performance with an AUROC of 0.89 (95% CI: 0.84-0.94) in the derivation cohort and 0.90 (95% CI: 0.86-0.95) in the validation cohort. Next best-performing biomarkers were: sFit-1, IL-8, Angpt-2, CHI3L1, and IL-6.
McDonald <i>et al.</i> (2022) [128]	Prospective cohort	Uganda	2012-2013	Hospital (Jinja Regional Hospital in Uganda)	2 months to 5 years	Clinical pneumonia	Same as in Leigodowicz <i>et al.</i> (2021) [169]. This is a subset of the cohort who met the IMCI diagnostic criteria for pneumonia.	805	48-hour mortality	55/805 (6.8%)	Angpt-1, Angpt-2, CHI3L1, CRP, IL-6, IL-8, IP-10, lactate, PCT, sFit-1, sICAM-1, sTNFR1, sTREM-1, sVCAM-1	sTREM-1 had the best performance with an AUROC of 0.89 (95% CI: 0.84-0.93). Next best-performing biomarkers were: IL-8, sFit-1, IL-6, Angpt-2, and CHI3L1.
Mufumba <i>et al.</i> (2023) [170]	Prospective cohort	Uganda	2014-2017	Hospital (Mulago National Referral Hospital and Jinja Regional Referral Hospital)	6 months to 5 years	Severe malaria	Positive malaria RDT or blood smear, hospitalization required, and one of the following: prostration, severe anemia, multiple seizures, respiratory distress, or coma. Exclusion criteria included history of chronic illness requiring medical care (including known HIV infection), history of coma, head trauma, known developmental delay, cerebral palsy, or prior hospitalization for malnutrition.	592	In-hospital mortality	43/592 (7.3%)	Angpt-2, CHI3L1, CRP, IL-6, IL-8, PCT, sFit-1, sICAM-1, sTNFR1, sTREM-1, sVCAM-1	sTREM-1 had the best performance with an AUROC of 0.78 (95% CI: 0.70-0.86). Next best-performing biomarkers were: sTNFR1, sFit-1, and CHI3L1.
Chandna <i>et al.</i> (2023) [162]	Prospective cohort	Thailand-Myanmar border	2007-2010	Primary care clinic located within a refugee camp	≤24 months	Clinical pneumonia	Consecutive pregnant women attending a medical clinic for refugees and internally displaced people were approached to participate in the study and children of consenting women were reviewed at birth and followed-up each month (routine visit) and during any intercurrent illness (illness visit) until 24 months of age. All illness visits meeting WHO pneumonia criteria were included in this analysis.	900	Receipt of supplemental oxygen at any time during the illness visit	49/900 (5.4%)	Angpt-1, Angpt-2, CHI3L1, CRP, IL-1ra, IL-6, IL-8, IL-10, IP-10, PCT, sFit-1, sTREM-1, sTNFR-1	Angpt-2 had the best performance with an AUROC of 0.81 (95% CI: 0.74-0.87). Next best-performing biomarkers were: IL-8, sFit-1, PCT, IL-1ra, and IL-6.

(Continued)

Table 2 (Continued). Large studies in low- and middle-income countries evaluating the prognostic performance of biomarkers of immune and endothelial activation

STUDY	STUDY DESIGN	COUNTRY	STUDY PERIOD	HEALTHCARE LEVEL	AGE GROUP	CLINICAL SYNDROME	INCLUSION & EXCLUSION CRITERIA	SAMPLE SIZE	PRIMARY OUTCOME	OUTCOME PREVALENCE	BIOMARKERS ANALYZED	BEST BIOMARKERS
Wright <i>et al.</i> (2020) [171]	Prospective cohort	Thailand	2013-2017	Hospital (Sunpasitthiprasong Hospital)	≥18 years	Suspected infection	Admitted with suspected infection and at least 3 documented systemic manifestations of infection as proposed by the 2012 Surviving Sepsis Campaign.	760	28-day mortality	110/760 (14.5%)	Angpt-1, Angpt-2, IL-8, sTNFR1, sTREM-1	sTREM-1 had the best performance with an AUROC of 0.81 (95% CI: 0.77–0.85). Next best-performing biomarkers were: IL-8, sTNFR-1, and Angpt-2.
Richard-Greenblatt <i>et al.</i> (2020) [172]	Prospective cohort	Tanzania	2013-2014	4 outpatient clinics (an outpatient department of a public hospital and 3 primary care health facilities)	≥18 years	Febrile illness	Presenting with fever (lympnic temperature ≥ 38°C), having a fever for ≤7 days, and receiving the first consultation for the present complaint. Exclusion criteria included refusal of HIV testing, injury or trauma as the main reason for consultation, hospitalization within 1 month, or delivery within 6 weeks of presentation.	507	28-day mortality	32/507 (6.3%)	Angpt-1, Angpt-2, CHI3L1, CRP, IL-6, IL-8, IP-10, PCT, sFlt-1, sICAM-1, sTNFR1, sTREM-1, sVCAM-1	sTREM-1 had the best performance with an AUROC of 0.87 (95% CI: 0.81–0.92). Next best-performing biomarkers were: sTNFR1, IL-6, and sFlt-1.
Chandha <i>et al.</i> (2022) [173]. (Complementary results in [174])	Prospective cohort	India	2020-2021	Hospital (All India Institute of Medical Sciences and Christian Medical College Hospital)	≥18 years	Moderate COVID-19	Presenting with clinically suspected COVID-19 of moderate severity: SpO2 ≥94% and respiratory rate <30 breaths/min, in the context of systemic symptoms (breathlessness or fever and chest pain, abdominal pain, diarrhea, or severe myalgia). Exclusion criteria included prior history of laboratory-confirmed SARS-CoV-2 infection and/or ≥1 dose of any COVID-19 vaccine.	420	Ordinal outcome reflecting the maximum level of respiratory support required within 14 days following enrollment	62/420 (14.8%) required supplemental oxygen, 15/420 (3.6%) received noninvasive ventilation, and 12/420 (2.9%) were mechanically ventilated or died	Angpt-2, CRP, D-dimer, IL-1Ra, IL-6, IL-8, IL-10, IP-10, NLR, PCT, platelets, sTREM-1, suPAR	IL-1Ra, IL-6, IL-10, and IL-8 exhibited dose-response relationships with subsequent disease progression.
Vuong <i>et al.</i> (2021) [175]	Nested case-control	Vietnam, Cambodia, Malaysia, El Salvador	2011-2016	Outpatient facilities	≥5 years	Dengue	Have fever or history of fever for <72h, and have symptoms consistent with dengue and no features strongly suggestive of another disease. This analysis was restricted to a subset of laboratory-confirmed dengue cases.	837	Development of severe/moderate dengue	281/837 (33.6%)	Angpt-2, CRP, ferritin, IL-1Ra, IL-8, IP-10, SDC-1, sCD163, sTREM-1, sVCAM-1	High levels of each biomarker were associated with an increased risk of developing severe/moderate dengue, except for ferritin in adults. Most robust associations were found for IL-1Ra in children, and SDC-1 and IL-8 in adults.

Abbreviations: Angpt-1=angiopoietin-1, Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, COVID-19=coronavirus disease 2019, HIV=human immunodeficiency virus, IMCI=Integrated Management of Childhood Illness, IL-1Ra=interleukin-1 receptor antagonist, IL-6=interleukin-6, IL-8=interleukin-8, IL-10=interleukin-10, IP-10=interferon gamma-induced protein 10, NLR=neutrophil-to-lymphocyte ratio, PCT=procalcitonin, RDT=rapid diagnostic test, SARS-CoV-2=severe acute respiratory syndrome coronavirus 2, sCD163=soluble CD163, SDC-1=syndecan-1, sFlt-1=soluble fms-like tyrosine kinase 1, sICAM-1=soluble intercellular adhesion molecule 1, SpO2=peripheral capillary oxygen saturation, sTNFR1=soluble tumor necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells-1, sVCAM-1=soluble vascular cell adhesion molecule 1, suPAR=soluble plasminogen activator receptor. Table created by the author.

Most of these studies quantified CRP and PCT and compared their prognostic performance to that of novel biomarkers of immune and endothelial activation. One study also included lactate as a comparator. Remarkably, CRP, PCT, and lactate were worse predictors of adverse outcomes in these cohorts when compared to the best-performing biomarkers identified in each study [128,162,169,170,172,173]. Furthermore, in almost all studies, biomarkers were compared with relevant clinical signs, combinations thereof, and clinical severity scores for each disease or syndrome. The best prognostic biomarkers in each study showed either equivalent or superior prognostic performance compared to clinical parameters, and integrating biomarkers with clinical parameters improved outcome prediction in terms of discrimination capacity or net benefit [128,162,169–172,174]. The characteristics of the populations under study and outcomes of interest varied in each study. However, most studies focused exclusively on inpatients, only one study examined whether prognostic performance varied by several subgroups of patients [170], and just one proposed tentative biomarker thresholds to guide clinical management [169].

4.3.4. Novel biomarkers of injury to the brain and other organs

Measurement of host biomarkers could also help identify and quantify specific organ injury. Given the severity of infections affecting the brain and the limitations and scarcity of current tools to assess brain injury in resource-limited settings, measuring biomarkers in cerebrospinal fluid (CSF) or blood could provide valuable insights. Different biomarkers indicative of neuronal and glial cell damage have been studied extensively in traumatic brain injury and neurodegenerative diseases. These include neuron-specific enolase (NSE), ubiquitin C-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), astrocyte calcium-binding protein β (S100 β), neurofilament light chain (NfL), and Tau [176,177]. These biomarkers have recently begun to be explored in the context of infections, especially following the emergence of new immunoassays such as Simoa and ELLA, which offer increased sensitivity and enable the quantification of low-abundant biomarkers in blood. Increased levels of neurobiomarkers in CSF and blood have been associated with neurological manifestations, brain damage observed through imaging, neurologic deficits, neurocognitive *sequelae*, overall disease deterioration, and mortality in various infections including sepsis, malaria, COVID-19, and meningitis [20,178–182]. However, scientific evidence is limited and many questions remain about clinical utility in different populations and use-cases, optimal timing for assessment, useful thresholds for abnormality, and impact of potential factors on baseline levels [20].

Biomarkers indicative of other specific organ injuries could also be valuable in acute infections for targeting specific interventions and for triage and risk stratification purposes. Infection can trigger acute kidney injury (AKI), a severe condition characterized by a sudden decline in kidney function that is associated with increased mortality rates [183,184]. AKI is defined based on changes in kidney function and requires multiple serum creatinine measures and urine output evaluation [185]. However, clinicians may fail to recognize the initial development of AKI due to a delayed increase in

serum creatinine or inaccurate monitoring of urine output [186]. Neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, and other emerging biomarkers in blood or urine may facilitate early detection of renal dysfunction and provide prognostic information in patients with AKI, supporting timely and appropriate clinical management [186,187].

Research into biomarkers indicative of intestinal injury and dysfunction is also a rapidly evolving field, with recent reports from cohorts of infectious diseases. Biomarkers of intestinal barrier integrity and microbial translocation include intestinal fatty acid-binding protein (I-FABP), trefoil factor 3 (TFF3), zonula occludens-1 (ZO-1), lipopolysaccharide binding protein (LBP), and soluble complement of differentiation 14 (sCD14). They have been recently explored in the context of malaria [188,189], but also in other infectious diseases like pneumonia or sepsis [190–192]. I-FABP is one of the most studied biomarkers and available evidence indicates that I-FABP levels during infections are associated with gastrointestinal symptoms, disease severity, and mortality [188–190]. Thus, measuring this or similar biomarkers could be useful for targeting the stabilization of the intestinal barrier and risk stratification of patients. A commentary article by our research group, discussing recent results on biomarkers of intestinal injury and dysfunction in pediatric malaria, can be found at the end of the introduction of this thesis [193].

4.3.5. Potential advantages of using novel host biomarkers

The field of host biomarkers is rapidly growing, showing great potential. Indeed, important advances have been made in the last decades. Using host biomarkers as indicators of disease severity, organ injury, and subsequent adverse outcomes is an innovative approach to improve triage and risk stratification strategies for patients with acute infections in resource-limited settings. Currently, host biomarkers are not routinely used for this purpose in these settings, and existing tools based on CRP, PCT, and lactate may be outperformed by future tools based on other novel host biomarkers [139]. In fact, the WHO has highlighted the importance of developing new biomarker-based POC tests to detect severe disease or risk of progression to severe illness for patients with febrile syndromes in resource-limited settings, irrespective of the causal agent [22]. Findings from stakeholder consultations also endorsed this idea [23,194]. Along with reinforcing the training of healthcare workers and promoting devices for measuring vital signs, developing POC tests based on host biomarkers (including those to support triage and risk stratification of severe disease) is considered a top priority that requires further research (**Figure 9**) [194].

"It would be helpful for primary care or remote settings. In this case it would be interesting to have a more sensitive and less specific test. A test that allows early identification of serious patients, that would allow less hospitalization time for the patient. Easy test to implement. This test should have an impact at the hospital."

Policymaker, LATAM

"Both are equally important. For example, there are children with cough and flu. If breathing not fast, IMCI advises to take plenty fluids and send home. If you use biomarkers that are predictors of severity, if elevated, the child qualifies for admission, as much as the IMCI says outpatient. But in the true clinics setting the child should be admitted for observation in case the patient goes in the other direction."

Policymaker, Africa

"But if someone is not giving an antibiotic and sending the child home, often they are saying did I make the right decision so having something that says this child also scored low on severity scores this would be quite helpful."

Researcher, Africa

Figure 9. Opinions of stakeholders on severity tests based on host biomarkers for acute febrile illnesses. Results of focus group discussions with key stakeholders to understand needs of healthcare providers diagnosing and managing acute febrile illness in resource-limited settings. Adapted from reference [31] by the author.

Measuring different host biomarkers in patients with acute infection in resource-limited settings could improve patient management, bringing benefits for both patients and the healthcare system in terms of clinical outcomes and economic savings (**Figure 10**). Host biomarkers can be quantified, in blood or other bodily fluids, using laboratory assays or potentially using simple and inexpensive POC rapid tests. These tests could be used during community visits by healthcare workers, at primary healthcare facilities or higher-level care facilities for patients actively seeking care, or in hospitals for already hospitalized patients. They could offer additional insights and lead to earlier and/or more accurate determination of disease severity, prediction of adverse outcomes, and evaluation of specific organ injury. Host biomarker data, integrated with relevant and context-specific clinical severity scores or decision algorithms, could improve patient triage and risk stratification and guide decisions on patient referral, hospitalization, resource prioritization, discharge decisions and timing, and follow-up requirements after discharge [22,26,99]. Especially at lower levels of the health system, having data on host biomarkers would reduce reliance on clinical judgment alone, increase healthcare workers' confidence in their decisions, and help them convince patients to accept recommendations (e.g. withholding antibiotics, or referral/admission when there is reticence) [31]. Overall, host biomarker-based strategies have the potential to inform actionable steps and reduce mortality and morbidity in survivors, improve healthcare resource allocation, decrease the pressure of antimicrobial resistance through optimized antimicrobial use (even if not pathogen-specific), and maximize equity in decision-making due to the objectivity inherent in quantifying biomarkers. A perspective article by our research group, in which we discuss the potential of prognostic host biomarkers to guide clinical decisions and review promising candidates, can be found at the end of the introduction of this thesis [135].

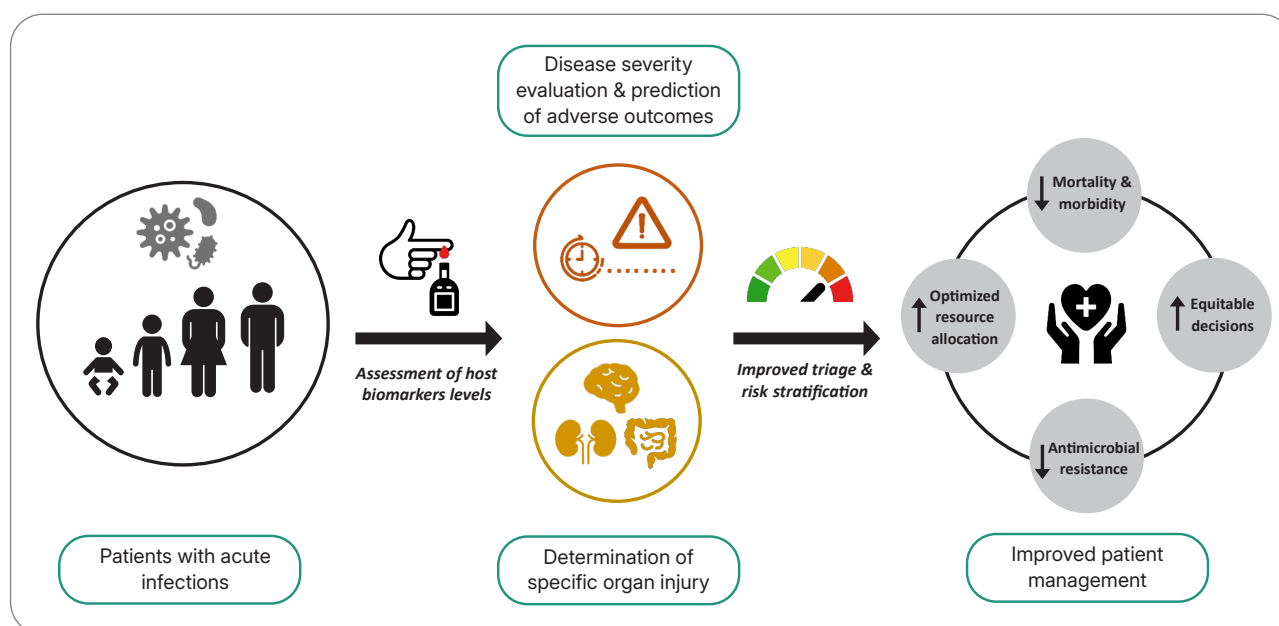


Figure 10. Conceptual framework of the potential impact of measuring host biomarkers in acute infections. Figure created by the author.

4.4. CONTEXT OF THE THESIS RESEARCH

4.4.1. Thesis rationale

Triage and risk stratification strategies in patients with acute infections in resource-limited settings need improvement. Back in 2019, in line with WHO recommendations and the perspectives of stakeholders, host biomarker-based tools were considered a promising solution to mitigate this problem. However, further scientific evidence was necessary [22,23,194]. It was deemed essential to conduct studies in large, diverse, and well-characterized populations with acute infections in resource-limited settings to better understand the relationship between different host biomarker levels and organ injury, severe disease, or adverse outcomes. Classic and novel host biomarkers involved in the host response to infection had seldom been studied and compared against each other in large cohorts in resource-limited settings, and their potential clinical utility remained largely unknown [115,139]. Additionally, biomarkers of brain injury had been scarcely investigated in the context of infectious diseases. It was within this context that this thesis was conceived. This thesis aimed to generate high-quality data on host biomarkers in populations with suspected acute infections from Mozambique, where previous evidence on this topic was very scarce, despite the extremely high burden of infections. As pathogen-related morbidity and mortality are concentrated in children, most studies were designed to focus on this population. The generated data were expected to support the development of new tools based on host biomarkers and contribute to global efforts to improve the management of acute infections. Furthermore, another goal was to

describe healthcare access and provision for sick pediatric populations in Mozambique, where this kind of host biomarker-based tools could have a significant impact, and to do so through a gender lens to explore potential gender-based disparities that such objective measurements could help to mitigate.

4.4.2. Location of studies – Mozambique & Manhiça district

All the original research presented in this thesis was conducted on children and adults in Mozambique. All studies were carried out in the district of Manhiça, where the Manhiça District Hospital is the highest-level health facility. Only one study of this thesis also included participants from General José Macamo Hospital, located in the capital city of Maputo (**Figure 11**).

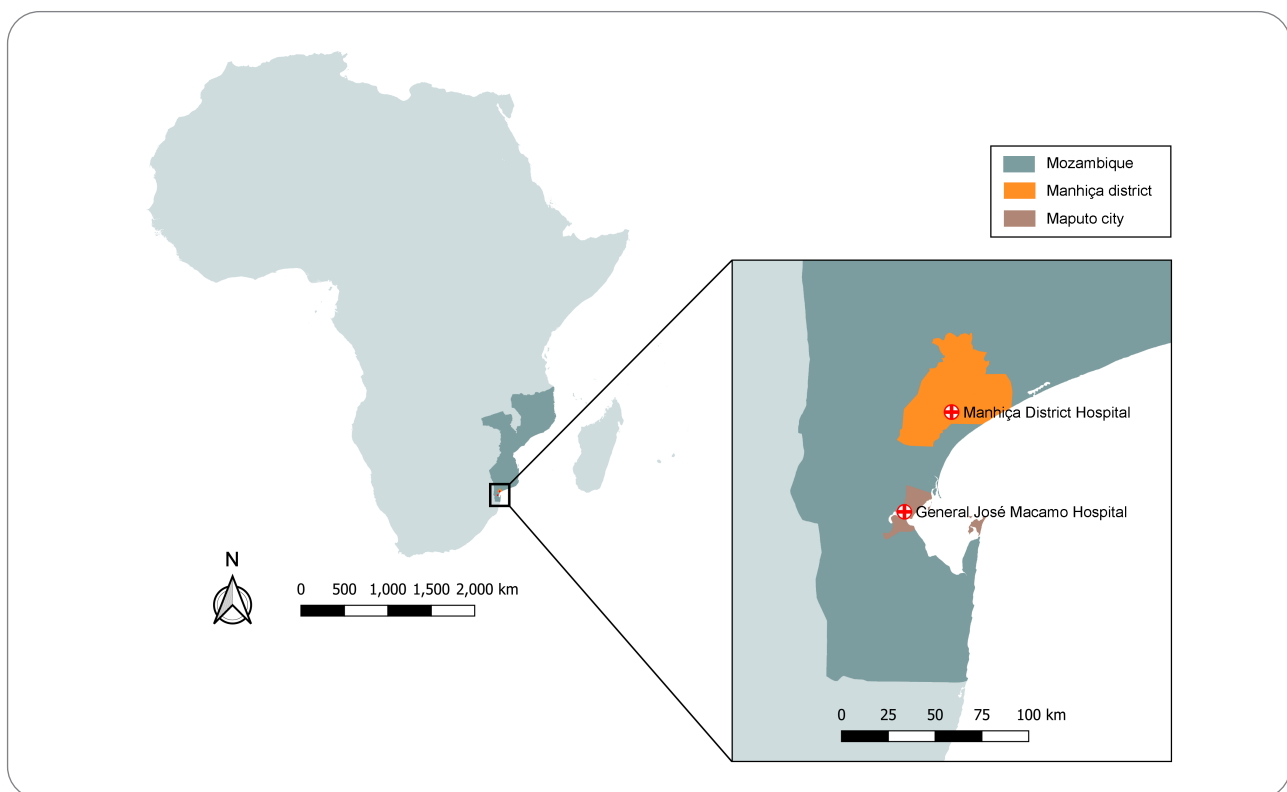


Figure 11. Map of Mozambique, Manhiça district, and Maputo city. Figure created by the author.

Mozambique is a low-income country located in southeast Africa. It has a population of 33 million as of 2022, with a life expectancy at birth of 57.7 years and a median age of 17.6 years. The gross domestic product (GDP) per capita in 2021 was US\$504, with 9.1% allocated to health [195]. Infectious diseases are among the leading causes of morbidity and mortality in the country. HIV/AIDS is currently the main cause of overall mortality, whereas malaria is the leading cause of death among children under the age of five [195,196]. In 2022, there were an estimated 316 new malaria cases per 1,000 population at risk, 361 tuberculosis cases per 100,000 population, and 3.2 new HIV infections per 1,000 uninfected population [195]. The neonatal mortality rate in 2022 was 25.7 per 1,000 live

births and the under-5 mortality rate was 66.2 per 1,000 live births [197], both of which exceed the Sustainable Development Goal (SDG) targets. Health infrastructure is limited in the country and medicine stockouts are common [198–201], with only 0.82 physicians per 10,000 population [195].

The district of Manhiça is located in Maputo province, southern Mozambique [202,203]. With ~209,000 inhabitants in 2022 and an area of 2,380 km² on a plain crossed by the Incomati river [204], it is the paradigm of a poor, resource-limited rural sub-Saharan setting. The population is predominantly young and there is a sharp reduction of males from ages 20 years and above, attributed to higher migration and mortality among adult males than among females. A large proportion of the population has no formal education, and most residents are engaged in small businesses, practice subsistence farming, or work in agricultural companies [202–204]. Major causes of morbidity and mortality include malaria, HIV/AIDS, tuberculosis, and invasive bacterial diseases [202,203,205,206]. The district of Manhiça is one of the most HIV-impacted areas in the world, with an HIV prevalence in adults of around 30–40% [207,208]. *Plasmodium falciparum* malaria transmission is perennial but peaks during the rainy season (November–April). However, an upscale of malaria control tools over the past two decades has contributed to a significant reduction in malaria cases [209–211]. The neonatal mortality rate in 2020 was 13.6 per 1,000 live births and the under-5 mortality rate was 31.3 per 1,000 live births, lower than in the entire Mozambique but still over the SDG targets [204]. A more detailed description of the Manhiça HDSS study area and population can be found elsewhere [202,203].

The Manhiça Health Research Centre (*Centro de Investigação em Saúde de Manhiça* [CISM]) was created in 1996 as part of a collaborative program between the Governments of Mozambique and Spain. CISM has received since its creation significant core funding from the Spanish Agency for International Development Cooperation (AECID), and technical support from the Barcelona Institute for Global Health (ISGlobal) and Hospital Clínic – University of Barcelona. The three pillars of CISM's activities are research in local priority areas, clinical assistance, and training of young investigators. CISM has run a Health and Demographic Surveillance System (HDSS) in the district of Manhiça since its creation, which comprises well-established surveillance platforms focusing on demography, pediatric morbidity, and microbiology (**Figures 12–15**).



Figure 12. Exterior of Manhiça District Hospital, Manhiça, Mozambique. Photo taken by Sara Ajanovic Andelic.

Figure 13. Pharmacy at the Manhiça District Hospital, Manhiça, Mozambique. Photo taken by Andalu Vila San Juan.



Figure 14. The Incomati river crossing Manhiça, Mozambique. Photo taken by the author.

Figure 15. The Manhiça Health Research Centre, Manhiça, Mozambique. Photo taken by the author.

4.5. PERSPECTIVE ARTICLE

Host-based prognostic biomarkers to improve risk stratification and outcome of febrile children in low- and middle-income countries





Host-Based Prognostic Biomarkers to Improve Risk Stratification and Outcome of Febrile Children in Low- and Middle-Income Countries

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Specialty section:

This article was submitted to
Pediatric Infectious Diseases,
a section of the journal
Frontiers in Pediatrics

Received: 16 April 2020

Accepted: 12 August 2020

Published: 18 September 2020

Citation:

Balanza N, Erice C, Ngai M, Varo R,
Kain KC and Bassat Q (2020)
Host-Based Prognostic Biomarkers to
Improve Risk Stratification and
Outcome of Febrile Children in
Low- and Middle-Income Countries.
Front. Pediatr. 8:552083.
doi: 10.3389/fped.2020.552083

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Fever is one of the leading causes for pediatric medical consultation and the most common symptom at clinical presentation in low- and middle-income countries (LMICs). Most febrile episodes are due to self-limited infections, but a small proportion of children will develop life-threatening infections. The early recognition of children who have or are progressing to a critical illness among all febrile cases is challenging, and there are currently no objective and quantitative tools to do so. This results in increased morbidity and mortality among children with impending life-threatening infections, whilst contributing to the unnecessary prescription of antibiotics, overwhelming health care facilities, and harm to patients receiving avoidable antimicrobial treatment. Specific fever origin is difficult to ascertain and co-infections in LMICs are common. However, many severe infections share common pathways of host injury irrespective of etiology, including immune and endothelial activation that contribute to the pathobiology of sepsis (i.e., pathogen “agnostic” mechanisms of disease). Importantly, mediators of these pathways are independent markers of disease severity and outcome. We propose that measuring circulating levels of these factors can provide quantitative and objective evidence to: enable early recognition of severe infection; guide patient triage and management; enhance post-discharge risk stratification and follow up; and mitigate potential gender bias in clinical decisions. Here, we review the clinical and biological evidence supporting the clinical utility of host immune and endothelial activation biomarkers as components of novel rapid triage tests, and discuss the challenges and needs for developing and implementing such tools.

Keywords: febrile syndrome, severe infection, sepsis, risk stratification, prognostic host-biomarkers, angiopoietin-2 (Ang-2), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1)

INTRODUCTION

In low- and middle-income countries (LMICs), it is common to have multiple infections throughout childhood that often manifest as febrile syndromes. It is estimated that a child under five years in a LMIC may experience around six episodes of fever per year (1). Consequently, fever is one of the leading causes for medical consultation and the most common pediatric symptom at clinical presentation (2). The vast majority of febrile illnesses are uncomplicated and self-limited. However, a small proportion of these may become severe and progress to sepsis, which is defined as life-threatening organ dysfunction due to a dysregulated host response to infection and considered to be agnostic to etiology (3, 4). While severe disease/sepsis needs urgent referral/admission and adequate treatment, febrile children without critical illness can be treated conservatively and often without antimicrobials once malaria has been ruled out (5, 6). The early recognition of children who have or are progressing to sepsis is challenging, because the initial presentation of a severe infection may be subtle and non-specific (2). Delays in the recognition and treatment of severe infections/sepsis increase child mortality and neurocognitive impairment in survivors (7, 8). Moreover, unnecessary admissions, referrals, and treatment of self-limited infections results in misallocation of health resources, overloading of health care facilities, contribution to development of antimicrobial resistance, and adverse events due to nosocomial infections and exposure to unnecessary investigations and treatments.

The underlying infectious etiologies of pediatric febrile syndromes are highly diverse and differ according to community (e.g., geographical location, season, or disease control measures implemented) and individual factors (e.g., age, immunity, or concomitant conditions) (2). Despite progress in recent years, the epidemiology of fever in LMICs remains poorly characterized (9, 10). The scarcity of available diagnostic tools and laboratory services in LMICs makes it challenging to establish whether a fever is viral, bacterial or parasitic in origin. Moreover, mixed or multiple infections, and high rates of asymptomatic pathogen carriers, are frequent in these settings (5, 11). The innovative use of minimally-invasive post-mortem methods, to investigate cause of death, also supports the co-existence of multiple infections and a progressive abandonment of the dogma “one pathogen—one disease—one cause of death” (12).

International guidelines for the management of childhood illnesses (i.e., Integrated Management of Childhood Illness [IMCI], integrated Community Case Management [iCCM]), which have been shown to improve child survival, give simplified algorithms to assist health care providers in fever management (13, 14). However, these are designed in such a broad way that may result in over-diagnosis, over-treatment, and over-referral (2). Pediatric disease severity scores (e.g., Lambaréné Organ Dysfunction Score [LODS] (15), Signs of Inflammation in Child that Kill [SICK] (16)) have also been developed, but their performance is highly dependent on the capacity of health care providers to identify danger signs and the availability of clinical and laboratory data. In parallel, current rapid diagnostic tests (RDTs) are largely pathogen-based (e.g., dengue and malaria

RDTs) and, while they can identify the presence or absence of infection, they do not have the ability to differentiate between an uncomplicated infection vs. a life-threatening one. Previous studies have reported sub-optimal management of severe febrile syndromes in children, including poor triage, referral, and admission practices, in different levels of healthcare (17–20).

Additionally, once a child is admitted in a health facility, it is still difficult to clearly determine disease prognosis and thus make adequate decisions in regards to discharge. In fact, death in the first weeks after discharge remains an important and poorly acknowledged contributor to child mortality in LMICs, and can be as common or even more frequent than in-hospital mortality (21). Although triaging algorithms that identify children at increased risk of post-discharge mortality exist (22, 23), it is necessary to enhance how discharge decisions are made and identify those children that could benefit most from a follow-up visit.

Consequently, there is a compelling need to improve risk stratification tools for febrile children, for both initial management and discharge decisions. Accordingly, the World Health Organization (WHO) has highlighted the importance of developing new point-of-care (POC) tests to identify febrile patients at risk of progressing to severe disease/sepsis (2). This perspective article aims to highlight the potential of using host-biomarkers of disease severity and prognosis as objective measurements to guide critical clinical decisions. The measurement of these biomarkers using POC tests could complement clinical scores in community settings, as well as add value to them in formal health care settings.

Pathogen “Agnostic” Mechanisms of Severe Infection

Host response has an important role in determining severity and outcome of infections. Moreover, it has become well-recognized that many different types of pathogens share common host response pathways of injury (i.e., pathogen “agnostic” mechanisms of disease) (24–26). Immune and endothelial activation lay upstream of endothelial destabilization, microvascular leak, multi-organ dysfunction, and death and, therefore, these pathways have been implicated in the pathogenesis of sepsis (27–29). A healthy endothelium must be adaptive in order to interact with its changing environment, alternating between activated and quiescent states by responding to host-derived cues. During an infection, the immune response triggers endothelium activation, meaning it becomes permeable and pro-inflammatory, in order to accommodate pathogen sequestration and elimination as well as vascular remodeling and healing. However, a dysregulated host response, in either step of this cascade of events, can result in endothelial dysfunction and tissue injury (25).

Evidence suggests that mediators of immune and endothelial activation are independent and quantitative markers of disease severity and prognosis in sepsis due to multiple causes, including: malaria, bacterial infections, dengue, or Ebola (25, 26, 28, 30–34). Therefore, measuring circulating levels of mediators of these pathways could be used for disease severity

and prognostic determination to guide clinical decisions. Additionally, they represent an alternative to using acute phase proteins procalcitonin (PCT) and C-reactive protein (CRP). The performance of PCT and CRP as disease severity biomarkers has been extensively studied in high-income countries (HIC), but less is known from LMIC populations (35). In children living in LMICs, PCT and CRP concentrations have been reported to be influenced by prevalent conditions like malnutrition, coexisting malaria infection, and HIV-related immunosuppression (36–38).

Mediators of Immune and Endothelial Activation as Prognostic and Severity Markers: Overview of Clinical Studies

A large body of literature exists for the potential use of immune and endothelial host-biomarkers as disease severity and prognostic tools. Here, we highlight and provide an overview of some of these biomarkers and concentrate on two specific promising examples (Figure 1).

Tumor necrosis factor (TNF) is the classic pro-inflammatory cytokine. Not surprisingly, elevated levels of TNF are observed in individuals with severe disease (i.e., severe malaria, bacteremia, and dengue) in comparison to individuals with uncomplicated disease (39–52). Increased TNF levels are also associated with neurological deficits and longer coma duration in children with cerebral malaria (53). Similarly, several studies have reported that individuals with severe disease and/or non-survivors have higher

levels of soluble TNF receptor 1 (sTNFR1) (30, 47, 48, 54–61). Likewise, interleukins (ILs) are key components of the immune system and circulating concentrations of IL-6, IL-8, and IL-10 have been extensively studied in disease severity and progression. Among them, IL-6 is perhaps the best characterized. Several lines of evidence indicate that high IL-6 levels are correlated with bacterial sepsis, severe malaria, severe dengue, and increased risk of dengue mortality (39, 41, 44, 46, 48, 50, 62–68). A study in febrile adults reported IL-6 to be a good predictor analyte between non-survivors and survivors (54). Interferon γ -induced protein 10 (IP-10) is an interesting pro-inflammatory chemokine, but studies of its association with disease severity have generated contradictory results. A few studies have found reduced IP-10 levels in severe dengue cases, but other observed the contrary (69–72). In the context of malaria, studies have reported an association between high IP-10 and mortality (73–75), while in febrile adults low levels were associated with death (54). Chitinase-3-like 1 (CHI3L1) has also been identified as a severity marker in different causes of sepsis, with increased levels associated with severe disease, complications and mortality (54, 76, 77).

Molecules indicative of endothelial activation and dysfunction include mediators of endothelial cell function, components of the coagulation pathway, soluble cell surface adhesion molecules, and regulators of vascular tone and permeability (25). Among them, a growing body of evidence demonstrates that elevated levels of soluble intercellular adhesion molecule-1 (sICAM-1)

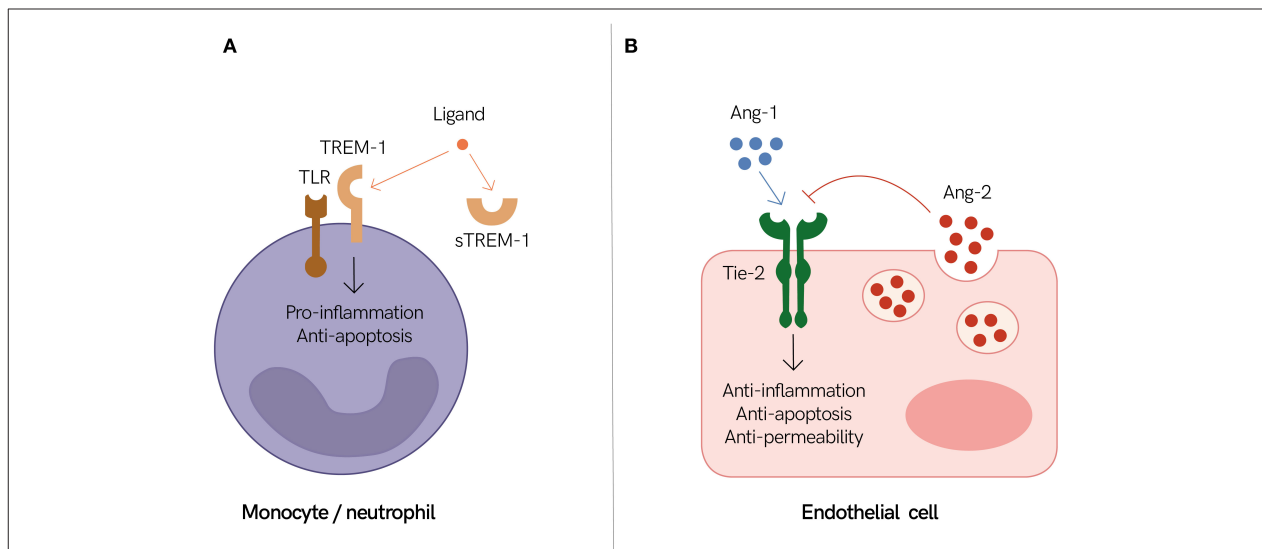


FIGURE 1 | Endothelial and immune mediators as prognostic biomarkers of severe infections. **(A)** Triggering receptor expressed on myeloid cells 1 (TREM-1) together with Toll-like receptors (TLR) promote inflammation during an active infection. It is speculated that high levels of circulating soluble TREM-1 (sTREM-1) inhibit TREM-1 signaling by sequestering its ligand. Therefore, individuals with high levels of circulating sTREM-1 may have a dysregulated immune response. **(B)** The endothelium shifts between states of quiescence (i.e., stable endothelium) and activation (i.e., permeable endothelium) to adapt and accommodate pathogen sequestration and elimination, as well as vascular remodeling and healing. The angiopoietin and tyrosine-protein kinase receptor (Ang/Tie) axis plays an important role in regulating these events. Angiopoietin-1 (Ang-1) activates Tie-2, which promotes endothelium stabilization, while angiopoietin-2 (Ang-2) inhibits these events, thereby promoting endothelium activation. Individuals with high levels of circulating Ang-2 may suffer from a dysregulated host response to infection resulting in excessive endothelium activation and vascular leak. **(A,B)** Both sTREM-1 and Ang-2 are associated with poor outcomes in different infectious diseases and thus are good candidates to help identify individuals with impending life-threatening infections.

are associated with disease severity and prognosis in malaria, dengue, and other causes of sepsis (72–74, 78–80). Soluble Fms-like tyrosine kinase 1 (sFlt-1) is another interesting marker of endothelial activation, which binds to vascular endothelial growth factor (VEGF). sFlt-1 has been reported to be good at discriminating between survivors and non-survivors in pediatric severe malaria (73, 74) and at predicting 28-day mortality in febrile adults (54). Moreover, other studies have shown that sFlt-1 correlates with severity in patients with bacterial sepsis (81).

sTREM-1 as a Promising Biomarker of a Dysregulated Immune Response

Triggering receptor expressed on myeloid cells 1 (TREM-1) is expressed on neutrophils and monocytes. Activation of TREM-1 signaling culminates in an enhanced pro-inflammatory response and induction of anti-apoptotic pathways. Soluble TREM-1 (sTREM-1) has the same extracellular region as TREM-1 but lacks its transmembrane region. Thus, sTREM-1 can combine with the same ligands, and it is thought to negatively regulate TREM-1 signaling pathways (Figure 1A) (82).

Several clinical studies show that increased sTREM-1 levels are correlated with disease severity, longer clinical recovery times, multi-organ dysfunction, and mortality in both children and adults (54, 61, 73, 74, 83–86). It is speculated that these adverse outcomes are reflective of an imbalance in TREM-1 signaling, resulting in excess immune effector cells death and immunosuppression (54). ROC curve analysis of a cohort study conducted in Cameroon showed that sTREM-1 had discriminatory power between children with severe malaria vs. uncomplicated malaria (AUC = 0.96 [95% CI: 0.92–0.99]) (85). This finding is complemented by a Tanzanian study in outpatient febrile adults, which found that sTREM-1 is a strong prognostic marker for 28-day mortality (AUC = 0.87 [95% CI: 0.81–0.92]) (54). Importantly, this same study showed that the mortality prognostic utility of sTREM-1 was superior to CRP and PCT ($p \leq 0.0001$). Another study conducted in rural Thailand in hospitalized patients with suspected infection also reported sTREM-1 to have strong prognostic 28-day mortality (AUC = 0.81 [95% CI: 0.77–0.85]) (61). Moreover, in these two studies, models combining sTREM-1 levels and validated clinical scoring systems (e.g., quick Sequential Organ Failure Assessment [qSOFA] and systemic Inflammatory Response Syndrome [SIRS]) had better prognostic accuracy than the clinical scoring systems alone (54, 61).

The Ang/Tie Axis as an Example of Endothelial Integrity Modulator

Microvascular function and permeability are regulated by several pathways, including the angiopoietin and tyrosine-protein kinase receptor (Ang/Tie) axis. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands of the Tie-2 receptor, which is present on endothelial cells. Ang-1 is constitutively produced and secreted by pericytes and smooth muscle cells.

Upon Ang-1 interaction with Tie-2, stabilization and maturation of blood vessels is triggered. Ang-2 is constitutively expressed at low levels and is co-localized with von Willebrand factor (vWF) within the Weibel Palade bodies (WPB) of endothelial cells. During an inflammatory response, both the expression of Ang-2 and its release from the WPB are increased, leading to an increased Ang-2:Ang-1 ratio. Ang-2 antagonizes Ang-1 downstream signaling by binding to Tie-2, resulting in endothelial destabilization, microvascular leakage, and organ dysfunction, which are common features of sepsis due to multiple causes (Figure 1B) (26).

Several studies have linked a dysregulated Ang/Tie axis to disease severity in malaria. Low levels of Ang-1, high levels of Ang-2, high Ang-2:Ang-1 ratios, as well as high soluble Tie-2 (sTie-2) levels are consistently reported in severe malaria in comparison to uncomplicated malaria (73, 78, 87–91). Elevated levels of Ang-2 and sTie-2 are associated with mortality, and Ang-2 has also been linked to multi-organ dysfunction, respiratory distress, impaired consciousness, and acute kidney injury among other complications (73, 74, 92–95). ROC curve analyses have demonstrated that Ang-1, Ang-2, and the ratio of Ang-2:Ang-1 have strong predictive power for disease severity and adverse outcomes, and some studies indicate that these markers are superior to lactate or parasitemia (73, 89, 91, 92). Interestingly, Ang-2 levels at presentation have also been reported to be good at predicting post-discharge mortality (AUC = 0.82 [95% CI: 0.71–0.93]) (74). Moreover, Ang-2 levels in combination with clinical predictors, have been shown to improve prognostic accuracy above that of clinical predictors alone (e.g., LODS) (74, 92). Levels of Ang-1, Ang-2, and sTie-2 have also been examined in other causes of sepsis. Results are mostly comparable to the malaria studies, as elevated Ang-2 and reduced Ang-1 levels have been associated with disease severity, intensive care unit (ICU) admission, shock, organ dysfunction, and death (54, 61, 79, 96–99).

CHALLENGES AHEAD AND THE NEED TO DEVELOP A RAPID TRIAGE TOOL

In the last decades, the number of published studies on biomarkers has increased exponentially. Recent scientific advances in high throughput “omics” technologies have driven the biomarker discovery and development field forward. Despite progress in the field and investment from major stakeholders, the number of biomarkers approved for any type of clinical use is limited (100, 101). Currently, the aforementioned biomarkers of immunological and endothelial origin hold promise, but they are not used in routine clinical practice in LMICs for risk-stratification of children.

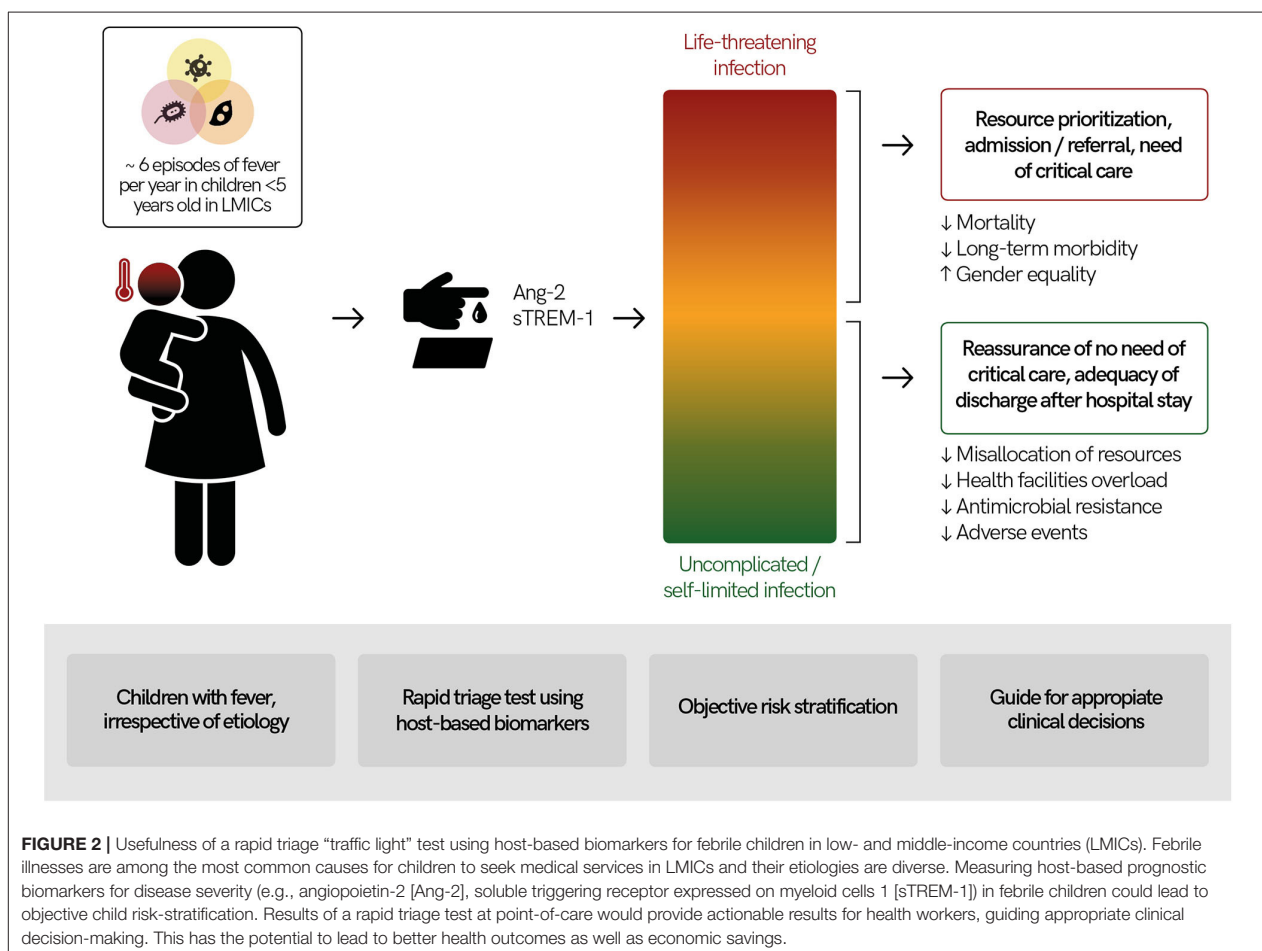
For future implementation, further research is necessary. Additional scientific evidence is needed regarding which biomarkers are most useful and which cut-off levels should be used. The ideal prognostic biomarker for severe infection/sepsis should be: capable of identifying the subset of patients at high risk of adverse outcomes before this risk is clearly evident,

correlated with disease severity over time, and useful regardless of disease microbiological etiology (25). Of the previously outlined biomarkers, sTREM-1 and Ang-2 are promising contenders to approximate this ideal. Besides, a combinational biomarker approach should also be considered, as it has been reported to enhance prognostic accuracy in some studies and it could be especially useful if biomarkers from different pathobiological pathways were measured (59, 73). Furthermore, rigorous and adequately powered randomized controlled trials will be essential to validate the clinical utility of adding biomarker measurements in guiding management decisions in both community-based and formal health care settings. Ideally, these studies should be carried out in different geographical areas in order to demonstrate reproducibility of findings and their added benefit to standard of care. If solid evidence of clinical benefit is provided, results could be used to adapt current international guidelines (i.e., iCCM, IMCI) (35).

Another crucial step is the development of rapid POC tests. This would allow measuring prognostic biomarkers at the community and peripheral level, where they could have a significant impact. These new rapid triage tests should fulfill

the WHO ASSURED criteria which include simplicity, low cost, rapidness and suitability for field use (102). Recently, a lateral flow immunoassay has been reported to detect IL-6 in a quantitatively manner and similar devices need to be developed (103). Malaria RDTs are already widely implemented in Asia and Africa for fever management, where they are culturally accepted by communities (104–107). Therefore, it is expected that analogous devices would also be culturally acceptable. Another approach would be the integration of prognostic biomarker measurements into existing malaria RDTs for use in malaria endemic areas, rather than developing separate rapid triage tests, as proposed by McDonald et al. (24). In both scenarios a training phase for health care providers would be important to ensure their correct usage and interpretation of results.

Finally, cost-effectiveness studies are also necessary to ascertain affordability of using these specific biomarkers in different use case scenarios during routine clinical practice in LMICs. Currently, there are no cost-effectiveness analyses for the biomarkers we propose and therefore they need to be conducted. These will need to take into account the potential



deaths, disability, adverse effects, and resource misallocation that could be averted. Importantly, in order to make this prognostic biomarker approach cost-effective, developing inexpensive rapid triage tests is essential.

BROAD POTENTIAL IMPACT

If current barriers are overcome, rapid triage tools measuring host-biomarkers of immunological and endothelial origin have the potential to transform child fever management in LMICs. Objective risk stratification of febrile children could bring benefits for child health and optimize the use of limited health resources (Figure 2). Several studies have reported that the roll out of malaria RDTs has resulted in reduced antimalarial overuse but increased antibiotic prescription for febrile syndromes (108). Antimicrobial stewardship programs aim to reduce antimicrobial resistance by, among others, optimizing antibiotic use (109). Therefore, identification of individuals with life-threatening infections from those with self-resolving infections could help guide the safe reduction of antibiotic prescriptions.

The proposed approach could also reduce inequalities in fever management related to gender. Gender-based health disparities have been reported in LMICs, especially in south Asia, and are attributed to societal norms and family practices that favor sons (110, 111). These include differences in length of time before seeking care, frequency in consulting a health professional during illness, type of treatment given, or duration of hospital stay. This is a major driver of adverse health outcomes in girls and is hypothesized to contribute to the inequality observed in some countries' sex-stratified child mortality rates (112). A quantitative and objective triage tool indicative of severity could help mitigate gender biases when determining prioritization of clinical care, and referral/admission or discharge decisions.

THE BIGGER PICTURE

Although the immediate benefit of host-biomarkers as prognostic tools is warranted for child risk stratification in LMICs, a similar triage tool for risk-stratification could also be useful in other scenarios, such as in pediatric health care settings in HIC and could also be applied to adult patient triage. In fact, some of the evidence we discuss comes from studies in HICs or from adults in LMICs. The current coronavirus disease (COVID-19) pandemic exemplifies the urgent need for accurate and rapid triage tools for early triage of severe disease, since most cases have self-

limited courses but high-risk individuals need identification and prioritization of care and follow-up. HICs and LMICs are moving toward integrated approaches to care, and in both settings the ultimate aim is the same: better care whilst reducing costs.

CONCLUSIONS

Mediators of endothelial and immunological pathways (e.g., sTREM-1 and Ang-2) have great potential as prognostic biomarkers for disease severity. Nevertheless, their clinical utility and cost-effectiveness needs further validation by prospective studies and randomized clinical trials. Their hypothetical implementation in LMICs via POC tests could provide actionable results for frontline health care workers facing the daily challenge of visiting children with fever. The proposed approach could be highly beneficial for child health, gender equality, appropriate use of antimicrobials, and optimization of limited health resources.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The manuscript was prepared with input from all authors. NB, CE, and MN contributed equally and share first co-authorship. KCK and QB share senior co-authorship. All authors read and approved the final manuscript.

FUNDING

This work was supported in part by grants from the Canadian Institutes of Health Research (CIHR FDN 148439 to KCK), the Canada Research Chairs program (KCK), and The Tesari Foundation. ISGlobal acknowledges support from the Spanish Ministry of Science and Innovation through the Centro de Excelencia Severo Ochoa 2019–2023 Program (CEX2018-000806-S) and support from the Generalitat de Catalunya through the CERCA Program. CISM is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID). NB is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260).

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Conflict of Interest: KCK is a named inventor on a patent “Biomarkers for early determination of a critical or life-threatening response to illness and/or treatment response” held by the University Health Network.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4.6. COMMENTARY ARTICLE

Biomarkers of intestinal injury and dysfunction: Adding new possibilities to current methods for risk stratification of children with malaria disease



Biomarkers of Intestinal Injury and Dysfunction: Adding New Possibilities to Current Methods for Risk Stratification of Children with Malaria Disease

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ABSTRACT Malaria remains, in 2022, a major cause of pediatric preventable mortality, with its major burden disproportionately circumscribed to sub-Saharan African countries. Although only ~1 to 2% of malaria cases can be considered severe and potentially life threatening, it is often challenging to identify them so as to prioritize adequate health care and resources. In a recent investigation, M. L. Sarangam, R. Namazzi, D. Datta, C. Bond, et al. (mBio 13:e01325-22, 2022, <https://journals.asm.org/doi/10.1128/mbio.01325-22>) studied intestinal barrier dysfunction and injury in Ugandan children hospitalized with severe malaria and in healthy community controls. By measuring circulating levels of four different and complementary biomarkers of gut barrier dysfunction and microbial translocation, they demonstrated that intestinal injury is common in pediatric severe malaria (18% of all cases) and is associated with increased mortality, acute kidney injury, acidosis, and endothelial activation. This commentary discusses the prognostic implications of these results, knowledge gaps that remain to be filled, and how findings could be potentially translated into effective interventions to improve outcomes in children with malaria.

KEYWORDS malaria, intestinal injury, gut barrier dysfunction, pediatrics, mortality

Malaria remains a major global health problem, and the COVID-19 pandemic has further aggravated a situation which, since 2015, has certainly been at a crossroads (1). Of the circa 241 million cases of malaria that were estimated to occur in 2020, a minimum of 2.5 million were severe ones, which led to an estimated 627,000 deaths worldwide. Children under 5 years of age are the group at most risk, disproportionately accounting for up to 77% of all deaths (2). In a delicate equilibrium between the exposure to *Plasmodium* parasites (determined by intensity of transmission), host and parasite factors, and the natural progressive acquisition of partial immunity, malaria shows a wide spectrum of clinical presentations, ranging from purely asymptomatic infections to rapidly evolving life-threatening disease. Severe malaria (SM) is a multisystem disease, which manifests as several overlapping syndromes such as impaired consciousness, respiratory distress, severe anemia, and acute kidney injury (AKI). Nevertheless, our current understanding of SM pathogenesis remains far from complete. Additionally, it is often challenging to predict or identify which patients with malaria are at higher risk of developing severe disease, given the unspecific and overlapping nature of clinical symptoms, the scarcity of prognostic tools in the settings where malaria is endemic, and the potential for this disease to evolve rapidly. Thus, any rapid triaging tool that could help determine, objectively, quantitatively, and with high precision, which malaria patients are at high risk of dying so as to prioritize their care would be an invaluable addition to our limited arsenal to decrease malaria-associated mortality.

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The authors declare no conflict of interest.

For the article discussed, see <https://doi.org/10.1128/mbio.01325-22>.

The views expressed in this article do not necessarily reflect the views of the journal or of ASM.

Published 31 October 2022

Gastrointestinal symptoms can be frequent in malaria (3, 4), yet there has been little interest in exploring intestinal injury and its role in SM pathogenesis. Histopathological studies of fatal cases have demonstrated intense sequestration of infected erythrocytes in the intestinal microvasculature and small bowel intussusceptions (5, 6). *In vivo* studies in malaria cases have shown increased gastrointestinal permeability (7) and metabolomic analysis suggested compromised intestinal barrier function in SM (8). Studies in mice have described transient gut dysbiosis after *Plasmodium* infection, as well as an association between distinct gut microbiomes and malaria severity (9). Actually, translocation of enteric bacteria is hypothesized to be a common explanation of concomitant invasive bacterial infection during malaria, which substantially worsens prognosis (10, 11). Could gut barrier dysfunction be a key but insufficiently recognized piece of the puzzle in SM pathogenesis?

The recent investigation by Sarangam and colleagues (12) explored intestinal barrier dysfunction in 598 Ugandan children hospitalized with SM and 120 healthy community controls, aged 6 months to 4 years. They measured in serum or plasma a biomarker of enterocyte damage (intestinal fatty acid binding protein [I-FABP]), a biomarker of intestinal mucosal maintenance and repair (trefoil factor 3 [TFF3]), and two biomarkers that may reflect microbial translocation from the gut lumen into the circulation (lipopolysaccharide binding protein [LBP] and soluble complement of differentiation 14 [sCD14]). In order to further explore biomarker kinetics, measurements were conducted upon admission and repeated after 1 month during the follow-up clinical visit. The authors showed that intestinal injury was a common feature at hospital admission in this SM pediatric cohort, with 18% of children fulfilling the definition of either elevated TFF3 or I-FABP compared to the population reference levels (i.e., >99th percentile for the healthy population). As expected, intestinal injury was associated with gastrointestinal symptoms. All four biomarker levels were significantly elevated in children with SM upon admission compared to healthy community children. All, except TFF3, decreased after 1 month to reach levels comparable to those of community children. These findings support the notion that an important proportion of children with SM suffer from intestinal barrier dysfunction when admitted to the hospital, which rapidly improves in the weeks after, leaving only evidence of mucosal healing.

Children with intestinal injury at admission were more likely to have clinical signs consistent with increased disease severity, AKI, acidosis, and elevated biomarkers reflecting endothelial activation. Importantly, TFF3 and I-FABP levels at admission were associated with in-hospital mortality, and TFF3 levels also were associated with 12-month postdischarge mortality. In pediatric SM, as in other infectious diseases, few biomarkers indicative of endothelial and immune activation have been consistently reported to be associated with mortality (13–15). Biomarkers hold potential as risk stratification tools, as they can be measured by standard laboratory methods or be incorporated into easier-to-use point-of-care devices and inform about risk of adverse outcomes in an objective and quantitative manner. However, whether TFF3 and I-FABP have better prognostic accuracy than other promising prognostic markers remains unknown. Unfortunately, the authors did not use their extensive biomarker data to answer this question in their report. Future research should include comparative studies and evaluate if TFF3 and I-FABP could effectively be used to improve risk stratification of children with SM and determine whether other less invasive samples beyond peripheral blood (for instance, stool) could be used to ascertain biomarker levels and subsequent risk.

Intestinal injury in SM is hypothesized to be caused by parasite sequestration in intestinal capillaries, hypoperfusion secondary to different pathophysiological processes, malaria-induced systemic immune and endothelial activation, underlying factors related to the host microbiome, or a combination of these and other complex mechanisms. A significant highlight of the work of Sarangam et al. is undoubtedly the description of the interrelation of intestinal injury, AKI, and acidosis, in an attempt to further understand the role of intestinal injury in SM. Interestingly, they reported that intestinal injury overlapped significantly with AKI and acidosis, and those children presenting with all three features had an increased risk of in-hospital mortality. Different shared pathways could explain this three-way interaction, as discussed in detail by the authors. Of note, markers of intestinal injury and markers of microbial translocation (including blood culture positivity) were surprisingly not correlated in this cohort. Although

further studies are warranted to disentangle the underlying pathological mechanisms, these findings are already key and importantly advance our current knowledge on pediatric SM pathogenesis.

Remarkably, Ngai and colleagues (16) recently published a similar study that complements, validates, and further expands the findings of Sarangam et al. The biomarkers I-FABP, LBP, and sCD14 (in addition to zonula occludens-1 [ZO-1], another marker of intestinal injury) were studied in a different cohort of 523 Ugandan children hospitalized with SM. That study showed that severe intestinal injury, defined slightly differently (i.e., I-FABP levels of ≥ 5.6 ng/mL), was present in 1 out of every 10 sick children. I-FABP was also associated with gastrointestinal symptoms, impaired tissue perfusion, AKI, central nervous system dysfunction, inflammation, and endothelial activation. Moreover, I-FABP was associated with in-hospital death as well. Given the high prevalence of intestinal injury and its association with mortality demonstrated by both studies, findings hint toward the need to not only explore the prognostic accuracy of intestinal injury markers but also use the current unveiling mechanistic and pathophysiological understanding to conduct clinical trials of agents that stabilize the intestinal barrier. Mitigating intestinal damage, as an adjunctive therapy strategy in pediatric SM, could improve recovery and survival in the most vulnerable children.

Nevertheless, multiple questions remain unanswered and should be addressed by future analogous biomarker studies. Does this phenomenon of intestinal injury happen in populations of older children or adults with SM? Is it already present to some extent in uncomplicated malaria cases? Is this also occurring in other life-threatening infections, and therefore, could this be a “pathogen-agnostic” mechanism of severe disease? Even if there is much work ahead, one thing is clear: we are now one step closer to understanding the mysteries of SM and to improving outcomes in malaria patients by turning these new insights into innovative and successful interventions.

ACKNOWLEDGMENTS

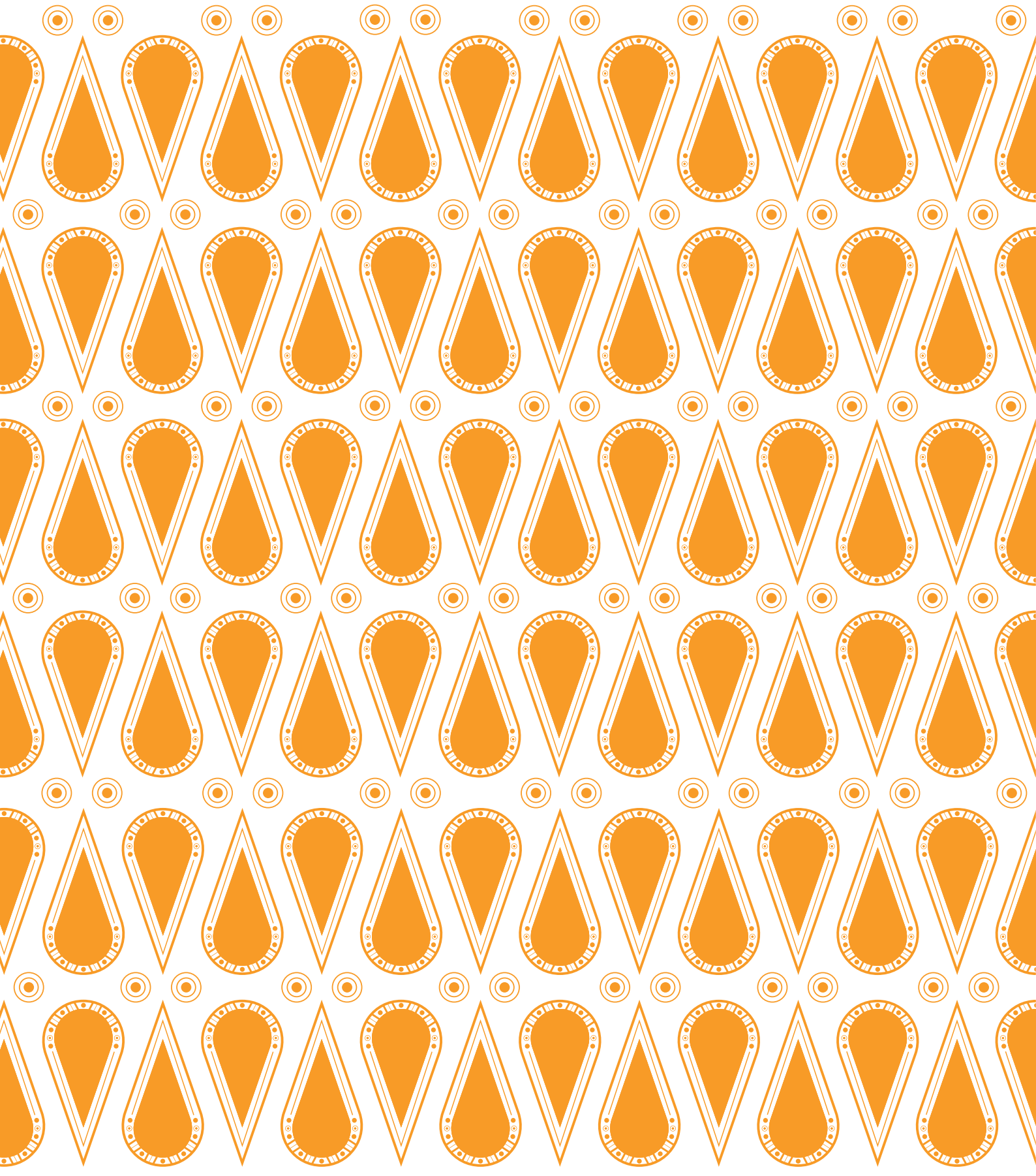
ISGlobal receives support from the Spanish Ministry of Science and Innovation through the Centro de Excelencia Severo Ochoa 2019–2023 Program (CEX2018-000806-S) and receives support from the Generalitat de Catalunya through the CERCA Program. CISM is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID). N.B. is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260). E.L.-V. has received funding from the European Respiratory Society and the European Union's H2020 research and innovation program under Marie Skłodowska-Curie grant agreement no. 847462. B.B. is supported by a Beatriz de Pinós postdoctoral fellowship granted by the Government of Catalonia's Secretariat for Universities and Research and is also supported by the Marie Skłodowska-Curie Actions COFUND Program (BP3, 801370).

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5. HYPOTHESES

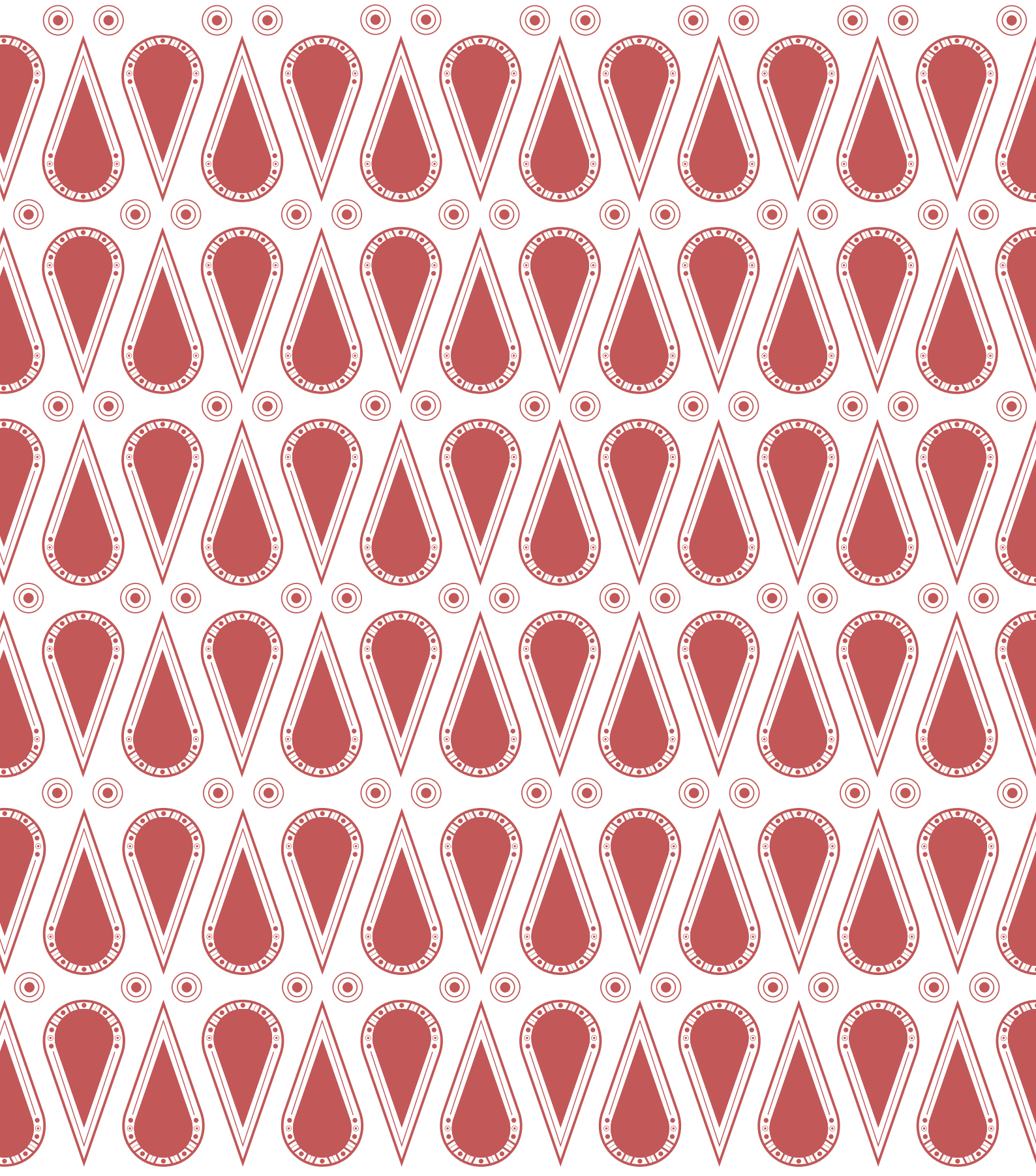


5. HYPOTHESES

The overarching hypothesis of this thesis is that measuring host biomarkers in patients with acute infections would improve current patient triage and risk stratification practices in resource-limited settings and trigger more equitable management decisions, with a positive impact on clinical outcomes.

- **Hypothesis 1:** Boys and girls in Manhiça district, southern Mozambique, differ in access to and provision of healthcare.
- **Hypothesis 2:** In pediatric and adult patients with suspected acute infections in Mozambique, measuring host biomarkers can help identify individuals at risk of adverse outcomes or with specific organ injury.

6. OBJECTIVES



6. OBJECTIVES

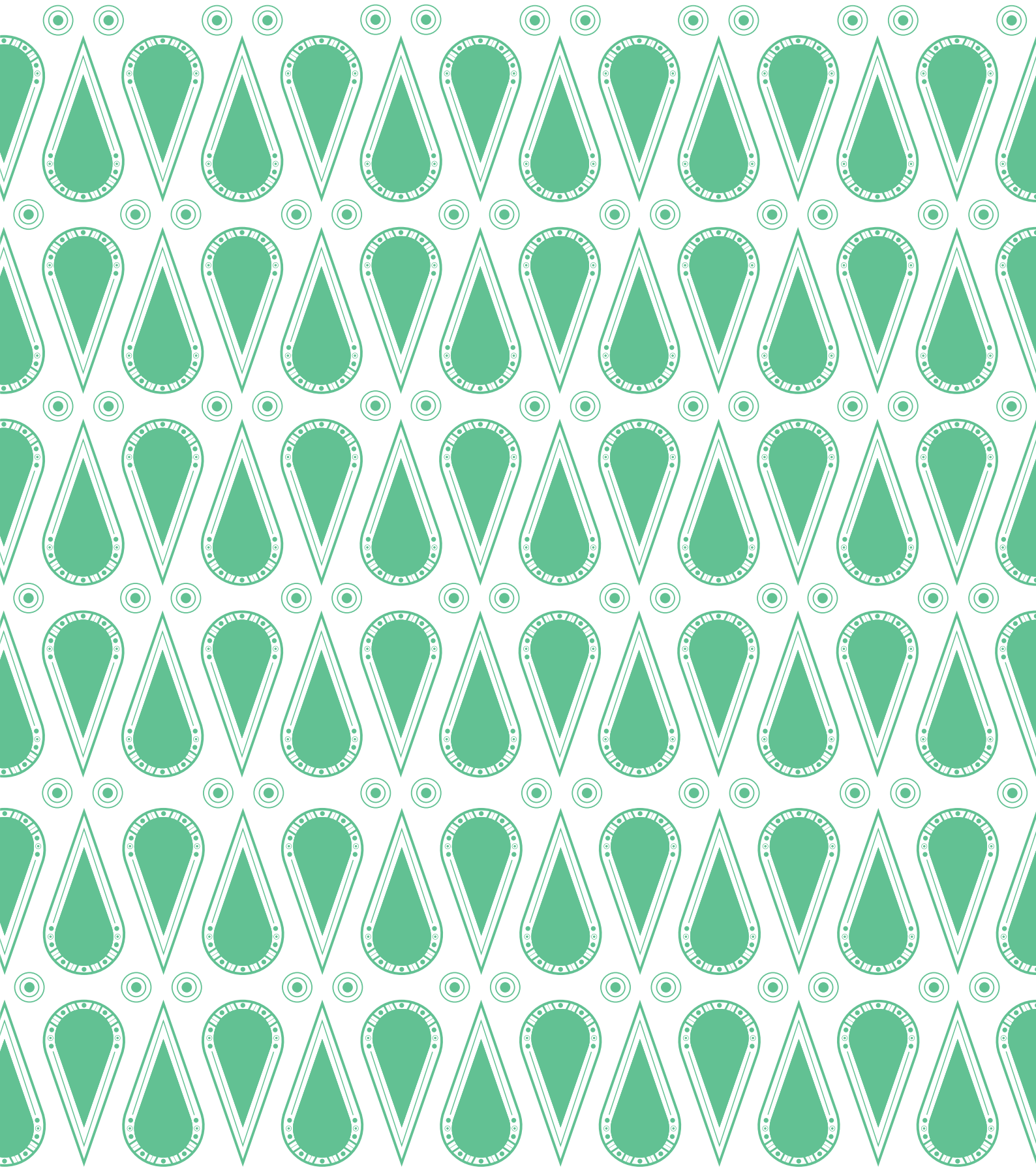
Objective 1: To describe pediatric healthcare in Manhiça district, southern Mozambique, through a gender lens.

- **Specific objective:** To compare different indicators of healthcare access and provision between boys and girls in Manhiça district, southern Mozambique → **Article 1.**

Objective 2: To evaluate whether measuring host biomarkers can help identify individuals at risk of adverse outcomes or with specific organ injury in diverse clinical scenarios involving pediatric and adult patients with suspected acute infections in Mozambique.

- **Specific objective:** To evaluate the prognostic performance of immune and endothelial activation biomarkers in plasma at clinical presentation to predict in-hospital, 28-day, and 90-day mortality in Mozambican children hospitalized with clinical pneumonia → **Article 2.**
- **Specific objective:** To evaluate the prognostic performance of immune and endothelial activation biomarkers in plasma at clinical presentation to predict 28-day mortality and other adverse outcomes in children and adults with all-cause febrile illness presenting to hospital in Mozambique → **Article 3.**
- **Specific objective:** To explore whether plasma levels of NfL could support identification of brain injury in Mozambican children with malaria → **Article 4.**

7. MATERIAL, METHODS, AND RESULTS



ARTICLE

1

Paediatric healthcare in Manhiça district through a gender lens: a retrospective analysis of 17 years of morbidity and demographic surveillance data

Electronic supplementary material:
The online version of this article contains supplementary material.

journal of
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Cite as: Balanza N, Hunguana A, Ajanovic S, Varo R, Bramugy J, Matsena T, Nhampossa T, Ouchi D, Nhacolo A, Dalsuco J, Siteo A, Quintó L, Acácio S, Nhacolo A, Maixenchs M, Munguambe K, Mandomando I, Aide P, Saúte F, Guinovart C, Sacoó C, Bassat Q. Paediatric healthcare in Manhica district through a gender lens: a retrospective analysis of 17 years of morbidity and demographic surveillance data. *J Glob Health*. 2025;15:04010.

PAPERS



Paediatric healthcare in Manhica district through a gender lens: a retrospective analysis of 17 years of morbidity and demographic surveillance data

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Background Sex and gender are important determinants of health. Gender-based health inequities in the paediatric population have been reported in various countries, but data remain limited. In Mozambique, research on this topic is very scarce. Here we aimed to explore whether boys and girls in Manhica district, southern Mozambique, differ in access to and provision of healthcare.

Methods This retrospective analysis includes data on all paediatric (<15 years old) visits to six outpatient clinics and admissions to one hospital in Manhica district from 2004 to 2020, collected through the morbidity surveillance system of the Manhica Health and Demographic Surveillance System (HDSS). We compared characteristics and outcomes between boys and girls using descriptive statistics, standardised mean differences, and logistic regression. Post-discharge events were analysed using Cox proportional hazards regression and Fine-Gray competing risk regression. Minimum community-based incidence rates of outpatient clinic visits and hospitalisations were calculated using demographic surveillance data from the Manhica HDSS and analysed with negative binomial regression.

Results Girls represented 49.2% (560 630 out of 1 139 962) of paediatric visits to outpatient clinics and 45.1% (18 625 out of 41 278) of hospitalisations. The girls-to-boys incidence rate ratio (IRR) for hospitalisations was 0.81 (95% confidence interval (CI)=0.79–0.84). Both boys and girls experienced symptoms for a median duration of one day (interquartile range (IQR)=1–2) before seeking care. Severe manifestations at presentation to an outpatient clinic or upon hospitalisation tended to be less frequent in girls (girls-to-boys odds ratios (ORs)=0.71–1.11). Girls were less frequently referred or admitted to hospital after an outpatient clinic visit (OR=0.82; 95% CI=0.79–0.86 and OR=0.85; 95% CI=0.84–0.87, respectively). The hospital case fatality ratio was 4.1% in boys and 4.2% in girls. The median duration of hospitalisation was three days (IQR=2–5) and did not differ between boys and girls. Revisits to outpatient clinics, hospital readmissions, and hospital post-discharge mortality were similar in both groups.

Conclusions Girls had fewer referrals and admissions to hospital in Manhica district, but they were also less likely to present with severe manifestations. Other studied indicators of healthcare access and provision were overall similar for boys and girls. Further research is needed to continue assessing potential gender biases and sex differences in paediatric healthcare in Mozambique.

Sex and gender are major drivers of health and well-being. Sex, defined as the biological and physiological differences between males, females, and intersex people, can influence disease susceptibility and progression. Gender refers to the socially constructed norms, roles, and behaviours for men/boys, women/girls, and other gender identities, which affects other determinants of health, health risk behaviours, and access to and quality of healthcare [1]. Understanding the impact of sex-specific differences and gender inequities on health is crucial for informing interventions and policies aimed at improving health outcomes and leaving no one behind. However, research in this area has been predominantly focused on adults, with limited attention given to the paediatric population.

Male infants are at a higher risk of death than their female counterparts during the first months of life [2], which is generally attributed to biological differences that make them more vulnerable to congenital diseases, perinatal illnesses, and infectious diseases [3–6]. Yet this survival advantage in female children might be lost or even reversed in settings where girls are at an increased social risk. A few studies have investigated whether gender-driven inequities exist in children's care and feeding practices, access to health services, timely seeking of healthcare, and management by healthcare providers [7]. Most of this research comes from Asia, where some studies have described that girls face disadvantages compared to boys which might be explained by societal norms and family or cultural practices [7–10]. Studies on this topic including data from sub-Saharan African countries are very scarce. Some have reported that girls are admitted to hospital less frequently, have higher in-hospital mortality, or that their caregivers seek healthcare for them less often, but others did not find significant differences, with findings varying by study and country [11–13].

Mozambique ranked 118 out of 166 countries in the Gender Inequality Index in 2022, indicating remarkable inequalities between women and men in health, empowerment, and labour market [14]. Nevertheless, there is a lack of data on the impact of gender on health among Mozambique's paediatric population. Only two multi-country reports assessed healthcare-seeking behaviour for children under five in Mozambique using data from the Demographic and Health Surveys programme. According to these, caregivers took boys and girls to a healthcare provider with similar frequency in the presence of diarrhoea, fever, or suspected pneumonia [11,12]. Some studies have suggested an excess of female under-five mortality in Mozambique, but interpretation of findings is complex and results vary depending on the methodology used and the specific years analysed, with no such excess observed in older children [15–20]. Therefore, further country-specific research is needed to understand potential sex- and gender-based differences in health during childhood and early adolescence.

This study aimed to explore whether there are differences in access to and provision of healthcare between boys and girls younger than 15 years in the district of Manhica, southern Mozambique. We compared different indicators using surveillance data from the Manhica Health and Demographic Surveillance System (HDSS) of the Manhica Health Research Centre (*Centro de Investigação em Saúde de Manhica* (CISM)) during a 17-year period (2004–2020).

METHODS

Study site

The district of Manhica is located in Maputo province, southern Mozambique, 80 km north of the capital city of Maputo [21,22]. With ~209 000 inhabitants in 2022 and an area of 2380 km² on a plain crossed by the Incomati river [23], it is the paradigm of a poor, resource-limited rural sub-Saharan setting. The population is predominantly young and there is a sharp reduction of males from 20 years of age and above. Most residents are engaged in small businesses, practice subsistence farming, or work in agricultural companies. A more detailed description of the study area and population can be found elsewhere [21–23].

The prevalence of human immunodeficiency virus (HIV) in Manhica is among the highest in the world, with an adult community prevalence of around 30–40% and an HIV vertical transmission rate that has decreased over the years [24–27]. *Plasmodium falciparum* malaria transmission is perennial in the area and peaks during the rainy season (November–April), but an upscale of malaria control tools in the last two decades has contributed to a notable decrease in malaria cases [28]. Major causes of paediatric morbidity and mortality include malaria, pneumonia, HIV/acquired immunodeficiency syndrome, diarrheal diseases, and malnutrition [29].

The district of Manhica has 21 healthcare facilities, which include the Manhica District Hospital (MDH), a rural hospital in Xinavane, and several outpatient clinics [23]. The MDH is the main healthcare facility in the area, used for primary healthcare by nearby population and one of the two referral health centres for Manhica district. It has a 110-bed inpatient ward, an outpatient clinic, a maternal and child health clinic with a small surgical room, a round-the-clock emergency department, and basic laboratory facilities. Children visiting the outpatient clinic at MDH can be admitted to the paediatric ward of the hospital, immediately or after being under observation for some hours in a day-care unit. Severely ill patients admitted to MDH can be transferred to Maputo Central Hospital, a tertiary healthcare facility in the capital city of Maputo. Outpatient clinics have basic medical equipment and limited supplies. They offer primary healthcare services and not inpatient care, but they may have a few beds available for observation and post-delivery. All clinical management and treatment are free of charge, except for a standard subsidised fee for the outpatient medication to be taken at home.

The Manhica HDSS

The CISM has run an HDSS in the district of Manhica since 1996. It comprises both a demographic surveillance system covering the general population and a morbidity surveillance system monitoring paediatric patients attending healthcare facilities in the study area [21,22].

Demographic surveillance

The catchment area of the demographic surveillance gradually expanded since its creation to capture the entire district of Manhica, with the largest increase occurring in 2014. Each person living within the HDSS surveillance area is issued a unique HDSS permanent identification number. Households are visited twice a year to collect sociodemographic information at the individual and household levels, including data on births, deaths, and migrations. This information is supplemented by daily hospital visits, weekly updates from key community informants, and, more recently, notifications via a toll-free call centre that operates 24 hours a day, seven days a week, 365 days a year. Data was collected on paper forms and double-entered until 2011, after which electronic collection was implemented. Information validation rules, input constraints, and error-checking scripts are established to ensure data quality.

Paediatric morbidity surveillance

Seven healthcare facilities in Manhica district are currently involved in monitoring paediatric morbidity. The number of participating facilities has increased over the years in line with the demographic surveillance area expansions. Each time a child under 15 years attends one of these healthcare facilities, a standardised paper-based form is completed with the information about the health event (a two-page form for outpatient clinic visits and a four-page form for hospital admissions). These include demographic data, signs and symptoms upon presentation, basic laboratory investigations, multiple diagnoses, treatments prescribed, and outcomes. The HDSS permanent identification number of the patients is also collected in these questionnaires, enabling demographic and illness events to be linked for children residing within the Manhica HDSS study area. Data are collected using paper questionnaires and double-entered. Similarly to the demographic surveillance system, data quality control measures are established during and after data entry.

Study design

In this retrospective analysis, we included all visits by children younger than 15 years to the outpatient clinics of MDH, Maragra, Malavele, Palmeira, Taninga, and Ilha Josina Machel, as well as all hospitalisations of children younger than 15 years at MDH, from 1 January 2004 to 31 December 2020 (Figure S1 in the [Online Supplementary Document](#)). The Taninga outpatient clinic was incorporated into the morbidity surveillance system in 2005, while the Palmeira and Malavele clinics were added in 2009. The period 2004–2020 corresponds to a timeframe during which the same standardised questionnaires have been used in the morbidity surveillance system. We compared various indicators reflecting access to and provision of healthcare between boys and girls. These included percentages and minimum community-based incidence rates (MCBIRs) of outpatient clinic visits and hospitalisations, duration of symptoms before visiting an outpatient clinic and before hospitalisation, presence of severe manifestations at presentation to an outpatient clinic and upon hospitalisation, referrals or admissions to hospital, hospital case fatality ratios (CFRs), time from hospitalisation to in-hospital death, length of hospital stay, revisits to outpatient clinics within seven days, hospital readmissions within 28 days, post-discharge mortality within 28 days, and paediatric mortality rates in the Manhica HDSS study area. Demographic surveillance data were used to identify paediatric patients residing in the area, estimate population denominators, and retrieve additional mortality data for deaths occurring outside the hospital. The databases used from the Manhica HDSS included male and female sex data for children, but did not provide information on intersex biological status or gender identities. In the context of this study, male children were considered boys and female children were considered girls, and are indicated as such throughout the text, in line with our focus on understanding gender as a social determinant of health.

Data analysis

Descriptive data were reported as counts (%), median (interquartile range (IQR)), or mean (standard deviation). Continuous variables were compared between two groups using standardised mean differences [30]. Univariable logistic regression models were fitted to estimate girls-to-boys odds ratios (ORs) of presenting with severe manifestations or having certain outcomes. An OR greater than one denotes the odds are greater in girls. Definitions used for severe manifestations can be found in Table S1 in the [Online Supplementary Document](#). For outcomes, discharge home after an outpatient clinic visit or after hospitalisation was used as the reference category. We also calculated CFRs during hospitalisation, excluding patients who absconded or were transferred to the Maputo Central Hospital.

The MCBIRs of outpatient clinic visits and hospitalisations were calculated by dividing the number of events among children residing in the Manhica HDSS study area (i.e. those with an HDSS permanent identification number in the clinical questionnaire) by the total time at risk for the paediatric population of Manhica residents inferred from demographic surveillance data. Children did not contribute to the numerator or denominator when they were living outside the study area. The periods of hospitalisation at MDH were also excluded from the time at risk. For girls-to-boys incidence rate ratios (IRR), we used mixed-effects negative binomial regression models, with individual as a random effect and no adjustment for other covariates.

We studied revisits to outpatient clinics within seven days, hospital readmissions within 28 days, and hospital post-discharge mortality within 28 days in children living in the Manhica HDSS study area. For revisits and readmissions, we defined index visits/admissions as those in which children were either discharged home or absconded. We studied time to the first-or-only event within the determined time at risk (recurrent events occurred in <0.1% of children). After the follow-up period (seven or 28 days) any subsequent visit or admission was considered unrelated to a previous one and modelled as a new index visit/admission. Fine-Gray competing risk regression models, considering death as a competing event, were used to estimate girls-to-boys subdistribution hazard ratios (SHRs) and cumulative incidence functions of revisits and readmissions [31].

For hospital post-discharge mortality, we defined index hospitalisations as those with a known outcome different from in-hospital death. We used a single-discharge approach, considering the first hospital admission as reference and excluding subsequent readmissions within the following 28 days, consistent with previous analyses in Manhiça district [32]. Cox proportional hazards regression models were used to calculate girls-to-boys hazard ratios (HRs) of post-discharge mortality, and cumulative incidences were estimated as 1 - Kaplan-Meier survival function. Deaths occurring outside the healthcare facilities were retrieved from demographic surveillance data. In all analyses of post-discharge outcomes, children who migrated outside the Manhiça HDSS study area before the end of the follow-up period were censored at that moment. Standard errors were adjusted for within-child correlation, but the models did not include additional covariates.

Mortality rates for boys and girls in Manhiça district were calculated using demographic surveillance data. The number of infants, under-5, and under-15 deaths in each calendar year was divided by the total number of live births that occurred in the same year, expressed per thousand live births.

We conducted all data analyses both on the complete set and by stratifying it into four age groups: <1 year, 1–4 years, 5–9 years, and 10–14 years. Most variables had a small percentage of missing values and those were excluded when computing the different statistics. The statistical software Stata version 16.1 (StataCorp, College Station, TX, USA, 2019) and R version 4.2 or above (R Core Team, Vienna, Austria, 2022) were used.

Ethical approval

The CISM's HDSS was approved by the local administrative authorities (government of Manhiça district and president of Manhiça municipality), by the Institutional Ethics Review Board, and by the National Committee for Bioethics in Health of Mozambique. All residents of the Manhiça HDSS study area gave signed individual informed consent to be enrolled in the ongoing HDSS. For resident children, parents or guardians gave signed informed consent and children assented if aged 12–17 years.

RESULTS

Study population characteristics

Over the study period, from January 2004 to December 2020, there were 1 147 471 visits across the six outpatient clinics and 41 354 admissions to MDH by children younger than 15 years. Of these, 1 147 441 (99.9%) and 41 278 (99.8%) had available data on the child's sex, respectively. A total of 7479 visits to the outpatient clinic at MDH were identified as direct referrals from the other five outpatient clinics. To avoid double counting these same events, only the first available contact with the healthcare system was retained, resulting in a final sample size of 1 139 962 paediatric outpatient clinic visits. The total number of outpatient clinic visits and hospitalisations by calendar year and age group can be found in Figure S2 in the [Online Supplementary Document](#).

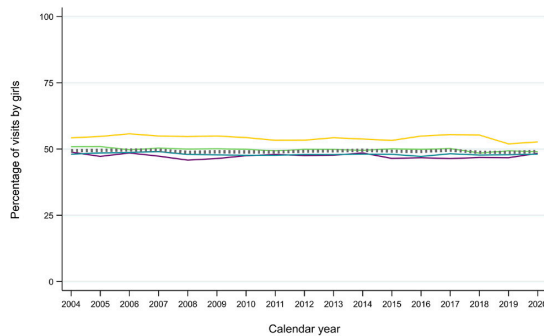
The median age of children visiting an outpatient clinic was 3.4 years (IQR=1.4–7.3), with a median age in boys of 3.3 years (IQR=1.3–7.0) and in girls of 3.6 years (IQR=1.4–7.7). Among outpatient clinic visits, the most frequent diagnoses were upper respiratory tract infections (32.1% for boys and 33.5% for girls) and malaria (24.5% for boys and 24.4% for girls). In children admitted to MDH, the median age was 1.6 years (IQR=0.7–3.3), the same for boys and girls. The most frequent diagnoses in inpatients included malaria (43.0% for boys and 45.0% for girls), lower respiratory tract infections (27.0% for boys and 26.4% for girls), and gastrointestinal diseases (13.8% for boys and 12.4% for girls).

Frequency of outpatient clinic visits and hospitalisations

Girls accounted for 49.2% (560 630 out of 1 139 962) of the total outpatient clinic visits. With respect to admissions to MDH, girls represented 45.1% (18 625 out of 41 278). These overall percentages were constant over time, with no marked downward or upward trend during the 17 years of the

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A) Visits to six outpatient clinics
(N=1,139,962)



B) Admissions to Manhica District Hospital
(N=41,278)

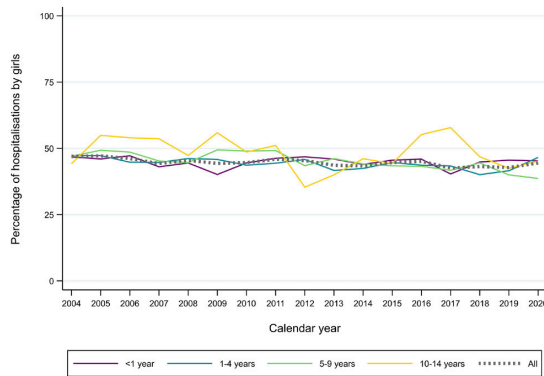


Figure 1. Percentage of paediatric outpatient clinic visits and hospitalisations by girls from 2004–2020 in Manhica district.

study. Results remained similar across age groups, except for children aged 10–14 years. In this oldest group, girls consistently accounted for a higher percentage of outpatient clinic visits, and there was more variability in the percentage of hospitalisations by girls over time (Figure 1).

When restricting the analysis to children living in the Manhica HDSS study area, the overall MCBIRs of outpatient clinic visits and hospitalisations were lower in girls compared to boys (Table S2 in the [Online Supplementary Document](#)). The overall girls-to-boys IRR for outpatient clinic visits was 0.97 (95% CI=0.96–0.98). This ratio remained consistent across age groups, except for children aged 10–14 years, where the MCBIR for outpatient clinic visits was higher for girls and the IRR was 1.09 (95% CI=1.07–1.11). The overall girls-to-boys IRR for hospitalisation was 0.81 (95% CI=0.79–0.84), consistent when stratifying by age group (Table S2 in the [Online Supplementary Document](#)).

Duration of symptoms before visiting an outpatient clinic and before hospitalisation

Caregivers were enquired about the number of days that their child had experienced fever, cough, diarrhoea, or vomiting during the current disease before visiting an outpatient clinic or being admitted to MDH. Both boys and girls had any of these symptoms for a median duration of one day (IQR=1–2) before visiting an outpatient clinic, and a median duration

of two days (IQR=1–3) before hospitalisation. For each symptom individually, the median duration was also the same for boys and girls. Results were consistent when stratified by age group, with all standardised mean differences <0.1 (Table 1).

Presence of severe manifestations at presentation to an outpatient clinic and upon hospitalisation

We selected 17 severe general manifestations among children visiting outpatient clinics and 27 among hospitalised children. When visiting an outpatient clinic, girls had lower odds for 13 out of 17 severe manifestations (girls-to-boys OR=0.71–0.96). For instance, these include dehydration (OR=0.71; 95% CI=0.67–0.76), seizures (OR=0.73; 95% CI=0.69–0.77), and jaundice (OR=0.76; 95% CI=0.68–0.85). Girls only had higher odds for increased respiratory rate (OR=1.03; 95% CI=1.02–1.04) (Figure 2, Panel A). Upon admission to MDH, severe manifestations were more common. Girls had lower odds for 11 out of 27 severe manifestations (girls-to-boys OR=0.78–0.93). However, ORs were closer to one compared to those for same manifestations observed at presentation to an outpatient clinic. The odds of three out of 27 severe manifestations were higher in girls: increased respiratory rate (OR=1.04; 95% CI=1.00–1.08), pallor (OR=1.09; 95% CI=1.03–1.14), and tachycardia (OR=1.11; 95% CI=1.06–1.17) (Figure 2, Panel B). All ORs for the entire cohort and each age group are presented in Figure 2. Nevertheless, the differences between boys and girls in the percentages of patients with severe manifestations were small in absolute terms (Figure S3 in the [Online Supplementary Document](#)).

Table 1. Reported symptoms in children and their duration before visiting an outpatient clinic and before hospitalisation in Manhiça district*

		N	Any symptom		Fever		Cough		Diarrhoea		Vomiting	
			Median days (IQR)	%	Median days (IQR)	%	Median days (IQR)	%	Median days (IQR)	%	Median days (IQR)	%
Visits to six outpatient clinics												
All	Boys	579 332	1 (1–2)	73.1	1 (1–2)	52.6	2 (1–3)	7.7	1 (1–1)	7.5	1 (1–1)	
	Girls	560 630	1 (1–2)	74.2	1 (1–2)	53.4	2 (1–3)	6.9	1 (1–1)	7.3	1 (1–1)	
<1 y	Boys	110 343	1 (1–2)	62.1	1 (1–2)	67.8	2 (1–3)	15.9	1 (1–1)	9.5	1 (1–1)	
	Girls	99 158	1 (1–2)	60.6	1 (1–2)	67.8	2 (1–3)	14.4	1 (1–1)	9.0	1 (1–1)	
1–4 y	Boys	256 752	1 (1–2)	73.8	1 (1–2)	57.3	2 (1–3)	8.8	1 (1–1)	7.6	1 (1–1)	
	Girls	236 980	1 (1–2)	74.1	1 (1–2)	59.2	2 (1–3)	8.2	1 (1–1)	7.5	1 (1–1)	
5–9 y	Boys	138 130	1 (1–2)	78.5	1 (1–2)	42.2	2 (1–3)	2.0	1 (1–1)	6.6	1 (1–1)	
	Girls	137 064	1 (1–2)	80.4	1 (1–2)	44.7	2 (1–3)	2.1	1 (1–1)	6.9	1 (1–1)	
10–14 y	Boys	74 107	1 (1–2)	77.4	1 (1–2)	32.9	2 (1–3)	2.3	1 (1–1)	5.5	1 (1–1)	
	Girls	87 428	1 (1–2)	80.7	1 (1–2)	35.2	2 (1–2)	2.4	1 (1–1)	5.5	1 (1–1)	
Admissions to Manhiça District Hospital												
All	Boys	22 653	2 (1–3)	84.7	1 (1–3)	58.5	2 (1–3)	20.6	1 (1–2)	22.2	1 (1–2)	
	Girls	18 625	2 (1–3)	85.5	1 (1–3)	58.8	2 (1–3)	19.1	1 (1–2)	22.7	1 (1–2)	
<1 y	Boys	8 096	2 (1–3)	77.4	1 (1–3)	62.6	2 (1–3)	28.5	1 (1–2)	24.5	1 (1–2)	
	Girls	6 619	2 (1–3)	78.9	1 (1–3)	61.7	2 (1–3)	25.8	1 (1–3)	23.6	1 (1–2)	
1–4 y	Boys	11 271	2 (1–3)	89.7	1 (1–3)	59.9	2 (1–3)	18.4	1 (1–2)	20.8	1 (1–1)	
	Girls	9 203	2 (1–3)	89.8	2 (1–3)	60.6	2 (1–3)	17.7	1 (1–2)	21.0	1 (1–1)	
5–9 y	Boys	2 516	2 (1–3)	87.2	1 (1–3)	44.4	2 (1–4)	8.1	1 (1–2)	21.0	1 (1–1)	
	Girls	2 105	2 (1–3)	89.7	2 (1–3)	47.4	2 (1–4)	7.5	1 (1–2)	25.0	1 (1–2)	
10–14 y	Boys	770	2 (1–3)	79.7	2 (1–3)	40.4	3 (2–6)	10.3	1 (1–3)	22.4	1 (1–1)	
	Girls	698	2 (1–3)	80.1	2 (1–3)	41.1	3 (1–7)	9.8	1 (1–2)	31.0	1 (1–2)	

IQR – interquartile range.

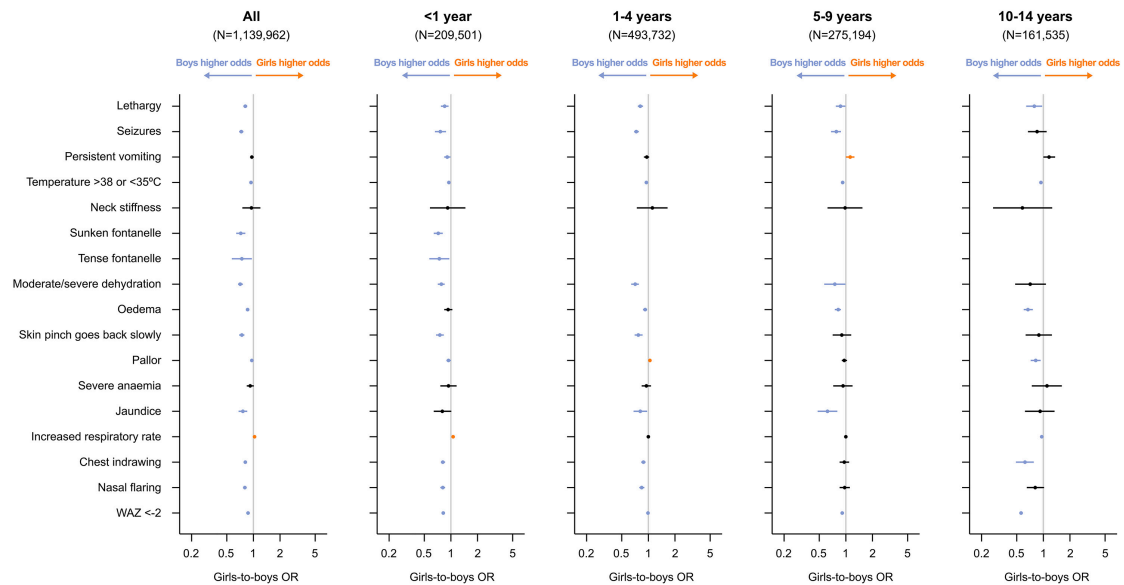
*The median (IQR) number of prior days with any symptom was calculated for all children who visited an outpatient clinic or were hospitalised. The median (IQR) number of prior days for each specific symptom was calculated for children who reported experiencing that symptom. All standardised mean differences in the duration of symptoms between boys and girls are <0.1. Missing data in all variables is <1%.

Patient management and outcomes of outpatient clinic visits and hospitalisations

In all outpatient clinics other than the one at MDH, 1.8% of boys and 1.5% of girls were referred to a higher-level healthcare facility (usually to MDH) after a clinical evaluation (girls-to-boys OR=0.82; 95% CI=0.79–0.86). In children who visited directly the MDH outpatient clinic, a decision for admission to MDH was reached in 8.0% of boys and 6.9% of girls (girls-to-boys OR=0.85; 95% CI=0.84–0.87). Similar results were obtained when stratifying by age group, with smaller ORs in older age groups (Table S3 in the [Online Supplementary Document](#)). Other possible outcomes at the outpatient clinic level (i.e. absconder, transfer from the MDH outpatient clinic to Maputo Central Hospital, or death) were uncommon in both boys and girls ([Figure 3](#), Panels A–B; Table S3 in the [Online Supplementary Document](#)).

Upon admission to MDH, the median length of hospital stay did not differ between boys and girls (three days (IQR=2–5) for each group), regardless of stratification by age group or restriction to only children who were finally discharged home (Table S4 in the [Online Supplementary Document](#)). An explorative breakdown of diagnostic tests conducted and treatments given in boys and girls can be found in Table S5 in the [Online Supplementary Document](#). The hospital CFR was 4.1% in boys and 4.2% in girls (girls-to-boys OR=1.02; 95% CI=0.92–1.13). Similarly, there were no significant differences in terms of absconders or transfers to Maputo Central Hospital. This was also observed when stratifying by age group ([Figure 3](#), Panel C; Table S3 in the [Online Supplementary Document](#)). Time to in-hospital death was similar overall in boys and girls, with both groups having a median of two days (IQR=1–5) from hospitalisation to in-hospital death (Table S6 in the [Online Supplementary Document](#)).

A) Visits to six outpatient clinics



B) Admissions to Manhica District Hospital

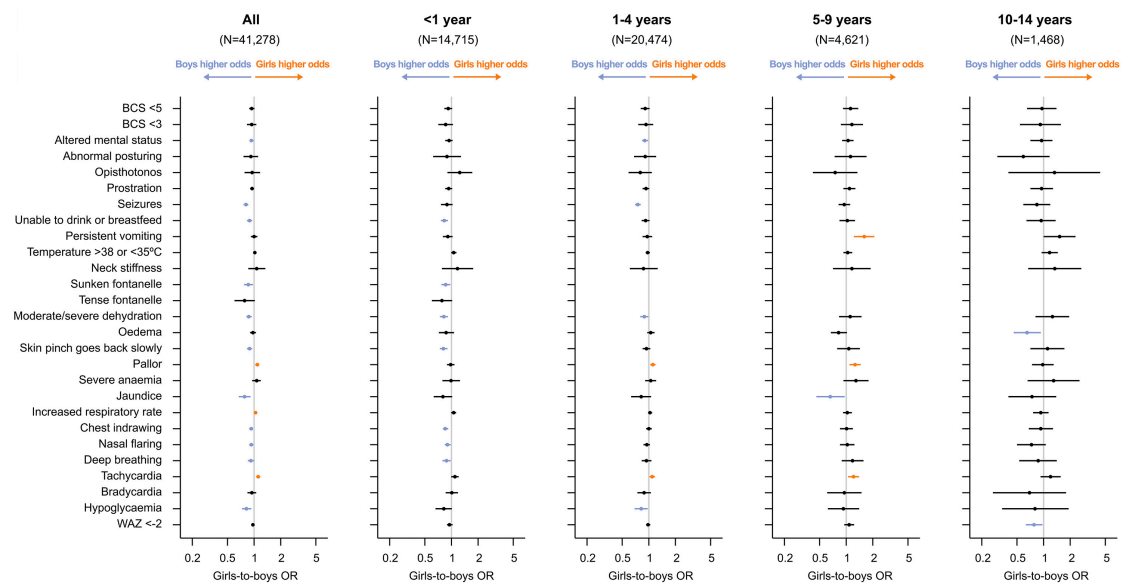


Figure 2. Girls-to-boys odds ratios of severe manifestations at presentation to an outpatient clinic and upon hospitalisation in Manhica district. Odds ratios and 95% CIs from univariable logistic regression models are shown on a logarithmic scale. Points to the right indicate that girls have higher odds than boys of having a certain severe manifestation when visiting an outpatient clinic or being admitted to Manhica District Hospital, whereas points to the left indicate that boys have higher odds than girls of having it. Estimates are highlighted in orange or blue if the 95% CIs do not cross one. Definitions used for severe manifestations can be found in Table S1 in the [Online Supplementary Document](#). Sunken or tense fontanelle was calculated only in children aged <1 year. Missing data in all variables is <5%, except for sunken/tense fontanelle (5% in outpatient clinic visits, 8% in hospitalisations), severe anaemia (45% in outpatient clinic visits, 9% in hospitalisations), and hypoglycaemia (9% in hospitalisations). BCS – Blantyre Coma Scale, CI – confidence interval, OR – odds ratio, WAZ – weight-for-age z-score.

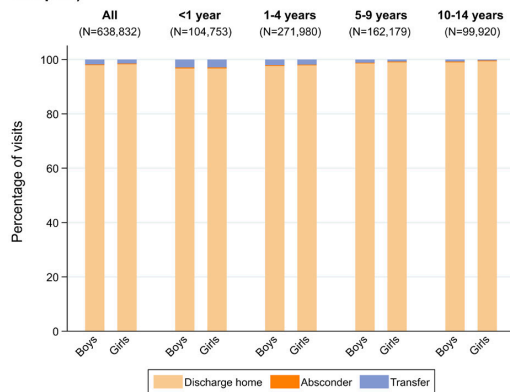
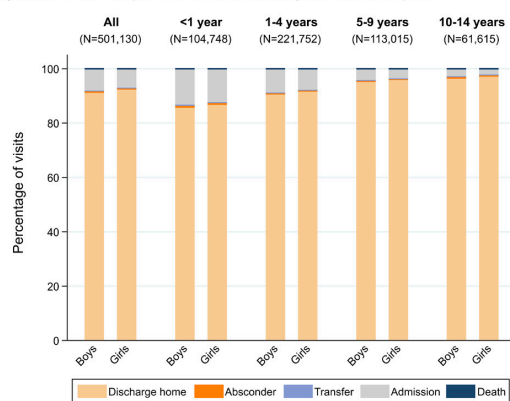
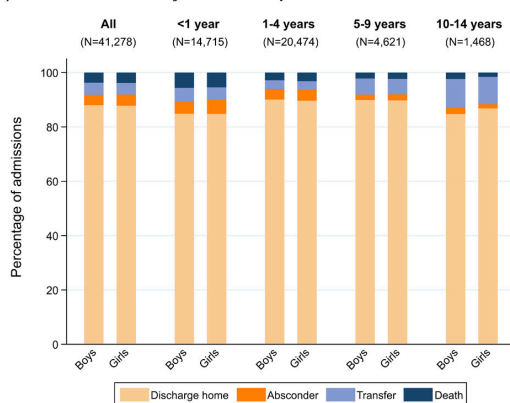
A) Visits to five outpatient clinics (excluding the one at Manhica District Hospital)**B) Visits to the outpatient clinic at Manhica District Hospital****C) Admissions to Manhica District Hospital**

Figure 3. Outcomes of paediatric outpatient clinic visits and hospitalisations in Manhica district. Outcomes of visits to the outpatient clinic at Manhica District Hospital (**Panel B**) were calculated separately from those of visits to the other five outpatient clinics (**Panel A**), as possible outcomes differ. Outcomes of visits to the outpatient clinic at Manhica District Hospital (**Panel B**) are a combination of the outcomes that occurred immediately after the clinical visit or after being under observation for some hours in a day-care unit of Manhica District Hospital. Missing data in outcomes is <1%.

Post-discharge outcomes after an outpatient clinic visit and after hospitalisation

Within seven days after visiting an outpatient clinic and being either discharged home or absconding, 4.5% (18 552 out of 414 777) of boys and 4.2% (16 756 out of 403 456) of girls revisited an outpatient clinic. The girls-to-boys SHR of revisiting was 0.93 (95% CI=0.90–0.95) (**Figure 4**, Panel A). Within 28 days after leaving the MDH due to being discharged home or absconding, 3.6% (413 out of 11 587) of boys and 3.4% (313 out of 9297) of girls were readmitted. The subdistribution hazard rates of hospital readmission were not different in the two groups (girls-to-boys SHR=0.94; 95% CI=0.81–1.10) (**Figure 4**, Panel B). Using the first admission to MDH as reference and excluding subsequent readmissions within the next 28 days, 2.3% (281 out of 12 038) of boys and 2.3% (225 out of 9652) of girls died within 28 days after hospital discharge. The hazard rates of post-discharge mortality did not differ between boys and girls (girls-to-boys HR=1.00; 95% CI=0.84–1.19) (**Figure 4**, Panel C). Results by age groups were similar and can be found in Table S7 and Figures S4–S6 in the **Online Supplementary Document**.

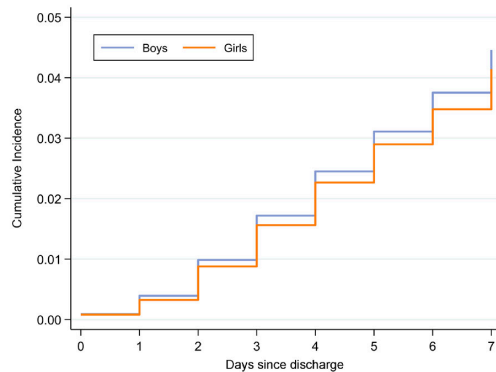
In addition, at the population level, the infant, under-5, and under-15 mortality rates in the Manhica HDSS study area over the study period were slightly higher for boys than girls (Figure S7 in the **Online Supplementary Document**).

DISCUSSION

In this paediatric cohort from southern Mozambique, comprising over 1.1 million visits to outpatient clinics and 41 000 hospitalisations from 2004 to 2020, we found no overall major differences between boys and girls in most studied indicators of access to and provision of healthcare. However, results indicate that girls were referred and admitted to hospital less frequently, and they were also less likely to present with severe manifestations. By using comprehensive morbidity and demographic surveillance data spanning 17 years we were able to assess multiple healthcare aspects separately for boys and girls, expanding our analysis beyond the healthcare facility level to encompass post-discharge outcomes and provide additional insights at the community level.

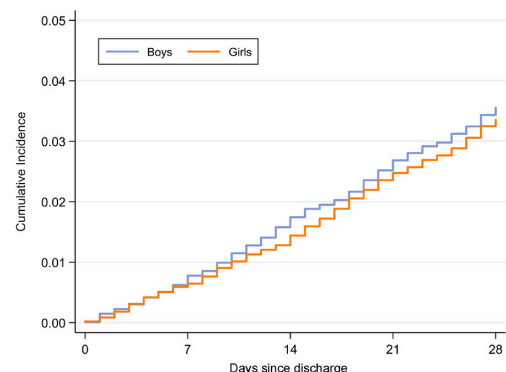
Gender bias by caregivers favouring boys over girls could manifest in differences in access to healthcare, with girls being taken to healthcare facilities less fre-

A) Revisits to outpatient clinics



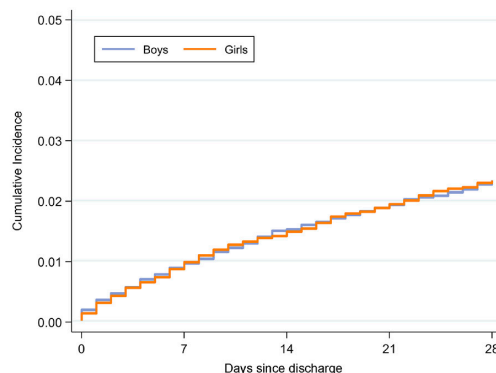
Patients at risk								
Boys	414777	414370	413060	410537	407445	404369	401587	398871
Girls	403456	403092	402074	399783	396982	394083	391479	389109
Events								
Boys	0	1636	2466	3050	3044	2739	2678	2939
Girls	0	1313	2243	2761	2854	2556	2349	2680
Competing events								
Boys	0	51	26	17	10	14	19	7
Girls	0	35	17	22	25	18	5	14

B) Readmissions to Manhica District Hospital



Patients at risk								
Boys	11587	11452	11290	11145	10998			
Girls	9297	9186	9076	8937	8828			
Events								
Boys	0	90	112	109	102			
Girls	0	60	74	96	83			
Competing events								
Boys	0	65	43	30	34			
Girls	0	59	36	35	22			

C) Manhica District Hospital post-discharge mortality



Patients at risk								
Boys	12038	11928	11848	11794	11739			
Girls	9652	9563	9506	9454	9413			
Events								
Boys	0	115	68	49	49			
Girls	0	94	49	44	38			

Figure 4. Cumulative incidence of post-discharge outcomes in children after an outpatient clinic visit and after hospitalisation in Manhica district. For revisits to outpatient clinics (**Panel A**) and hospital readmissions (**Panel B**), index visits or hospitalisations were defined as those in which children were either discharged home or absconded, and death was treated as a competing event. For hospital post-discharge mortality, index hospitalisations were defined as those with a known outcome different from in-hospital death.

quently, at later stages of illness, and with more severe symptoms. In our study population, girls accounted for 49.2% of visits to outpatient clinics and the girls-to-boys IRR of visits at the population level was 0.97 (95% CI=0.96–0.98). Frequency of utilisation of healthcare services is difficult to interpret in our study, as we do not know how many children did not seek care but needed it. However, our results suggest no important differences regarding healthcare-seeking behaviour according to referred days with symptoms before visiting an outpatient clinic. This is in line with the two previous reports using Demographic and Health Surveys data from Mozambique, which showed no difference in the proportion of boys and girls with symptoms of common illnesses who were taken to a healthcare provider. One study reported a boys-to-girls healthcare-seeking ratio of 1.02 (95% CI=0.93–1.10), while the other reported no significant differences in percentages [11,12]. In other sub-Saharan African countries, the limited studies comparing healthcare utilisation and time to seek healthcare between boys and girls when they face illness have usually shown either no significant differences or suggest an advantage for boys [11,12,33–42]. In our study, we also found that severe manifestations at presentation to an outpatient clinic were generally more frequently observed in boys. However, since these differences do not seem to be related to delays in seeking healthcare, they could be attributed to variations in how symp-

toms are assessed or to sex-based differences in pathophysiological mechanisms. Boys might be more susceptible to severe disease and rapid progression due to biological factors, such as having weaker immune systems during infancy [6]. In addition, we did not observe differences in the frequency of absconding decisions between boys and girls.

Gender-based inequities in the care provided by healthcare workers may lead to differences in clinical decisions and patient outcomes. Especially in resource-limited settings, with scarce laboratory and imaging services, the assessment of severity and prediction of adverse outcomes in paediatric conditions rely largely on symptom and sign-oriented clinical guidelines. This may introduce subjectivity, with the potential for sociocultural influences to affect the evaluation process and introduce a gender bias. If boys are favoured, girls may be less frequently referred or admitted to hospital, experience longer hospital stays due to complications, or have higher in-hospital mortality. In our study population, a slightly higher percentage of boys than girls (1.8 vs. 1.5%) were referred to a higher-level healthcare facility from outpatient clinics or were hospitalised (8.0 vs. 6.9%) if directly visiting the MDH outpatient clinic. This led to girls accounting for 45.1% of the hospital admissions and a girls-to-boys IRR for hospitalisations of 0.81 (95% CI=0.79–0.84). Once hospitalised, the length of hospital stay, CFR, and time to in-hospital death were similar for boys and girls. Previous studies in children with pneumonia, diarrhoea, or fever have also shown that girls tend to be admitted to hospital less frequently than boys, but their CFRs are similar or higher in girls [12,13,42]. Regarding post-discharge outcomes (i.e. revisits to outpatient clinics, hospital readmissions, or hospital post-discharge mortality), which could reflect poorer clinical care and inadequate management decisions, we did not observe major differences. Similarly, studies conducted in sub-Saharan African countries studying hospital readmission or post-discharge mortality have reported no differences between boys and girls [43–50].

The observed differences in referrals and admissions to hospital favouring boys, along with the finding that certain severe manifestations are less frequently observed in girls at clinical presentation, warrant further investigation. Differences in referrals and admissions to hospital may be a result of gender-based inequities in clinical evaluation and decision-making, but could also reflect the need for care and be explained by variation in signs and symptoms at presentation. The clinical impact of lower hospitalisation rates in girls remains unclear, but we did not observe more revisits to outpatient clinics or mortality among girls. In fact, mortality rates were slightly higher for boys in the entire Manhica HDSS study area in the same period, as generally expected under conditions of equitable resource allocation and care [7,19]. Additionally, the results of this study should be interpreted in the light of analysing very large databases, where many significant relative effect measures were close to one and absolute differences in percentages between boys and girls were usually small.

One of the main strengths of our analysis is the stratification of all results in four distinct age groups, as sociocultural and biological influences on health vary with age [8,20,51]. For instance, from a biological perspective, the survival advantage in females decreases between 6–12 months after birth [6]. Also, older children could seek healthcare on their own when feeling sick and express their symptoms. In our study cohort, some results substantially differed between age groups, but differences between boys and girls remained largely consistent across all age groups. Nevertheless, we observed that girls aged 10–14 years had a higher MCBIR for outpatient clinic visits compared to boys and accounted for a larger percentage of outpatient visits, different from what was observed in other age groups. Furthermore, larger differences were observed between boys and girls in the decision to refer or admit older children to hospital from outpatient clinics.

Despite the high-quality and comprehensive data collected over 17 years, this study has some limitations. We employed an exploratory approach to examine various characteristics and outcomes, stratifying all results by age group. However, factors not considered in the analyses could act as confounders or effect modifiers and may impact the results. For instance, we did not examine how gender intersects and interacts with other dimensions of inequity (e.g. socioeconomic status, ethnicity, or disability) for which we lacked homogeneous and complete data

for our entire study population [51,52]. Moreover, differences between boys and girls can arise because of both biological factors and gender-based roles. Determining the extent to which each impacts our results is challenging, as the two do not act independently but are intertwined. We acknowledge that the retrospective analysis of routine surveillance data may introduce some degree of information bias. To mitigate these, data quality control measures are established and CISM undertakes awareness and engagement activities within the community to encourage accurate reporting of events. Not all formal healthcare facilities in Manhica district are part of the paediatric morbidity surveillance. The number of participating facilities has increased over the years, alongside demographic surveillance area expansions and an increasing number of children having an HDSS permanent identification number [21,22]. While this allows comparing outcomes for boys vs. girls, it may have introduced selection bias and limits the generalisability of findings to the entire district population and the comparison between different time periods. Moreover, we did not evaluate differences between the various outpatient clinics or specific areas within the district. We also lacked data on utilisation of informal or traditional medicine, a common resource in response to health problems in children in Manhica district and Mozambique [53,54]. We explored differences in curative medicine but were unable to investigate disparities in preventive medicine, vaccination status, or feeding practices, which could have offered complementary information. However, previous evidence describes high levels of immunisation in Manhica district, being similar for boys and girls [55,56].

Disaggregating data by sex or gender identity is a first critical step to address inequities in healthcare, since aggregated data sets can mask differences between boys and girls and result in the assumption that they share the same experiences and outcomes. This exploratory flagging exercise can be used as a trigger to spark further research on how and why differences arise. Although in our study population we did not observe major differences between boys and girls in most of the studied indicators of access to and provision of healthcare, similar studies in other areas of Mozambique could provide additional and complementary evidence. Further in-depth research focused on certain syndromes or aetiologies is warranted, as sex differences and gender biases during childhood can be disease-specific [6]. For example, in paediatric pneumonia, some prognostic scores for mortality incorporate sex and give a higher risk to females [57–59]. In addition, parallel qualitative research could provide valuable, context-specific insights into complex gender dynamics, offer perspectives from caregivers and healthcare providers, and help interpret the differences observed in referrals and admissions to hospital and severe manifestations at clinical presentation in Manhica district. Lastly, the incorporation of a gender perspective and specific gender-related items in questionnaires used in the Manhica HDSS could also aid in continuing to study potential gender disparities in children's care and health and their underlying causes [60].

CONCLUSIONS

The results of this study show that girls had fewer referrals and admissions to hospital in Manhica district, but severe manifestations at clinical presentation were also less frequent in girls. Other studied indicators of healthcare access and provision were overall similar for boys and girls. Despite these generally encouraging findings, further research is needed to continue evaluating whether inherent sex differences and gender biases affect paediatric healthcare in Mozambique. Along with efforts to reduce child mortality and morbidity in the country, we must ensure sustained gender equity in the health and well-being of the paediatric population.

Acknowledgements: We extend our gratitude to all the participating children and their families. We also wish to acknowledge the initial designers of the Manhica HDSS and all contributors to its design and implementation. We thank all health workers, laboratory staff, and demographers for their work and participation in data collection.

Data availability: Data are available upon formal request to Vasco Sambo (vasco.sambo@manhica.net), accompanied by a proposal that will be analysed by CISM's internal scientific and ethical committees.

Funding: The Manhica Health Research Centre (CISM) is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID). The Barcelona Institute for Global Health (ISGlobal) receives support from the grant CEX2023-0001290-S funded by MCIN/AEI/10.13039/501100011033, and support from the Generalitat de Catalunya through the CERCA Program. NB is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260). JB is supported by a career development grant from the EDCTP2 programme supported by the European Union (TMA2019SFP-2836 TB lung-FACT2). The article publication charge was funded by the Department of Research and Universities of the Generalitat de Catalunya 2021 SGR 01513. The funders of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or submission of this article for publication.

Authorship contributions: The HDSS in Manhica was set up in 1996 and has received numerous contributions to its design and implementation since then. During the study period, SAJ, RV, JB, TN, JD, AS, SAC, AriN, IM, PA, FS, CS, and QB, among many others, contributed to data collection and/or coordinated the Manhica HDSS. NB and QB conceived the original idea for this study. NB led the data analysis, interpretation of results, and write-up of the manuscript, and received input from all authors. NB, AH, TM, ArsN, and LQ were involved in data management. DO and TM contributed to data analysis. SAJ, RV, LQ, and CG assisted in the study design and clinical data curation. NB, DO, AH, LQ, and TM had access to and verified the underlying data. MM and KM contributed to the socio-behavioural interpretation of study results. All authors read and approved the final manuscript.

Disclosure of interests: The authors completed the ICMJE Disclosure of Interest Form (available upon request from the corresponding author) and disclose no relevant interests.

Additional material

Online Supplementary Document

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SUPPLEMENTARY MATERIAL

Paediatric healthcare in Manhiça district through a gender lens: a retrospective analysis of 17 years of morbidity and demographic surveillance data

I. SUPPLEMENTARY FIGURES

Figure S1. Healthcare facilities covered by the morbidity surveillance of Manhiça HDSS and included in this study

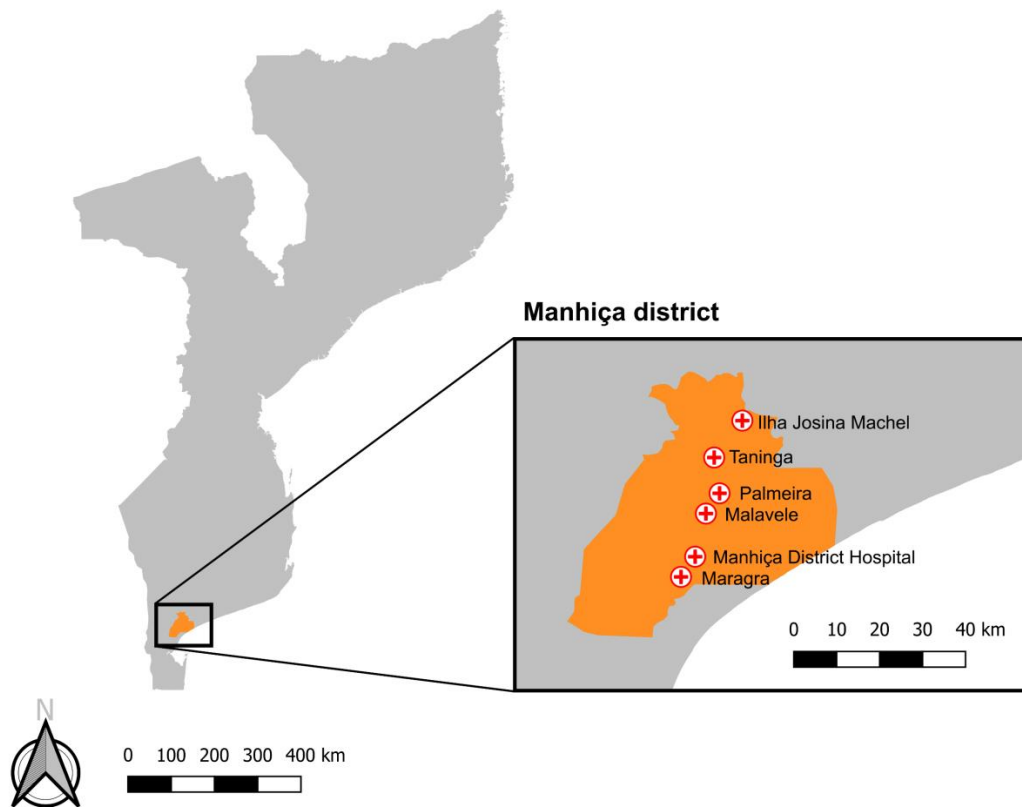
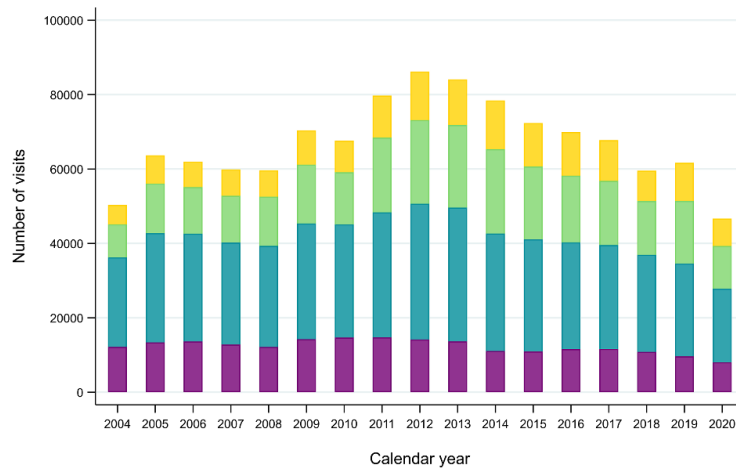
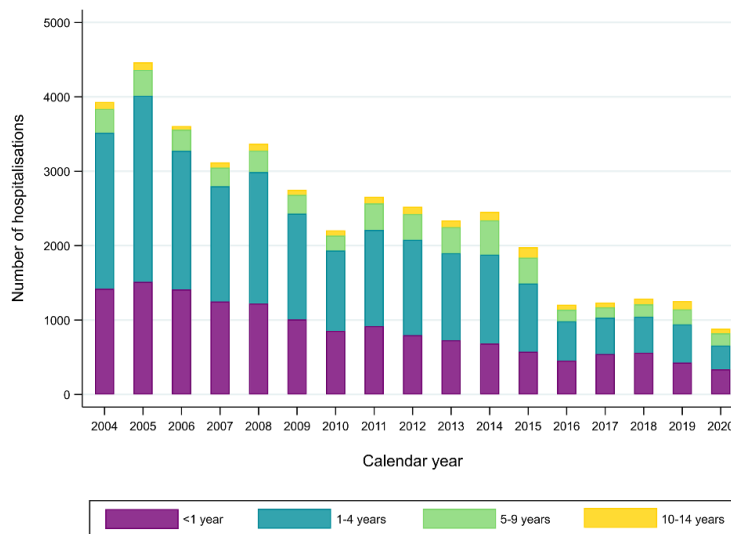


Figure S2. Number of paediatric outpatient clinic visits and hospitalisations from 2004 to 2020 in Manhiça district

A) Visits to six outpatient clinics



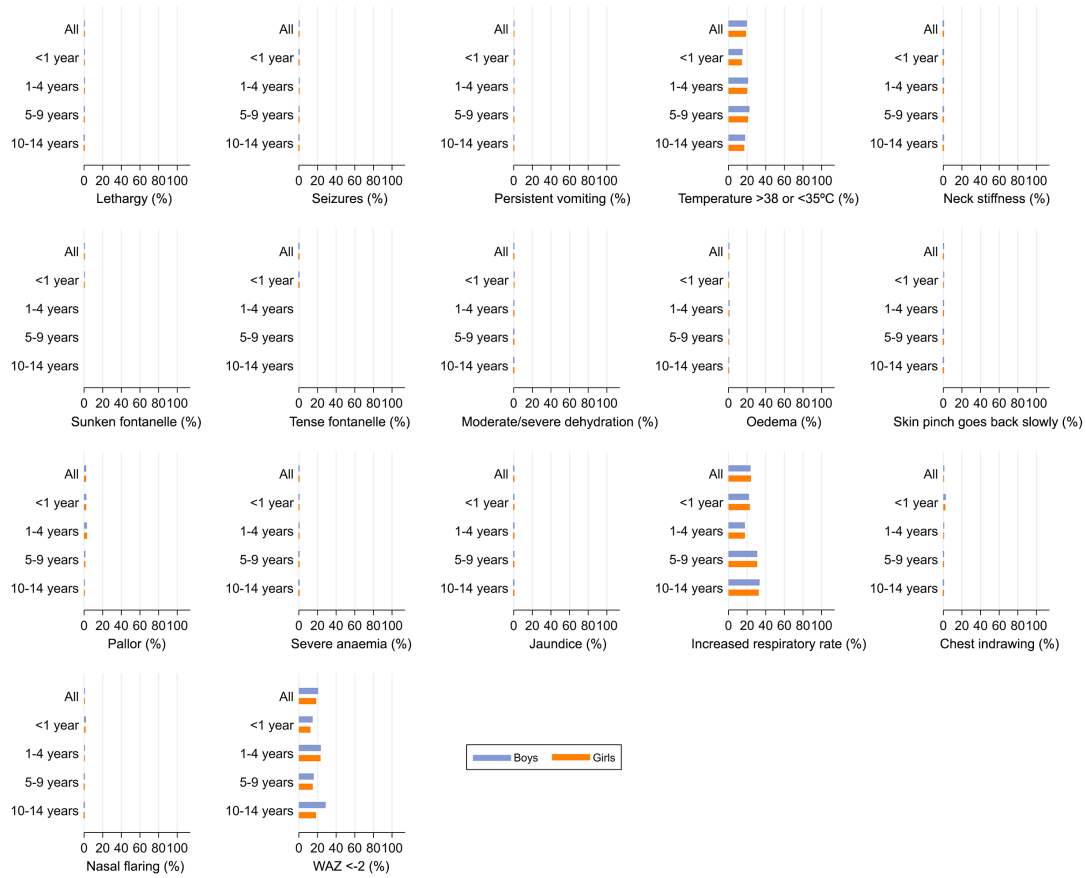
B) Admissions to Manhiça District Hospital



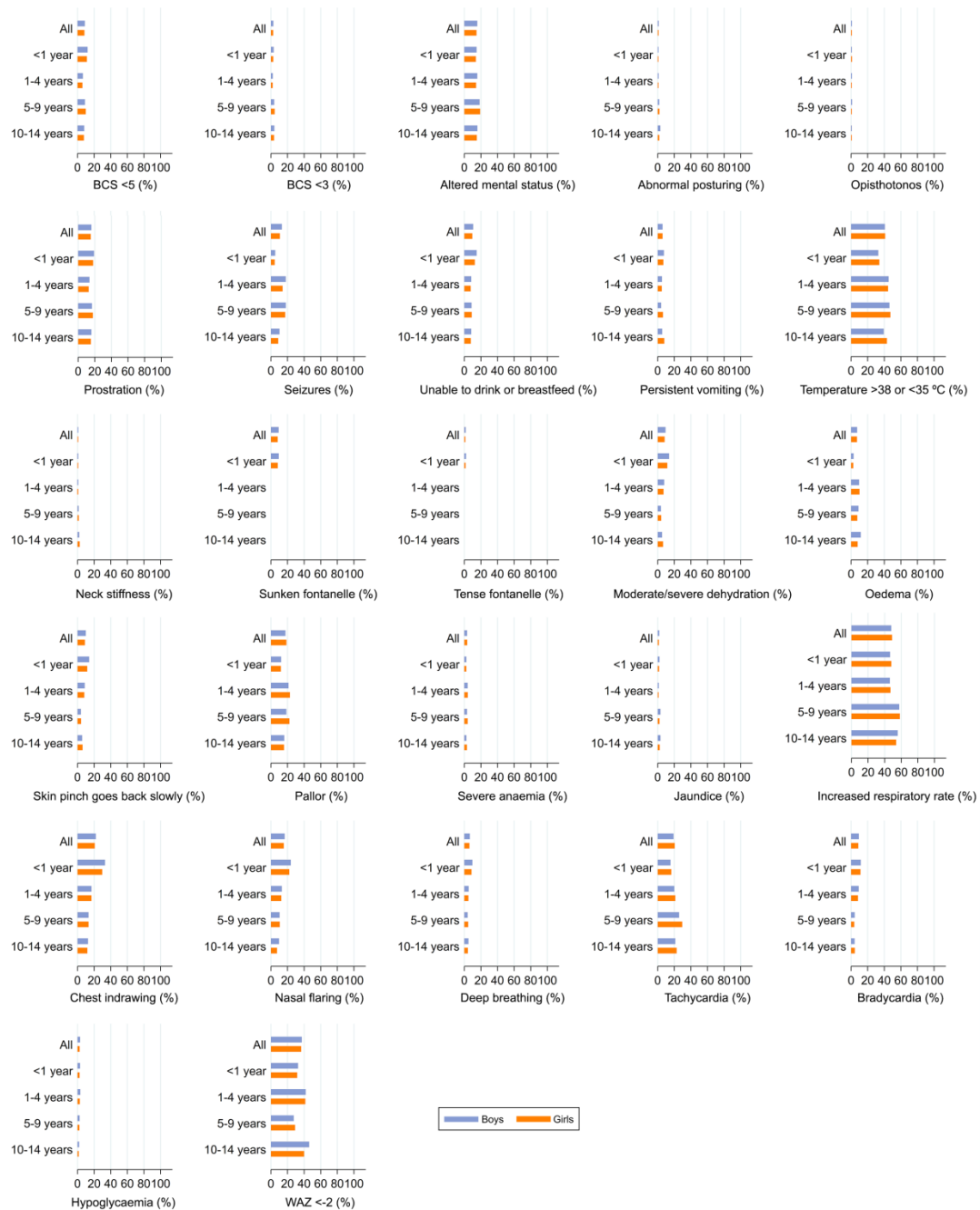
Data on outpatient clinic visits were collected from: Manhiça District Hospital (2004-2020), Maragra (2004-2020), Ilha Josina Machel (2004-2020), Tanninga (2005-2020), Palmeira (2009-2020), and Malavele (2009-2020) outpatient clinics.

Figure S3. Percentage of children with severe manifestations at presentation to an outpatient clinic and upon hospitalisation in Manhica district

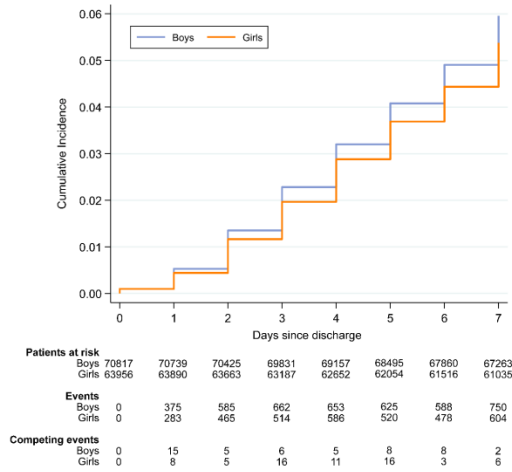
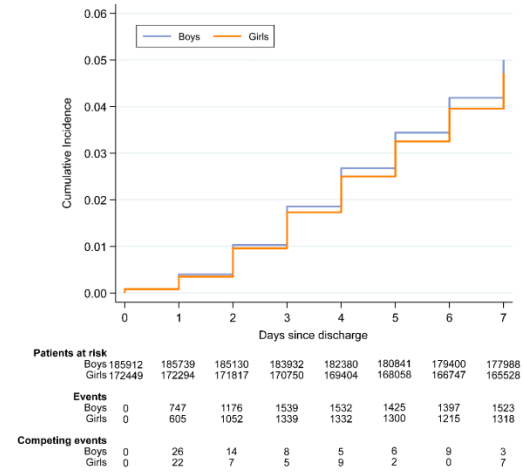
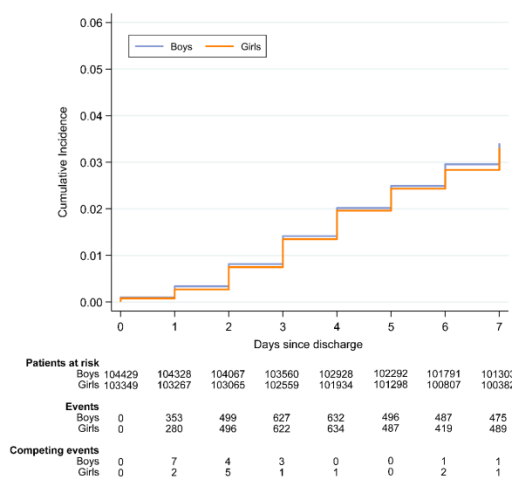
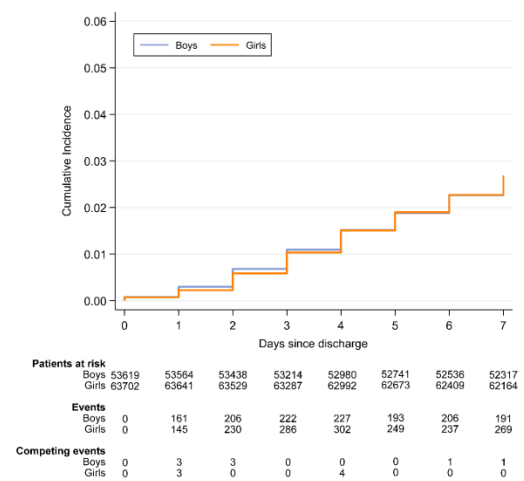
A) Visits to six outpatient clinics



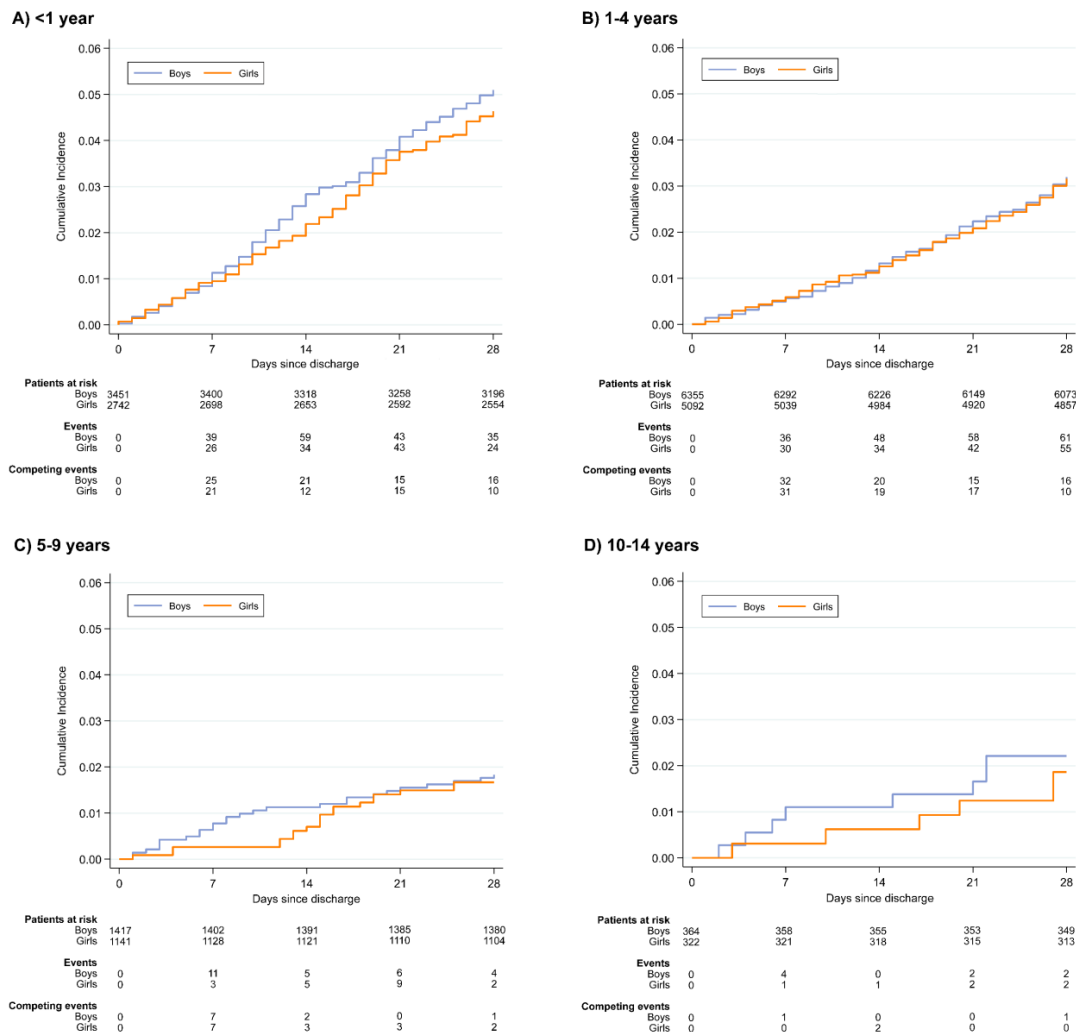
B) Admissions to Manhiça District Hospital



Definitions used for severe manifestations can be found in Table S1. Sunken or tense fontanelle was calculated only in children aged <1 year. Missing data in all variables is <5%, except for sunken/tense fontanelle (5% in outpatient clinic visits, 8% in hospitalisations), severe anaemia (45% in outpatient clinic visits, 9% in hospitalisations), and hypoglycaemia (9% in hospitalisations). Abbreviations: BCS=Blantyre Coma Scale, WAZ=weight-for-age z-score.

Figure S4. Cumulative incidence of outpatient clinic revisits in children in Manhica district**A) <1 year****B) 1-4 years****C) 5-9 years****D) 10-14 years**

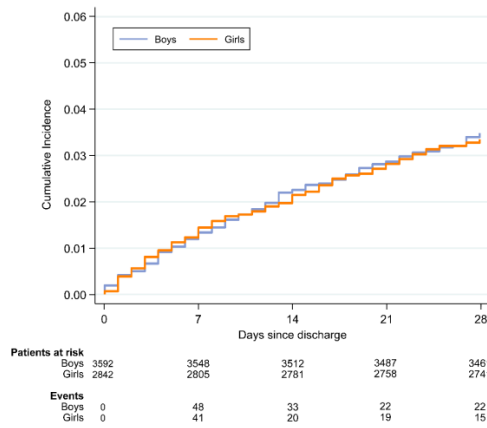
Index visits were defined as those in which children were discharged home or absconded, and death was treated as a competing event.

Figure S5. Cumulative incidence of hospital readmissions in children in Manhica district

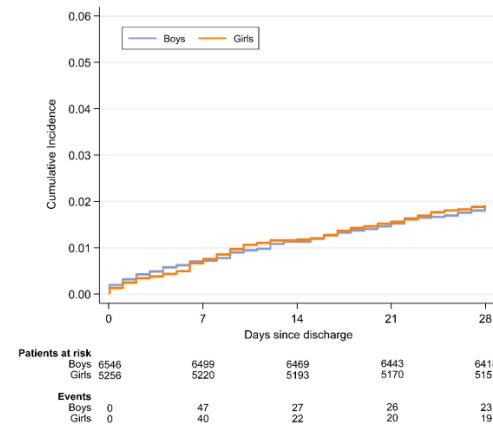
Index hospitalisations were defined as those in which children were discharged home or absconded, and death was treated as a competing event.

Figure S6. Cumulative incidence of hospital post-discharge mortality in children in Manhíça district

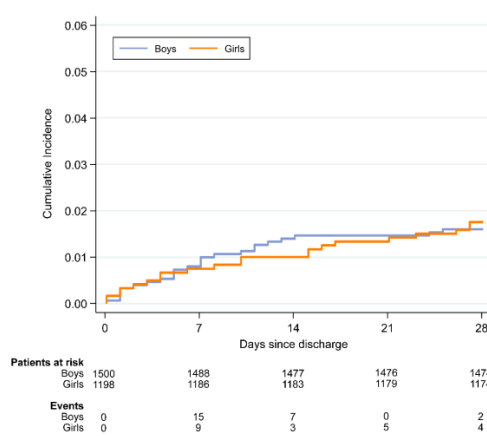
A) <1 year



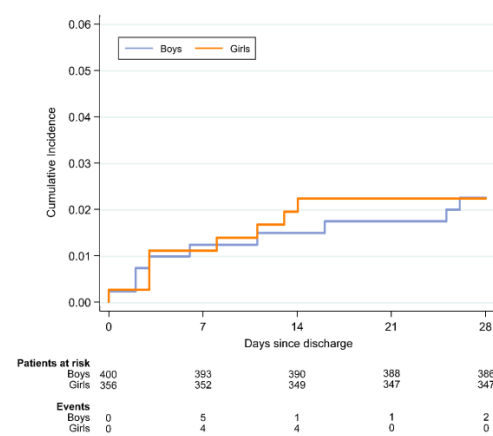
B) 1-4 years



C) 5-9 years

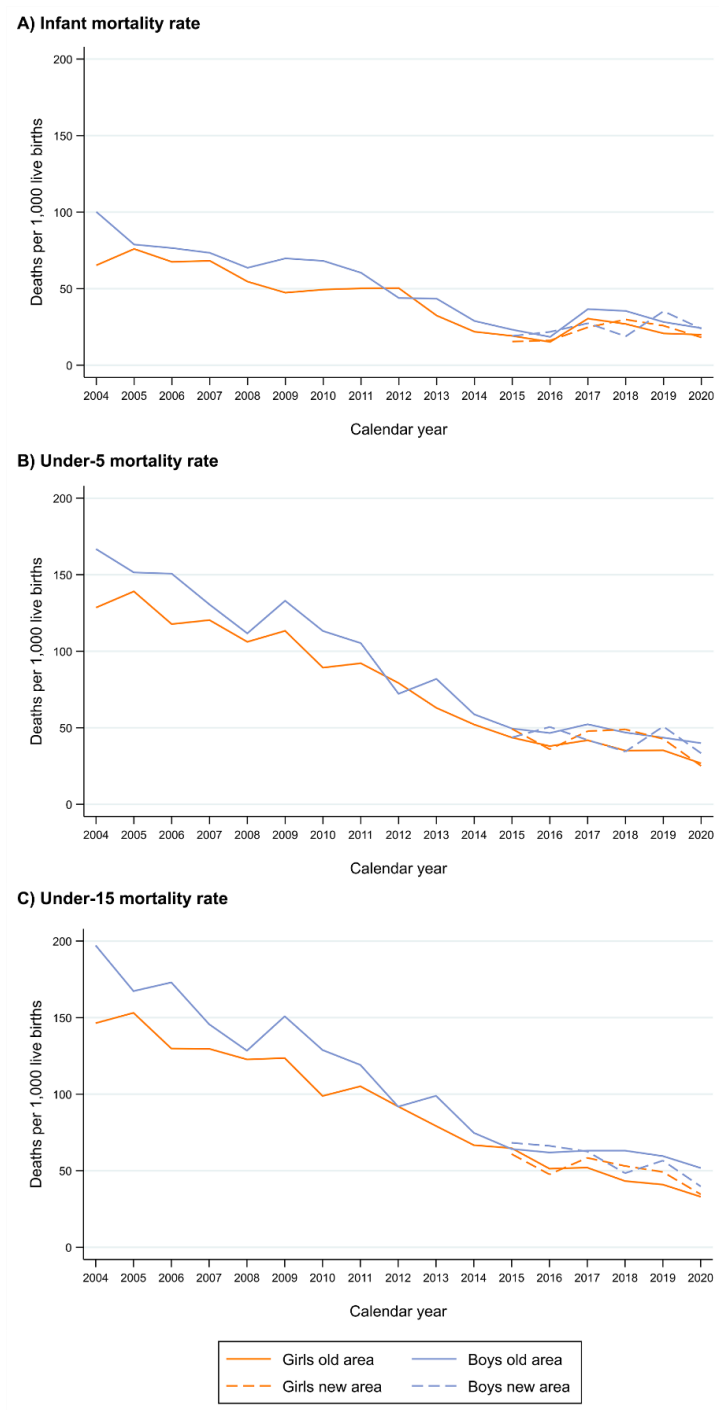


D) 10-14 years



Index hospitalisations were defined as those with a known outcome different from in-hospital death.

Figure S7. Infant, under-5, and under-15 mortality rates in the Manhiça HDSS study area from 2004 to 2020



Mortality rates were computed as the number of deaths (in infants, children younger than five years, or children younger than fifteen years) in each calendar year divided by the total number of live births that occurred in the same year, expressed per thousand live births. Data are shown stratified by old and new Manhiça HDSS demographic surveillance areas.

II. SUPPLEMENTARY TABLES

Table S1. Definitions used for severe manifestations

Severe manifestation	Definition
Persistent vomiting	Reported vomiting for ≥ 2 days.
Prostration	Inability to sit unaided or look for a mother's breast/feed in children who are not yet able to sit.
Severe anaemia	Haematocrit $< 25\%$ in children ≤ 28 days and haematocrit $< 15\%$ in children > 28 days.
Increased respiratory rate	≥ 60 breaths/min in children aged < 2 months, ≥ 50 breaths/min in children aged 2–11 months, ≥ 40 breaths/min in children aged 12–59 months, ≥ 30 breaths/min in children aged 5–12 years, and ≥ 20 breaths/min in children aged 13–14 years.
Tachycardia and bradycardia	Above or under the normal ranges of: 100–180 beats/min in children aged < 1 month, 100–160 beats/min in children aged 1–11 months, 90–150 beats/min in children aged 1–2 years, 80–140 beats/min in children aged 3–5 years, 70–120 beats/min in children aged 6–12 years, and 60–100 beats/min in children aged 13–14 years.
Hypoglycaemia	Glycemia < 2.5 mmol/L.
Weight-for-age z-score	Calculated using the LMS method and the 2000 US Centers for Disease Control and Prevention Growth Reference data (Vidmar <i>et al.</i> , 2013).

Table S2. Minimum community-based incidence rates of outpatient clinic visits and hospitalisations in children living in the Manhiça HDSS study area from 2004 to 2020

	Visits to six outpatient clinics		Admissions to Manhiça District Hospital	
	MCBIR per 1,000 child-years at risk	IRR (95% CI)	MCBIR per 1,000 child-years at risk	IRR (95% CI)
All				
Boys	906.05	1	26.18	1
Girls	878.44	0.97 (0.96–0.98)	21.10	0.81 (0.79–0.84)
<1 year				
Boys	2282.57	1	113.43	1
Girls	2091.86	0.96 (0.95–0.96)	91.57	0.82 (0.78–0.86)
1–4 years				
Boys	1463.20	1	50.22	1
Girls	1364.72	0.96 (0.96–0.97)	41.00	0.83 (0.80–0.87)
5–9 years				
Boys	643.82	1	9.24	1
Girls	632.80	0.97 (0.96–0.99)	7.37	0.80 (0.74–0.86)
10–14 years				
Boys	370.14	1	2.86	1
Girls	439.14	1.09 (1.07–1.11)	2.50	0.85 (0.73–0.98)

IRRs calculated by fitting mixed-effects negative binomial regression models, with individual as a random effect. Abbreviations: CI=confidence interval, IRRs=incidence rate ratios, MCBIR = minimum community-based incidence rate.

Table S3. Girls-to-boys odds ratios of outcomes of paediatric outpatient clinic visits and hospitalisations in Manhiça district

Visits to five outpatient clinics (excluding the one at Manhiça District Hospital):															
	All			<1 year			1-4 years			5-9 years			10-14 years		
	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)
Discharge home	314,877 (97.9%)	310,820 (98.3%)	1	52,791 (96.7%)	48,221 (96.7%)	1	137,294 (97.7%)	128,273 (97.9%)	1	79,716 (98.6%)	80,397 (99.1%)	1	45,076 (99.0%)	53,929 (99.4%)	1
Absconder	844 (0.3%)	825 (0.3%)	0.99 (0.90-1.09)	199 (0.4%)	193 (0.4%)	1.06 (0.87-1.29)	329 (0.2%)	354 (0.3%)	1.15 (0.99-1.34)	182 (0.2%)	153 (0.2%)	0.83 (0.67-1.03)	134 (0.3%)	125 (0.2%)	0.78 (0.61-1.00)
Transfer	5,793 (1.8%)	4,707 (1.5%)	0.82 (0.79-0.86)	1,604 (2.9%)	1,450 (2.9%)	0.99 (0.92-1.06)	2,918 (2.1%)	2,467 (1.9%)	0.90 (0.86-0.96)	933 (1.2%)	592 (0.7%)	0.63 (0.57-0.70)	338 (0.7%)	198 (0.4%)	0.49 (0.41-0.58)
Visits to the outpatient clinic at Manhiça District Hospital:															
	All			<1 year			1-4 years			5-9 years			10-14 years		
	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)
Discharge home	233,701 (91.1%)	224,428 (92.3%)	1	47,435 (85.7%)	42,455 (86.7%)	1	104,651 (90.5%)	96,550 (91.6%)	1	54,218 (95.1%)	53,361 (95.8%)	1	27,397 (96.4%)	32,062 (97.1%)	1
Absconder	1,380 (0.5%)	1,220 (0.5%)	0.92 (0.85-0.99)	402 (0.7%)	331 (0.7%)	0.92 (0.79-1.06)	564 (0.5%)	485 (0.5%)	0.93 (0.83-1.05)	244 (0.4%)	224 (0.4%)	0.93 (0.78-1.12)	170 (0.6%)	180 (0.6%)	0.90 (0.73-1.12)
Transfer	788 (0.3%)	598 (0.3%)	0.79 (0.71-0.88)	221 (0.4%)	169 (0.4%)	0.85 (0.70-1.04)	294 (0.3%)	242 (0.2%)	0.89 (0.75-1.06)	187 (0.3%)	116 (0.2%)	0.63 (0.50-0.79)	86 (0.3%)	71 (0.2%)	0.71 (0.51-0.97)
Admission	20,509 (8.0%)	16,829 (6.9%)	0.85 (0.84-0.87)	7,293 (13.2%)	5,992 (12.2%)	0.92 (0.88-0.95)	10,104 (8.7%)	8,156 (7.7%)	0.87 (0.85-0.90)	2,338 (4.1%)	1,975 (3.6%)	0.86 (0.81-0.91)	774 (2.7%)	706 (2.1%)	0.78 (0.70-0.86)
Death	57 (0.02%)	53 (0.02%)	0.97 (0.67-1.41)	20 (0.04%)	19 (0.04%)	1.06 (0.57-1.99)	20 (0.02%)	26 (0.02%)	1.41 (0.79-2.52)	11 (0.02%)	7 (0.01%)	0.65 (0.25-1.67)	6 (0.02%)	1 (<0.01%)	0.14 (0.02-1.18)
Admissions to Manhiça District Hospital:															
	All			<1 year			1-4 years			5-9 years			10-14 years		
	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)	Boys, N (%)	Girls, N (%)	OR (95% CI)
Discharge home	19,773 (88.0%)	16,214 (87.8%)	1	6,819 (84.9%)	5,573 (84.8%)	1	10,082 (90.1%)	8,174 (89.6%)	1	2,228 (89.9%)	1,869 (89.8%)	1	644 (84.7%)	598 (86.8%)	1
Absconder	856 (3.8%)	756 (4.1%)	1.08 (0.97-1.19)	356 (4.4%)	334 (5.1%)	1.15 (0.98-1.34)	433 (3.9%)	365 (4.0%)	1.04 (0.90-1.20)	49 (2.0%)	46 (2.2%)	1.12 (0.74-1.68)	18 (2.4%)	11 (1.6%)	0.66 (0.31-1.40)
Transfer	998 (4.4%)	786 (4.3%)	0.96 (0.87-1.06)	410 (5.1%)	305 (4.6%)	0.91 (0.78-1.06)	361 (3.2%)	294 (3.2%)	1.00 (0.86-1.17)	147 (5.9%)	118 (5.7%)	0.96 (0.75-1.23)	80 (10.6%)	69 (10.0%)	0.93 (0.66-1.31)
Death	841 (3.7%)	706 (3.8%)	1.02 (0.92-1.13)	452 (5.6%)	358 (5.5%)	0.97 (0.84-1.12)	317 (2.8%)	288 (3.2%)	1.12 (0.95-1.32)	54 (2.2%)	49 (2.4%)	1.08 (0.73-1.60)	18 (2.4%)	11 (1.6%)	0.66 (0.31-1.40)

All girls-to-boys odd ratios were estimated from fitting univariable multinomial logistic regression models. Abbreviations: CI=confidence interval, OR=odds ratio.

Table S4. Length of hospital stay in children admitted to Manhica District Hospital

	All children		Children discharged home	
	N	Median days (IQR)	N	Median days (IQR)
All				
Boys	22,474	3 (2-5)	19,646	3 (2-5)
Girls	18,465	3 (2-5)	16,104	3 (2-5)
<1 year				
Boys	8,022	4 (2-6)	6,764	4 (3-6)
Girls	6,545	4 (2-6)	5,524	4 (3-6)
1-4 years				
Boys	11,198	3 (2-5)	10,033	3 (2-5)
Girls	9,147	3 (2-5)	8,134	3 (2-5)
5-9 years				
Boys	2,491	3 (2-4)	2,210	3 (2-4)
Girls	2,082	3 (2-5)	1,851	3 (2-5)
10-14 years				
Boys	763	3 (2-5)	639	3 (2-5)
Girls	691	3 (2-5)	595	3 (2-5)

All standardized mean differences in length of hospital stay between boys and girls are <0.1. Abbreviations: IQR=interquartile range.

Table S5. Diagnostic tests and treatments in children admitted to Manhica District Hospital

	All		<1 year		1-4 years		5-9 years		10-14 years	
	Boys, (%) (N=22,653)	Girls, (%) (N=18,625)	Boys, (%) (N=8,096)	Girls, (%) (N=6,619)	Boys, (%) (N=11,271)	Girls, (%) (N=9,203)	Boys, (%) (N=2,516)	Girls, (%) (N=2,105)	Boys, (%) (N=770)	Girls, (%) (N=698)
Antibiotics	68.5%	66.8%	82.6%	80.5%	61.5%	60.0%	56.5%	56.1%	63.3%	59.5%
Antimalarials	44.2%	46.4%	26.4%	29.4%	54.2%	55.9%	56.9%	58.2%	44.8%	48.2%
Blood culture	81.9%	81.2%	93.0%	93.3%	80.6%	79.4%	60.7%	60.6%	53.6%	51.3%
Blood smear	96.3%	96.9%	94.4%	95.1%	97.8%	98.1%	96.3%	97.7%	94.1%	94.0%
Blood transfusion	6.6%	7.4%	4.5%	4.9%	8.4%	9.3%	5.9%	8.0%	4.7%	4.9%
Chest X-ray	27.3%	27.0%	30.3%	28.1%	26.8%	27.3%	20.8%	23.4%	24.8%	24.6%
HIV test	24.0%	23.5%	18.7%	17.2%	26.6%	27.0%	28.1%	27.0%	29.3%	26.9%
Lumbar puncture	13.6%	12.3%	14.7%	14.4%	12.9%	10.9%	15.1%	12.9%	9.2%	8.2%

Missing data in all variables is <2%.

Table S6. Time to death in children admitted to Manhiça District Hospital who died in-hospital

	All children	
	N	Median days (IQR)
All		
Boys	838	2 (1-5)
Girls	703	2 (1-5)
<1 year		
Boys	450	2 (1-5)
Girls	356	2 (1-5)
1-4 years		
Boys	316	2 (1-7)
Girls	287	2 (1-6)
5-9 years		
Boys	54	1 (0-3)
Girls	49	1 (0-3)
10-14 years		
Boys	18	0.5 (0-2)
Girls	11	0 (0-5)

All standardized mean differences in length of hospital stay between boys and girls are <0.2. Abbreviations: IQR=interquartile range.

Table S7. Hazard ratios and subdistribution hazard ratios for post-discharge outcomes in children after an outpatient clinic visit and hospitalisation in Manhiça district

	Revisits to outpatient clinics within 7 days, SHR (95% CI)	Hospital readmissions within 28 days, SHR (95% CI)	Hospital post-discharge mortality within 28 days, HR (95% CI)
All			
Boys	1	1	1
Girls	0.93 (0.90-0.95)	0.94 (0.81-1.10)	1.00 (0.84-1.19)
<1 year			
Boys	1	1	1
Girls	0.90 (0.86-0.94)	0.91 (0.72-1.14)	0.96 (0.74-1.25)
1-4 years			
Boys	1	1	1
Girls	0.94 (0.91-0.97)	0.99 (0.80-1.22)	1.02 (0.79-1.33)
5-9 years			
Boys	1	1	1
Girls	0.97 (0.92-1.02)	0.91 (0.50-1.64)	1.10 (0.61-1.96)
10-14 years			
Boys	1	1	1
Girls	1.03 (0.95-1.11)	0.84 (0.29-2.41)	0.99 (0.39-2.56)

For revisits to outpatient clinics and hospital readmissions, index visits or admissions were defined as those in which children were either discharged home or absconded. Fine-Gray competing risk regression models were used to calculate SHRs for revisits to outpatient clinics and hospital readmissions, considering death as a competing event. For hospital post-discharge mortality, index hospitalisations were defined as those with a known outcome different from in-hospital death. Cox proportional hazards regression models were used to calculate HRs for hospital post-discharge mortality. In all analyses, children who migrated outside the Manhiça HDSS study area before the end of the follow-up period were censored at that moment. Standard errors were adjusted for within-child correlation. Abbreviations: CI=confidence interval, HR=hazard ratio, SHR=subdistribution hazard ratio.

ARTICLE



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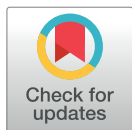
Prognostic accuracy of biomarkers of immune and endothelial activation in Mozambican children hospitalized with pneumonia

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OPEN ACCESS

Citation: Balanza N, Erice C, Ngai M, McDonald CR, Weckman AM, Wright J, et al. (2023) Prognostic accuracy of biomarkers of immune and endothelial activation in Mozambican children hospitalized with pneumonia. *PLOS Glob Public Health* 3(2): e0001553. <https://doi.org/10.1371/journal.pgph.0001553>

Editor: Sadia Shakoor, Aga Khan University, PAKISTAN

Received: November 1, 2022

Accepted: January 24, 2023

Published: February 23, 2023

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Data Availability Statement: De-identified data analyzed as part of this study is available at CORA research data repository through the following link <https://doi.org/10.34810/data595>.

Funding: We acknowledge funding support from the Bill and Melinda Gates Foundation (OPP50092), the Canadian Institutes of Health Research (CIHR) Foundation grant FDN-148439 (KCK), the Canada Research Chair Program (KCK), and donations from Kim Kertland and the Tesari Foundation.

Abstract

Pneumonia is a leading cause of child mortality. However, currently we lack simple, objective, and accurate risk-stratification tools for pediatric pneumonia. Here we test the hypothesis that measuring biomarkers of immune and endothelial activation in children with pneumonia may facilitate the identification of those at risk of death. We recruited children <10 years old fulfilling WHO criteria for pneumonia and admitted to the Manhiça District Hospital (Mozambique) from 2010 to 2014. We measured plasma levels of IL-6, IL-8, Angpt-2, sTREM-1, sFlt-1, sTNFR1, PCT, and CRP at admission, and assessed their prognostic accuracy for in-hospital, 28-day, and 90-day mortality. Healthy community controls, within same age strata and location, were also assessed. All biomarkers were significantly elevated in 472 pneumonia cases versus 80 controls ($p < 0.001$). IL-8, sFlt-1, and sTREM-1 were associated with in-hospital mortality ($p < 0.001$) and showed the best discrimination with AUROCs of 0.877 (95% CI: 0.782 to 0.972), 0.832 (95% CI: 0.729 to 0.935) and 0.822 (95% CI: 0.735 to 0.908), respectively. Their performance was superior to CRP, PCT, oxygen saturation, and clinical severity scores. IL-8, sFlt-1, and sTREM-1 remained good predictors of 28-day and 90-day mortality. These findings suggest that measuring IL-8, sFlt-1, or sTREM-1 at hospital presentation can guide risk-stratification of children with pneumonia, which could enable prioritized care to improve survival and resource allocation.

ISGlobal receives support from the Spanish Ministry of Science and Innovation through the “Centro de Excelencia Severo Ochoa 2019-2023” Program (CEX2018-000806-S), and support from the Generalitat de Catalunya through the CERCA Program. CISM is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID). NB is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260). BB is a Beatriz de Pinos postdoctoral fellow granted by the Government of Catalonia’s Secretariat for Universities and Research, and by Marie Skłodowska-Curie Actions COFUND Program (BP3, 801370). EL-V has received funding from the European Respiratory Society and the European Union’s H2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 847462. CRM, AMW, and JW are supported by CIHR Doctoral Awards. Funding bodies played no role in study design; collection, analysis, or interpretation of the data; writing of the report, or in submission of this article for publication.

Competing interests: I have read the journal’s policy and the authors of this manuscript have the following competing interests: KCK is a named inventor on a patent “Biomarkers for early determination of a critical or life-threatening response to illness and/or treatment response” held by the University Health Network. EL-V is an editorial board member of PLOS Global Public Health. The remaining authors declare no competing interests.

1. Introduction

Pneumonia is a leading cause of illness and death in children. In 2019, pneumonia resulted in an estimated 740,000 deaths in children under the age of five, accounting for nearly 14% of all deaths in this age group [1]. Although childhood pneumonia is a global problem, it disproportionately affects low and middle-income countries.

Identifying children with pneumonia who are at high risk of severe or fatal disease is essential to reduce mortality, as such cases need early and adequate clinical management. Nevertheless, this is challenging in settings without radiographic imaging, basic or inexistent laboratory facilities, and limited qualified medical personnel. The World Health Organization (WHO) has set clinical algorithms for resource-constrained settings to diagnose pneumonia as a clinical syndrome and identify severe cases to guide patient case management [2–4]. Yet, these guidelines have limitations including subjective signs recognition. Clinical signs and laboratory measures have also been combined to generate prognostic scores for children with pneumonia or, more broadly, febrile illness [5–8]. However, these scores have not been well validated nor implemented for childhood pneumonia. New simple, accurate, objective, and inexpensive triage and risk-stratification tools are needed.

Immune and endothelial activation are implicated in the pathogenesis of severe and fatal infections. Previous studies have shown that circulating analytes of these pathways, measured at clinical presentation, have good prognostic accuracy in a range of infections, including pneumonia. They have been associated with mortality and other adverse outcomes, and they may enable improved childhood pneumonia risk-stratification [9–13]. However, only a few studies have assessed some of these biomarkers in children with pneumonia in sub-Saharan Africa [14–21].

Here we evaluated the prognostic accuracy of eight biomarkers for in-hospital and longer-term (28-day and 90-day) mortality in Mozambican children hospitalized with WHO-defined clinical pneumonia versus clinical signs and severity scores. Their biomarker levels were also compared to those in healthy community controls.

2. Methods

2.1 Study site

This study was conducted in Manhiça district, a rural area located in Maputo province, southern Mozambique. Studies conducted in the district of Manhiça have consistently described significant percentages of child hospital admissions and mortality due to pneumonia [22–24], which are exacerbated by suboptimal pneumonia management and referral practices [25]. A full description of the Manhiça population and study area can be found in previous publications [26, 27].

2.2 Study design

This analysis is part of a larger study designed to identify etiological biomarkers in children with respiratory symptoms [28–30]. Between 2010 and 2014, children presenting to the Manhiça District Hospital with symptoms of clinical pneumonia and fulfilling criteria for hospital admission were assessed for recruitment. Children were invited to participate in the study if they were <10 years old, had fever ($\geq 37.5^{\circ}\text{C}$ axillary temperature) or reported a history of fever in the preceding 24 hours, and met the 2005 WHO case definition for clinical pneumonia (cough or/and difficulty breathing plus increased respiratory rate for age) [31]. Exclusion criteria included use of antimalarials in the preceding two weeks, established or suspected tuberculosis (based on history of cough of >2 weeks or close contact with a documented case),

marked hypoxemia ($\text{SpO}_2 \leq 85\%$, as a proxy of *Pneumocystis jirovecii* infection), and participation in conflicting studies. Patients were managed in accordance with Mozambique's national standard of care [22].

Community controls <10 years old and living in the district of Manhica were recruited from 2010 to 2012. The Health and Demographic Surveillance System (HDSS) running in the district allowed the selection of a random sample of children within the same age group and geographic area than study patients. Controls were not considered healthy if they presented with fever, had taken any medication during the preceding 30 days, had a positive malaria microscopy exam, or showed any other important sign of disease. Human immunodeficiency virus (HIV) testing was not performed in this group.

2.3 Clinical, laboratory, and radiological data

Standard clinical questionnaires were completed for all subjects. Upon recruitment and prior to initiating treatment, venous blood and a nasopharyngeal aspirate were collected from pneumonia patients. Briefly, blood was used on-site for hematocrit, full blood count, blood smears for malaria diagnosis, blood culture, and HIV-1 testing. Dried blood spots were collected and used for molecular screening for *Streptococcus pneumoniae* and *Haemophilus influenzae* type B. The nasopharyngeal aspirate was used for PCR-based determination of influenza virus (A, B and C), respiratory syncytial virus (A and B), adenovirus, parainfluenza (1, 2, 3 and 4), coronavirus (229E and OC43), rhinovirus, enterovirus, human metapneumovirus, and bocavirus. Cerebrospinal fluid and pleural effusion were collected and cultured if clinically indicated. The detection of a non-contaminating bacterial organism in cerebrospinal fluid, pleural effusion, and/or blood was defined as an invasive bacterial disease. Pneumonia patients underwent anteroposterior chest radiography and X-rays were interpreted by two experienced clinicians following a WHO-designed protocol [32]. From community controls, venous blood was collected and used for blood smears for malaria diagnosis. Further details on all procedures can be found elsewhere [30].

2.4 Biomarker quantification

A volume of ~2 mL of the venous blood was collected in EDTA coated tubes, centrifuged, and the derived plasma was isolated and stored at -80°C until shipment to University Health Network, Canada. Plasma concentrations of angiopoietin-2 (Angpt-2), interleukin-6 (IL-6), interleukin-8 (IL-8), procalcitonin (PCT), soluble fms-like tyrosine kinase-1 (sFlt-1, also known as soluble vascular endothelial growth factor receptor-1 or sVEGFR-1), soluble tumor necrosis factor receptor 1 (sTNFR1), and soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) were quantified using the multiplex Luminex platform with custom-developed reagents from R&D Systems [33]. Individual Luminex plates included 10% duplicates. C-reactive protein (CRP) was quantified by enzyme-linked immunosorbent assay (ELISA) (R&D DuoSet) and all assays were run in duplicate. Procedures were performed according to manufacturers' instructions and blinded to clinical data. Biomarker concentrations outside the dynamic range were assigned a value of one-third of the lowest limit or the highest limit of the standard curve (S1 Table). If volume was insufficient for both assays, Luminex was prioritized over ELISA.

2.5 Post-discharge mortality data

Post-discharge mortality data were retrieved from the Manhica HDSS. Each person living in the surveillance area is issued a unique permanent identification number, allowing HDSS data to be linked to the study database. HDSS mortality data were obtained through semestral household visits, weekly updates from key community informants, and daily hospital visits [26, 27].

2.6 Data analysis

All statistical analyses and graphs were performed using Stata 16.0 (Stata Corp, College Station, TX, USA). Our primary outcome was in-hospital mortality during initial admission. Secondary outcomes were 28-day and 90-day mortality. Children who were transferred to a higher-level hospital or absconded (i.e., left against medical advice) were excluded when evaluating the primary outcome, and children without information on vital status at 28 or 90 days were excluded when assessing secondary outcomes. A sensitivity analysis was conducted to evaluate 28-day and 90-day mortality excluding children who died during initial hospital admission. Biomarker concentrations in two groups were compared using Mann-Whitney U tests. Areas under the receiver operating characteristic curve (AUROCs) for one parameter were analyzed nonparametrically. Logistic models were used to estimate odds ratios (ORs) and AUROCs for multivariable models. Biomarker data were log-transformed for inclusion in logistic regression models. Models and variables were compared in terms of discrimination, by comparing AUROCs using the algorithm suggested by DeLong *et al.* [34]. Best two-biomarker combination was determined based on highest AUROC, excluding CRP from this analysis. Biomarker correlations were explored using Spearman's rank correlation coefficients. Two-sided p-values <0.05 were considered statistically significant.

Severe pneumonia was defined, according to the WHO 2013 pocket book, as the presence of at least one of the following: oxygen saturation <90% or central cyanosis, severe respiratory distress (i.e., grunting, very severe chest indrawing), or any danger sign (i.e., inability to breast-feed or drink, lethargy or reduced level of consciousness, convulsions) [3]. This WHO severity definition, SpO₂ [35], the Respiratory Index of Severity in Children-Malawi (RISC-Malawi) score [7, 36], and the Lambaréné Organ Dysfunction Score (LODS) [37–39] were pre-specified as important mortality prognostic factors. The RISC-Malawi score takes into account degree of hypoxemia, severity of malnutrition (here using mid-upper arm circumference [MUAC]), sex, presence of wheeze, and level of consciousness; while the LODS considers deep breathing, prostration, and coma. Biomarker cut-offs were derived for in-hospital mortality using the Youden's index method ($J = \max[\text{sensitivity} + \text{specificity} - 1]$) and pre-specified likelihood ratios. A negative likelihood ratio (LR-) of 0.1 and a positive likelihood ratio (LR+) of 10 were used as they indicate large changes from pre-test to post-test probabilities. Lastly, we applied sTREM-1 cut-offs derived using same likelihood ratios for 7-day in-hospital mortality by Lelgudowicz *et al.* [13] in a cohort of Ugandan febrile children. Corresponding sensitivity, specificity, LR-, LR+, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each cut-off.

2.7 Ethical approval

This study was approved by the Mozambique National Bioethics Committee (reference No. CNBS-IRB00002657) and the Ethics Committee at the Hospital Clinic in Barcelona, Spain (reference No. 2010/5590), and performed in accordance with the principles of the Declaration of Helsinki. Parents or guardians provided written informed consent after a detailed explanation of the study. HIV counseling was provided to families of participating children undergoing HIV testing.

3. Results

3.1 Study population characteristics

A total of 576 pneumonia cases were recruited. Of these, 472 had a plasma sample for biomarker measurement and were included in this analysis. In 11 out of 472 pneumonia cases, CRP could not be assessed due to insufficient volume for all assays. Their clinical and

demographic characteristics are presented in [Table 1](#). Half (50.0%; 236/472) were females, median age was 14.1 months (interquartile range [IQR]: 6.1 to 27.1), 17.6% (83/472) had severe malnutrition (weight-for-age z-score (WAZ) < -3 SD), 18.6% (88/472) were HIV positive, 18.6% (88/472) had malaria, 13.1% (62/472) had an invasive bacterial disease, 65.1% (298/458) had a viral respiratory infection, and 37.4% (151/404) had a normal chest X-ray. A total of 15 (3.2%) died during hospital stay, 20 (4.2%) absconded, 20 (4.2%) were transferred to another hospital, and 417 (88.3%) were discharged alive.

Of 117 community controls examined, 80 fulfilled the definition of healthy community control and had a plasma sample for biomarker measurement. Of these, 56.3% (45/80) were female and median age was 39.0 months (IQR: 24.7 to 55.8) ([Table 1](#)).

3.2 Biomarkers as indicators of disease and severity

All biomarker concentrations were significantly higher in pneumonia cases versus healthy community controls ($p < 0.001$). Equivalent results were observed when comparing pneumonia cases discharged alive with healthy community controls ([Fig 1](#)).

Forty-six percent of pneumonia cases fulfilled the WHO definition of severe pneumonia. IL-8 was the only biomarker significantly increased in severe cases compared to non-severe cases ($p = 0.046$) ([S2 Table](#)).

3.3 Biomarkers as indicators of in-hospital death

There were 15 in-hospital deaths in children with pneumonia during initial hospital admission. These occurred between the day of admission and 16 days afterwards, with 40.0% (6/15) happening within the first 48 hours of hospitalization. IL-8, sFlt-1, and sTREM-1 were higher in in-hospital deaths compared to children discharged alive ($p < 0.001$), as well as sTNFR1 ($p = 0.002$) ([Fig 1](#)). The odds of in-hospital mortality increased significantly for a two-fold increase in IL-8, sFlt-1, sTREM-1, or sTNFR1 concentration ([Table 2](#)).

IL-8 had the best discriminatory capacity for in-hospital deaths, with an AUROC of 0.877 (95% confidence interval [CI]: 0.782 to 0.972) ([Fig 2A and 2B](#)). However, IL-8 was not significantly better than the next two most discriminative biomarkers, sFlt-1 and sTREM-1, with AUROCs of 0.832 (95% CI: 0.729 to 0.935) and 0.822 (95% CI: 0.735 to 0.908), respectively. These three biomarkers individually were significantly superior to CRP and PCT ([S3 Table](#)). IL-8 combined with sTREM-1 was the best two-biomarker combination (AUROC: 0.904 [95% CI: 0.820 to 0.988]), which performed slightly better than IL-8 alone ($p = 0.024$). Correlations between biomarkers can be found in [S1 Fig](#).

3.4 Biomarkers as indicators of 28-day and 90-day mortality

HDSS data provided long-term vital status information for 65.5% (309/472) of pneumonia cases. There were 22 deaths within 28 days of hospital admission, and 28 deaths within 90 days. These correspond to the 15 children dying during the initial hospital admission and 7 or 13 children dying after hospital discharge. Deaths happening after hospital discharge were from children either discharged alive (10/13) or transferred to another hospital (3/13). As per HDSS data, 5/13 post-discharge deaths occurred at home, 3/13 in a health facility, and 5/13 in an unspecified location.

IL-8, sFlt-1, sTREM-1 ($p < 0.001$) and sTNFR1 ($p = 0.004$) were significantly elevated in children who subsequently died at 28 days. IL-8, sFlt-1, and sTREM-1 remained most significantly elevated in those dying at 90 days ($p < 0.001$), as was sTNFR1 ($p = 0.011$) ([S4 Table](#)). ORs are provided in [Table 2](#). IL-8, sFlt-1, and sTREM-1 were the best-performing biomarkers for discriminating deaths at 28 or 90 days, with AUROCs ranging from 0.742 (95% CI: 0.645

Table 1. Demographic and clinical characteristics of the study population.

Variable ^a	Healthy community controls (n = 80)	All pneumonia cases (n = 472)	Pneumonia cases			
			Discharged alive (n = 417)	In-hospital deaths (n = 15)	Transferred (n = 20)	Absconders (n = 20)
Female sex	45 (56.3)	236 (50.0)	205 (49.2)	6 (40.0)	14 (70.0)	11 (55.0)
Age						
<2 months	1 (1.3)	27 (5.7)	22 (5.3)	1 (6.7)	2 (10.0)	2 (10.0)
2 to 11 months	8 (10.0)	178 (37.7)	155 (37.2)	7 (46.7)	7 (35.0)	9 (45.0)
12 to 59 months	59 (73.8)	231 (48.9)	209 (50.1)	5 (33.3)	8 (40.0)	9 (45.0)
5 to <10 years	12 (15.0)	36 (7.6)	31 (7.4)	2 (13.3)	3 (15.0)	0 (0)
Median (months)	39.0 [24.7, 55.8]	14.1 [6.1, 27.1]	14.9 [6.4, 27.7]	10.5 [3.0, 24.1]	13.8 [5.4, 19.8]	7.9 [4.3, 20.4]
WAZ ^b						
>-1	46/68 (67.6)	176 (37.3)	159 (38.1)	1 (6.7)	6 (30.0)	10 (50.0)
-1 to -3	22/68 (32.4)	213 (45.1)	192 (46.0)	6 (40.0)	9 (45.0)	6 (30.0)
<-3	0/68 (0)	83 (17.6)	66 (15.8)	8 (53.3)	5 (25.0)	4 (20.0)
Median	-0.6 [-1.3, 0.0], n = 68	-1.5 [-2.7, -0.5]	-1.5 [-2.5, -0.4]	-3.4 [-4.2, -1.6]	-1.7 [-3.2, -0.8]	-1.0 [-2.8, -0.4]
MUAC (cm)	15.0 [14.0, 16.0], n = 68	14.0 [13.0, 15.0], n = 464	14.0 [13.0, 15.0], n = 409	12.0 [10.0, 13.0]	13.0 [11.5, 15.0]	13.0 [12.0, 14.5]
HIV positive status	-	88 (18.6)	72 (17.3)	7 (46.7)	6 (30.0)	3 (15.0)
Malaria parasitemia	-	88 (18.6)	81 (19.4)	1 (6.7)	3 (15.0)	3 (15.0)
Invasive bacterial disease ^c	-	62 (13.1)	45 (10.8)	7 (46.7)	6 (30.0)	4 (20.0)
Viral respiratory infection ^d	-	298/458 (65.1)	269/405 (66.4)	7/14 (50.0)	10 (50.0)	12/19 (63.2)
Axillary temperature	-	38.1 [37.1, 39.1]	38.1 [37.1, 39.1]	37.6 [36.5, 38.7]	37.9 [37.2, 39.0]	37.6 [37.0, 38.4]
Fever ($\geq 37.5^{\circ}\text{C}$, axillary temperature)	-	323 (68.4)	290 (69.5)	8 (53.3)	14 (70.0)	11 (55.0)
SpO ₂ (%)	-	97 [96, 98], n = 448	97 [96, 98], n = 397	97 [91, 97]	96 [94, 97], n = 17	97 [96, 98], n = 19
Hypoxemia (SpO ₂ <90%)	-	11/448 (2.5)	8/397 (2.0)	1 (6.7)	1/17 (5.9)	1/19 (5.3)
Cyanosis	-	16 (3.4)	14 (3.4)	1 (6.7)	1 (5.0)	0 (0)
Respiratory rate	-	56 [50, 63]	56 [49, 62]	54 [44, 60]	58 [49, 69]	58.0 [52, 66]
Lower chest wall indrawing	-	315 (66.7)	278 (66.7)	11 (73.3)	12 (60.0)	14 (70.0)
Nasal flaring	-	226 (47.9)	198 (47.5)	7 (46.7)	8 (40.0)	13 (65.0)
Deep breathing	-	54 (11.4)	44 (10.6)	3 (20.0)	2 (10.0)	5 (25.0)
Grunting	-	54 (11.4)	45 (10.8)	3 (20.0)	3 (15.0)	3 (15.0)
Wheezing	-	105 (22.3)	99 (23.7)	2 (13.3)	0 (0)	4 (20.0)
Crackles	-	261 (55.3)	232 (55.6)	10 (66.7)	7 (35.0)	12 (60.0)
Ronchi	-	162 (34.3)	147 (35.3)	4 (26.7)	3 (15.0)	8 (40.0)
Inspiratory stridor	-	20 (4.2)	18 (4.3)	0 (0)	0 (0)	2 (10.0)
Altered consciousness (BCS<5)	-	18 (3.8)	15 (3.6)	1 (6.7)	1 (5.0)	1 (5.0)
Coma (BCS<3)	-	3 (0.6)	1 (0.24)	0 (0)	1 (5.0)	1 (5.0)
Prostration	-	44 (9.3)	35 (8.4)	2 (13.3)	2 (10.0)	5 (25.0)
Convulsions	-	26/462 (5.6)	24/408 (5.9)	1 (6.7)	1 (5.0)	0/19 (0)
Unable to drink/breastfeed	-	22/461 (4.8)	18/407 (4.4)	2 (13.3)	1 (5.0)	1/19 (5.3)
Dehydration	-	29/462 (6.3)	22/408 (5.4)	5 (33.3)	0 (0)	2/19 (10.5)
Radiological findings						
Normal	-	151/404 (37.4)	136/367 (37.1)	2/8 (25.0)	5/13 (38.5)	8/16 (50.0)
Other infiltrates	-	89/404 (22.0)	83/367 (22.6)	2/8 (25.0)	2/13 (15.4)	2/16 (12.5)
Endpoint pneumonia	-	164/404 (40.6)	148/367 (40.3)	4/8 (50.0)	6/13 (46.2)	6/16 (37.5)
Glucose (mmol/L)	-	6.0 [5.3, 7.0], n = 424	6.1 [5.3, 7.0], n = 377	5.9 [4.3, 7.7], n = 13	5.9 [5.4, 6.0], n = 18	5.8 [5.4, 7.0], n = 16

(Continued)

Table 1. (Continued)

Hematocrit (%)	-	27.4 [22.9, 31.1], n = 470	27.5 [23.1, 31.3], n = 415	24.3 [22.9, 26.8]	24.4 [19.6, 30.2]	28.6 [23.2, 31.2]
WBC count (10 ⁹ /L)	-	14.3 [10.1, 20.4], n = 448	14.2 [10.1, 20.4], n = 394	14.6 [12.2, 21.1]	11.8 [9.7, 17.1]	15.4 [12.9, 22.0], n = 19
WHO severity definition	-	220 (46.6)	193 (46.3)	10 (66.7)	7 (35.0)	10 (50.0)
LODS	-	0 [0, 0]	0 [0, 0]	0 [0, 1]	0 [0, 0]	0 [0, 1]
RISC-Malawi score	-	1 [0, 4], n = 441	1 [0, 4], n = 390	5 [2, 8]	4 [1, 8], n = 17	3 [1, 8], n = 19
Length of hospital stay (days)	-	3.9 [2.8, 5.8]	3.9 [2.9, 5.4]	3.1 [0.9, 6.8]	2.5 [1.1, 6.4]	4.5 [2.5, 5.9]
Antibiotic therapy	-	415/460 (90.2)	369/408 (90.4)	13/14 (92.9)	17 (85.0)	16/18 (88.9)
Antimalarial therapy	-	108/459 (23.5)	97/407 (23.8)	2/14 (14.3)	4 (20.0)	5/18 (27.8)
Previous admissions for pneumonia	-	16 (3.4)	12 (2.9)	3 (20.0)	0 (0)	1 (5.0)

^a Data presented as median [interquartile range] or frequency (percent) as appropriate. Number of subjects indicated if presence of missing values.

^b Calculated using the LMS method and the 2000 US CDC Growth Reference data [40].

^c Defined as the detection of a non-contaminating bacterial organism in blood, cerebrospinal fluid, and/or pleural fluid through culture or PCR analysis of dried blood spots for *Streptococcus pneumoniae* and *Haemophilus influenzae* type B.

^d At least one virus detected in nasopharyngeal aspirate. Virus targeted included: adenovirus, bocavirus, coronavirus (229E and OC43), enterovirus, influenza virus (A, B, and C), metapneumovirus, parainfluenza virus (1, 2, 3, and 4), respiratory syncytial virus (A and B), and rhinovirus.

Abbreviations: BCS (Blantyre Coma Scale), HIV (human immunodeficiency virus), LODS (Lambaréné Organ Dysfunction Score), MUAC (mid-upper arm circumference), RISC-Malawi (Respiratory Index of Severity in Children-Malawi), WAZ (weight-for-age z-score), WBC (white blood cell), WHO (World Health Organization).

<https://doi.org/10.1371/journal.pgph.0001553.t001>

to 0.839) to 0.812 (95% CI: 0.716 to 0.908) (Fig 2C, S3 Table). Best two-biomarker combination for 28-day mortality was IL-8 and sFlt-1 (AUROC: 0.847 [95% CI: 0.770 to 0.924]), while for 90-day mortality was IL-8 and sTREM-1 (AUROC: 0.828 [95% CI: 0.742 to 0.914]). However, two-biomarker models did not outperform models with only IL-8 ($p \geq 0.05$).

The sensitivity analysis evaluating secondary outcomes only for children not dying during initial hospital admission showed similar results. Best AUROCs for 28-day mortality also were from sFlt-1 (0.744 [95% CI: 0.523 to 0.965]), sTREM-1 (0.686 [95% CI: 0.447 to 0.925]), and IL-8 (0.659 [95% CI: 0.473 to 0.845]); and for 90-day mortality the best ones were from IL-8 (0.700 [95% CI: 0.584 to 0.816]), sTREM-1 (0.672 [95% CI: 0.516 to 0.833]), and sFlt-1 (0.628 [95% CI: 0.478 to 0.777]) (S5 Table).

3.5 Biomarkers compared to clinical parameters to predict in-hospital death

Of the four clinical variables selected *a priori*, the RISC-Malawi score (OR: 1.38 [95% CI: 1.18 to 1.61], $p < 0.001$) and SpO₂ (OR: 1.19 [95% CI: 1.02 to 1.41] per unit decreased, $p = 0.049$) were associated with in-hospital mortality in univariable analysis (S6 Table). IL-8, sFlt-1, and sTREM-1 ORs for in-hospital mortality remained similar when adjusting for one of the pre-specified clinical variables at a time.

IL-8 performed significantly better in discriminating in-hospital mortality compared to the WHO severity definition, LODS, and SpO₂ ($p < 0.001$) (Fig 2D). sFlt-1 and sTREM-1 also performed significantly better than these three clinical parameters ($p < 0.009$). IL-8, sFlt-1, and sTREM-1 were better than the RISC-Malawi score but differences were non-significant ($p = 0.087$, $p = 0.466$, and $p = 0.429$, respectively). When adding IL-8, sFlt-1, or sTREM-1 to a model with one of these clinical parameters, AUROCs improved on all occasions (non-

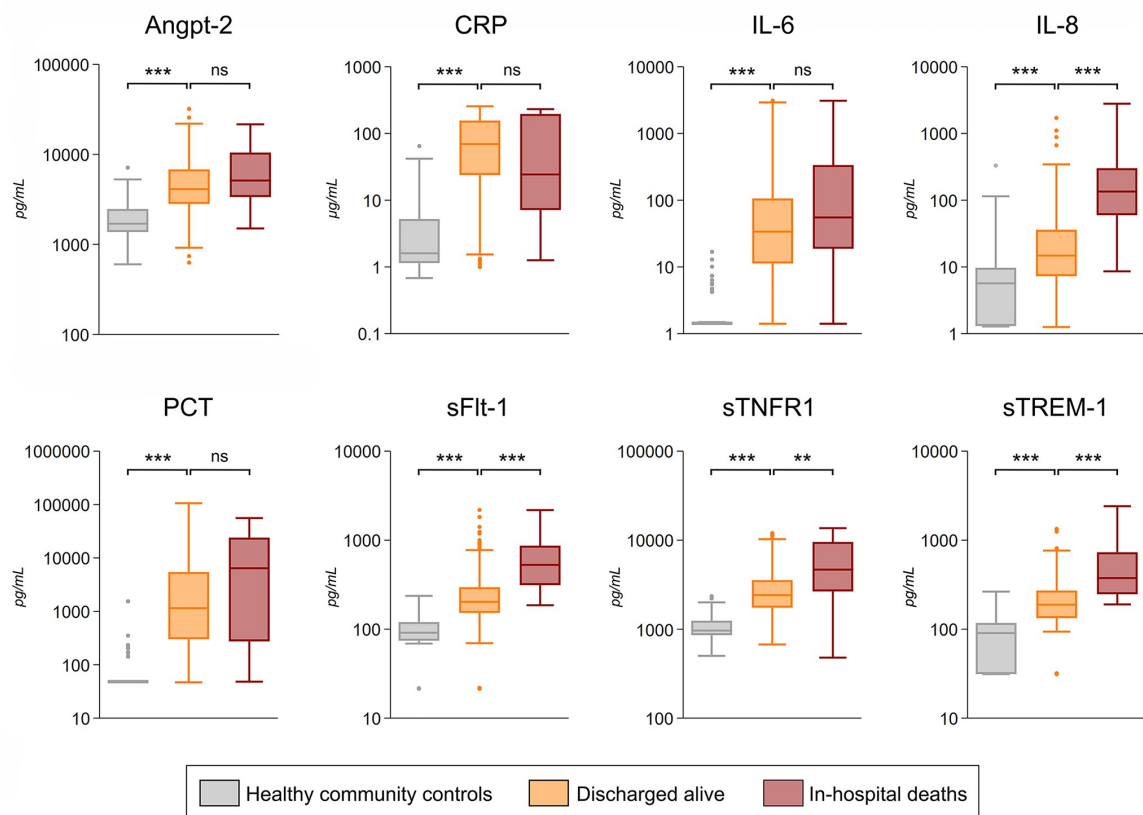


Fig 1. Plasma concentrations of immune and endothelial activation biomarkers at study enrolment in healthy community controls, pneumonia cases discharged alive, and pneumonia cases who died during hospital stay. Boxplots with median and interquartile range. Whiskers represent one and a half times the interquartile range, and outliers are plotted as individual points. Concentrations are in pg/mL, except CRP in µg/mL. p-values were computed using the Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns: non-significant. Abbreviations: Angpt-2 (angiopoietin-2), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

<https://doi.org/10.1371/journal.pgph.0001553.g001>

significantly for the addition of sTREM-1 to RISC-Malawi score) (Table 3). Of note, models with one of these three biomarkers alone performed as well as models combining the same biomarker with SpO_2 or a severity score/definition ($p > 0.05$).

3.6 Using biomarkers for risk-stratification

Cut-offs for in-hospital mortality maximizing sensitivity and specificity (Youden's index method) are reported in S7 Table. IL-8 was the biomarker with a cut-off associated with best performance estimators, as a 59.6 pg/mL threshold gave a sensitivity of 80.0 (95% CI: 51.9 to 95.7) and a specificity of 86.8 (95% CI: 83.2 to 89.9).

We also used a pre-specified LR+ of 10 and a LR- of 0.1 to define cut-offs and place children with pneumonia into low, intermediate and high-risk groups. We could only obtain these cut-offs for IL-8, sFlt-1, and sTREM-1, as other biomarkers were not good enough. Table 4 shows

Table 2. Associations of biomarkers with primary and secondary outcomes using univariable logistic regression.

Biomarker	In-hospital mortality		28-day mortality ^a		90-day mortality ^a	
	OR (95% CI) ^b	p-value	OR (95% CI) ^b	p-value	OR (95% CI) ^b	p-value
Angpt-2	1.51 (0.88, 2.62)	0.137	1.60 (1.01, 2.55)	0.047	1.48 (0.97, 2.23)	0.066
CRP	0.82 (0.64, 1.04)	0.102	0.82 (0.68, 1.01)	0.059	0.79 (0.66, 0.95)	0.010
IL-6	1.15 (0.94, 1.40)	0.189	1.12 (0.94, 1.32)	0.209	1.06 (0.91, 1.24)	0.428
IL-8	2.05 (1.56, 2.69)	<0.001	1.86 (1.47, 2.35)	<0.001	1.76 (1.42, 2.17)	<0.001
PCT	1.11 (0.94, 1.32)	0.229	1.07 (0.93, 1.23)	0.337	1.02 (0.90, 1.15)	0.785
sFlt-1	3.55 (2.13, 5.91)	<0.001	3.40 (2.12, 5.45)	<0.001	2.62 (1.73, 3.96)	<0.001
sTNFR1	3.11 (1.68, 5.73)	<0.001	2.52 (1.50, 4.22)	<0.001	2.03 (1.28, 3.22)	0.003
sTREM-1	3.66 (2.10, 6.37)	<0.001	2.88 (1.77, 4.67)	<0.001	2.63 (1.69, 4.08)	<0.001

^a Combining both in-hospital and post-discharge deaths after initial admission.

^b ORs, 95% CIs, and p-values are from univariable logistic regression models. ORs presented indicate the increase in odds of the outcome for a two-fold increase in each biomarker concentration.

Abbreviations: Angpt-2 (angiopoietin-2), CI (confidence interval), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), OR (odds ratio), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

<https://doi.org/10.1371/journal.pgph.0001553.t002>

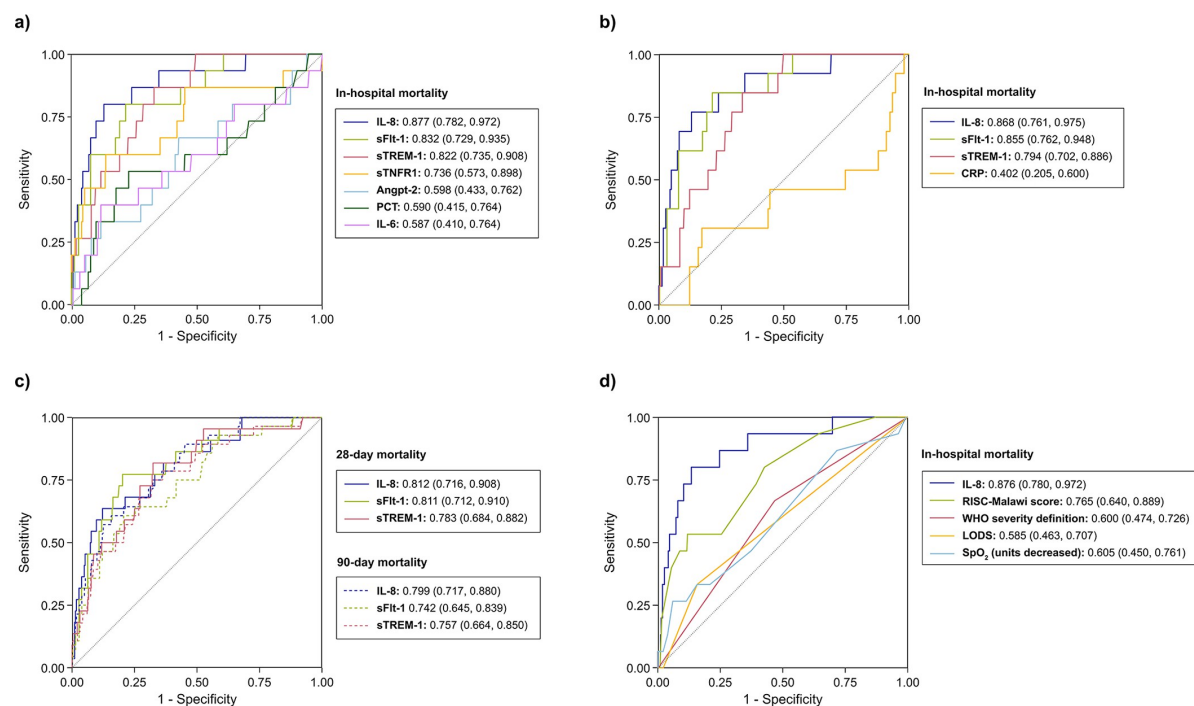


Fig 2. AUROCs of immune and endothelial activation biomarkers and clinical parameters. (A) IL-8, sFlt-1, sTREM-1, sTNFR1, Angpt-2, PCT, and IL-6 AUROCs for in-hospital deaths ($n = 432$). (B) IL-8, sFlt-1, sTREM-1, and CRP AUROCs for in-hospital deaths ($n = 421$). (C) IL-8, sFlt-1, and sTREM-1 AUROCs for 28-day and 90-day mortality (in-hospital & post-discharge) ($n = 309$). (D) IL-8 and clinical parameters AUROCs for in-hospital deaths ($n = 405$). Abbreviations: Angpt-2 (angiopoietin-2), AUROC (area under the receiver operating characteristic curve), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), LODS (Lambaréné Organ Dysfunction Score), PCT (procalcitonin), RISC-Malawi (Respiratory Index of Severity in Children-Malawi), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1), WHO (World Health Organization).

<https://doi.org/10.1371/journal.pgph.0001553.g002>

Table 3. AUROCs to discriminate between children discharged alive and in-hospital deaths for clinical variables alone or in combination with best-performing biomarkers.

Variables (n = 405)	AUROC (95% CI)	+ IL-8, AUROC (95% CI)	p-value ^a	+ sFlt-1, AUROC (95% CI)	p-value ^a	+ sTREM-1, AUROC (95% CI)	p-value ^a
RISC-Malawi score	0.765 (0.640, 0.889)	0.886 (0.790, 0.982)	0.023	0.896 (0.830, 0.962)	0.019	0.845 (0.754, 0.936)	0.062
SpO ₂ (units decreased)	0.605 (0.450, 0.761)	0.861 (0.754, 0.968)	<0.001	0.829 (0.721, 0.937)	<0.001	0.817 (0.723, 0.911)	0.004
WHO severity definition	0.600 (0.474, 0.726)	0.875 (0.774, 0.976)	<0.001	0.826 (0.716, 0.936)	<0.001	0.832 (0.731, 0.932)	<0.001
LODS	0.585 (0.463, 0.707)	0.876 (0.780, 0.972)	<0.001	0.836 (0.740, 0.932)	<0.001	0.816 (0.722, 0.909)	<0.001
IL-8	0.876 (0.780, 0.972)						
sFlt-1	0.830 (0.727, 0.933)						
sTREM-1	0.817 (0.730, 0.904)						

^a AUROCs comparison of the models with a clinical parameter alone versus the models with the same clinical parameter combined with either IL-8, sFlt-1, or sTREM-1. p-values were computed using the algorithm suggested by DeLong *et al.* [34].

Abbreviations: AUROC (area under the receiver operating characteristic curve), CI (confidence interval), IL-8 (interleukin-8), LODS (Lambaréné Organ Dysfunction Score), RISC-Malawi (Respiratory Index of Severity in Children-Malawi), sFlt-1 (soluble fms-like tyrosine kinase-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1), WHO (World Health Organization).

<https://doi.org/10.1371/journal.pgph.0001553.t003>

Table 4. Biomarker cut-offs for in-hospital mortality and performance estimators.

Biomarker	Cut-off (pg/mL)	LR+(95% CI)	LR-(95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	In-hospital deaths within each risk group		
								Low-risk (%)	Intermediate-risk (%)	High-risk (%)
IL-8 ^a	<25.4	2.67 (2.21, 3.22)	0.10 (0.02, 0.68)	93.3 (68.1, 99.8)	65.0 (60.2, 69.6)	8.8 (4.9, 14.2)	99.6 (98.0, 100.0)	1/272 (0.4)	6/130 (4.6)	8/30 (26.7)
	≥124.5	10.11 (5.42, 18.87)	0.49 (0.29, 0.85)	53.3 (26.6, 78.7)	94.7 (92.1, 96.7)	26.7 (12.3, 45.9)	98.3 (96.4, 99.3)			
sFlt-1 ^a	<197.7	1.75 (1.48, 2.05)	0.14 (0.02, 0.95)	93.3 (68.1, 99.8)	46.5 (41.7, 51.4)	5.9 (3.3, 9.7)	99.5 (97.2, 100.0)	1/195 (0.5)	8/215 (3.7)	6/22 (27.3)
	≥745.2	10.43 (4.76, 22.84)	0.62 (0.41, 0.94)	40.0 (16.3, 67.7)	96.2 (93.8, 97.8)	27.3 (10.7, 50.2)	97.8 (95.9, 99.0)			
sTREM-1 ^a	<197.6	1.97 (1.66, 2.33)	0.13 (0.02, 0.85)	93.3 (68.1, 99.8)	52.5 (47.6, 57.4)	6.6 (3.7, 10.8)	99.5 (97.5, 100.0)	1/220 (0.5)	10/197 (5.1)	4/15 (26.7)
	≥670.8	10.11 (3.64, 28.09)	0.75 (0.55, 1.02)	26.7 (7.8, 55.1)	97.4 (95.3, 98.7)	26.7 (7.8, 55.1)	97.4 (95.3, 98.7)			
sTREM-1 ^b	<239.0	2.57 (1.92, 3.43)	0.29 (0.11, 0.80)	80.0 (51.9, 95.7)	68.8 (64.1, 73.2)	8.5 (4.4, 14.3)	99.0 (97.0, 99.8)	3/290 (1.0)	8/125 (6.4)	4/17 (23.5)
	≥629.0	8.55 (3.16, 23.14)	0.76 (0.56, 1.03)	26.7 (7.8, 55.1)	96.9 (94.7, 98.3)	23.5 (6.8, 49.9)	97.3 (95.3, 98.7)			

^a Cut-offs derived from our dataset and associated with a LR+ of 10 or a LR- of 0.1.

^b Cut-offs applied from Leligdowicz *et al.* [13].

Abbreviations: CI (confidence interval), IL-8 (interleukin-8), LR+ (positive likelihood ratio), LR- (negative likelihood ratio), NPV (negative predictive value), PPV (positive predictive value), sFlt-1 (soluble fms-like tyrosine kinase-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

<https://doi.org/10.1371/journal.pgph.0001553.t004>

these values and their corresponding performance estimators. When we applied the cut-offs of <239 pg/mL and ≥ 629 pg/mL for sTREM-1 based on Leligdowicz *et al.* [13], this resulted in 1.0% (3/290) in-hospital deaths occurring among children placed in the low-risk group, 6.4% (8/125) among those in the intermediate-risk group, and 23.5% (4/17) among those in the high-risk group (Table 4).

4. Discussion

In this cohort of hospitalized Mozambican children fulfilling WHO diagnostic criteria for pneumonia, IL-8, sFlt-1, and sTREM-1 concentration determined at hospital admission showed good individual prognostic accuracy for in-hospital and longer-term mortality. They outperformed other commonly used clinical parameters and were unaltered in healthy community controls.

IL-8 had the highest discriminatory capacity for severity and in-hospital deaths. IL-8 is a chemokine produced by different blood cells upon stimulation with inflammatory stimuli, which attracts and activates neutrophils and other inflammatory cells. sFlt-1 and sTREM-1 were the next best prognostic biomarkers for in-hospital mortality. sFlt-1 regulates angiogenesis and inflammation-induced vascular permeability, and sTREM-1 is thought to contribute to immunosuppression via inhibition and apoptosis of neutrophils and monocytes. In accordance with our results, a previous study in 2,502 hospitalized Ugandan febrile children reported sTREM-1, followed by sFlt-1 and IL-8, as best prognostic biomarkers for 7-day in-hospital mortality [13]. When limiting the cohort to those children who met the IMCI diagnostic criteria for pneumonia, these three biomarkers remained the best ones for 48-hour in-hospital mortality [21]. Another study in Bhutanese children hospitalized with clinical pneumonia showed that sTREM-1 was the best prognostic biomarker for a composite outcome of poor prognosis [41]. In a study in India, IL-8 levels were also higher in fatal cases [42]. Moreover, IL-8 and sTREM-1 have been reported to be increased in severe childhood pneumonia cases [11, 43].

In this study, CRP and PCT were significantly elevated in pneumonia cases compared to controls, but they were neither good severity indicators nor prognosticators of death. A systematic review and meta-analysis found them to be significantly increased in children with severe pneumonia compared to those with non-severe pneumonia [11]. However, almost all included studies were conducted in high-income countries, and PCT and CRP have been suggested to be influenced by HIV, malaria, and malnutrition [14, 44, 45]. Other reviews reported limited or conflicting results [35, 46]. Studies conducted in sub-Saharan Africa have shown that they are poor indicators of childhood pneumonia severity and outcome [15–21], including a previous study in Manhiça [14].

Studies in children have found that biomarkers of immune and endothelial activation are better prognostic indicators than common clinical markers of pneumonia disease severity [21, 41]. Hypoxemia is considered a hallmark of pneumonia severity [35], but SpO₂ had poor discriminating performance for in-hospital mortality in our study cohort (with an exclusion criterion of SpO₂ $\leq 85\%$). The severity dichotomization based on the WHO 2013 pocket book [3] did not have good prognostic accuracy either. In fact, we did not find clear biomarker differences between these severity groups. Nonetheless, it is important to note that we applied this definition to children older than five years old. RISC-Malawi [7, 36] and LODS [37–39] were selected as relevant scores developed with data from African children to predict in-hospital mortality. The RISC-Malawi score demonstrated the best discrimination for in-hospital deaths among pre-specific clinical parameters and was the only one not significantly different from best-performing biomarkers. Remarkably, the addition of IL-8, sFlt-1, or sTREM-1 to a model

with one of these clinical parameters improved AUROCs. Hyperlactatemia has been identified as a promising prognostic indicator in children with pneumonia [47, 48], although inferior to sTREM-1 [21]. Unfortunately, we lacked lactate data, and further studies are needed to evaluate its prognostic accuracy in comparison to the studied biomarkers.

Children at high risk of adverse outcomes could be prioritized for resources and higher level of care as they are more likely to benefit, while those at low risk could be confidently discharged and iatrogenic harm prevented [12, 49]. Biomarker cut-offs were derived and tested to exemplify biomarker potential, but a larger sample size is needed to provide definitive values. Moreover, cut-offs would need to be adapted to the specific use-case and consider both benefits and costs based on decision theory. Interestingly, when calculating different biomarker cut-offs, optimal cut-offs of sTREM-1 based on pre-specified likelihood ratios were similar to those derived by Leligdowicz *et al.* in a cohort of hospitalized febrile children [13]. The potential of these biomarkers is not limited to pediatric pneumonia, but could be used in a myriad of situations where children are at risk of severe and fatal infections. In fact, in this study we included children with WHO-defined clinical pneumonia, a heterogeneous syndromic group in which we detected diverse pathogens (bacteria, viruses, and malaria parasites). Our study population does not include SARS-CoV-2 infections, but recent studies have also highlighted the utility of IL-8, sFlt-1, and sTREM-1 to risk-stratify adult patients with COVID-19 pneumonia [50–53].

IL-8, sFlt-1, and sTREM-1 had also the best predicting value for 28-day and 90-day mortality after initial hospital admission. When limiting analyses to deaths occurring after hospital discharge (sensitivity analysis), we obtained the same best-performing biomarkers. As in similar settings, the burden of post-discharge mortality in Manhiça is not trivial [54, 55]. Children discharged after treatment for severe pneumonia are at increased risk of relapse, morbidities and mortality [56, 57], but adequate follow-up of all children is often unfeasible and unaffordable in low-income settings. Host biomarkers could improve discharge follow-up by enabling the early identification of children at higher risk of post-discharge complications, therefore improving survival after hospitalization [12, 49].

Measuring a panel of biomarkers from different pathophysiological pathways could improve outcome prognostication compared to measuring individual biomarkers [58]. However, two-biomarker combinational approaches did not importantly increase mortality discrimination compared to a more parsimonious approach with only IL-8. Although the statistical power of this analysis is limited due to the low numbers of events in this cohort, these results are in agreement with previous studies evaluating similar biomarkers in children with pneumonia or febrile adult populations, where one biomarker provided comparable mortality discrimination to few biomarkers combined [21, 59, 60].

This study benefits from a prospective design and exhaustive clinical data collection. Major strengths were the addition of a healthy community control group and having post-discharge mortality data. Nevertheless, the number of deaths limited our statistical power. For this reason, we constructed models with only two independent variables, pre-specified few relevant clinical parameters for comparison with biomarkers, did not attempt to derive a full risk prediction model nor validate one, and did not explore if prognostic performance interacted with other factors (e.g., HIV status, malnutrition, age, or pathogens detected). This limitation, common in other studies, highlights the need for individual patient meta-analyses. Besides, HDSS mortality data were not specifically collected for the purpose of this study and this could have impacted the accuracy of the dates of post-discharge deaths.

In this study, we provide evidence that IL-8, sFlt-1, and sTREM-1 are promising candidates to be used in the risk-stratification of childhood pneumonia. Nonetheless, further studies are necessary to establish with assurance which biomarkers and cut-offs are most useful in

different use-cases and in combination with clinical parameters. Another important step is the incorporation of these promising biomarkers into rapid, easy-to-use, and inexpensive point-of-care triage tests to be used in low-resource settings. Their hypothetical implementation into clinical practice could transform risk-stratification and contribute in efforts to reduce pneumonia mortality among the most vulnerable children.

Supporting information

S1 Table. Luminex and ELISA biomarker dynamic ranges and distribution of values outside the dynamic range.

(DOCX)

S2 Table. Biomarker concentrations in pneumonia cases by severity group.

(DOCX)

S3 Table. Biomarker AUROCs for primary and secondary study outcomes.

(DOCX)

S4 Table. Biomarker concentrations in 28-day or 90-day deaths and survivors.

(DOCX)

S5 Table. Sensitivity analysis: Biomarker AUROCs for 28-day or 90-day deaths occurring only at post-discharge.

(DOCX)

S6 Table. Demographic and clinical characteristics associated with in-hospital mortality in pneumonia cases.

(DOCX)

S7 Table. Biomarker performance estimators to identify in-hospital mortality using cut-offs based on the Youden index.

(DOCX)

S1 Fig. Biomarker Spearman's correlation coefficients in pneumonia cases.

(DOCX)

Acknowledgments

We would like to express our gratitude to participant children and their families. We also thank Dyann Wirth and the HSPH and Broad Institute teams which supported this study, in addition to everyone else who contributed directly or indirectly through clinical, laboratory, or data entry support.

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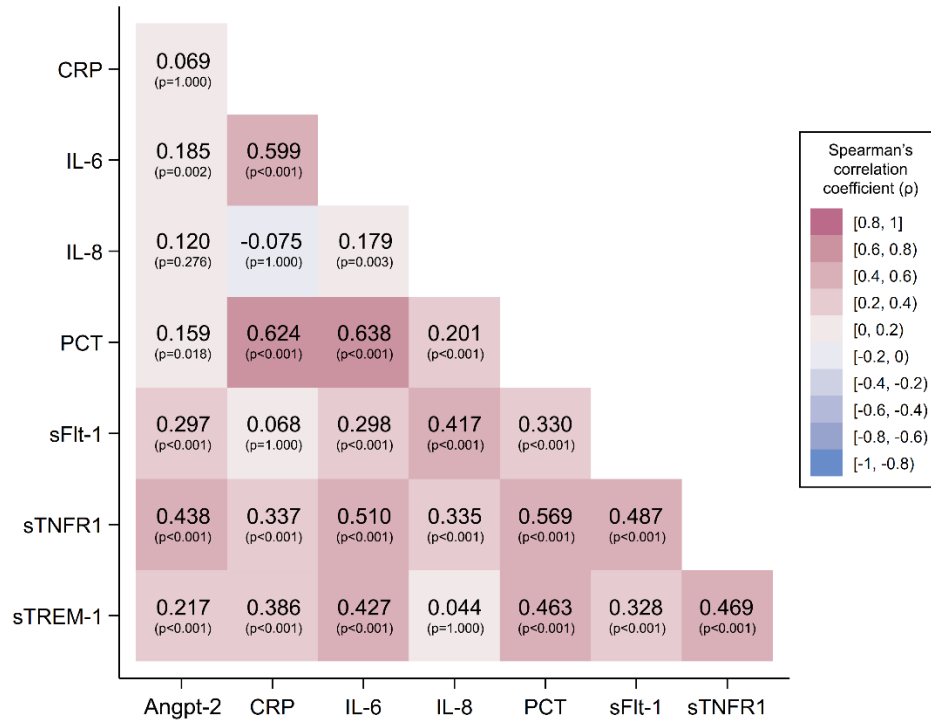
SUPPLEMENTARY MATERIAL

Prognostic accuracy of biomarkers of immune and endothelial activation
in Mozambican children hospitalized with pneumonia



I. SUPPLEMENTARY FIGURES

S1 Figure. Biomarker Spearman's correlation coefficients in pneumonia cases



* p-values were adjusted using the Bonferroni correction.

Abbreviations: Angpt-2 (angiopoietin-2), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

II. SUPPLEMENTARY TABLES

S1 Table. Luminex and ELISA biomarker dynamic ranges and distribution of values outside the dynamic range

Biomarker	All study participants (n=552)		Healthy controls (n=80)		Pneumonia cases (n=472)	
	Dynamic range (pg/mL)	Percentage of samples outside the dynamic range (n/N)	Number of samples below detection	Number of samples above detection	Number of samples below detection	Number of samples above detection
Luminex^a:						
Angpt-2	22.5 – 16430	0 (0/552)	0	0	0	0
IL-6	1.4 – 1030	18.5 (102/552)	68	0	27	7
IL-8	1.3 – 930	13.0 (72/552)	23	0	47	2
PCT	2.9 – 2110	23.2 (128/552)	71	0	55	2
sFlt-1	21.4 – 15610	3.3 (18/552)	13	0	5	0
sTNFR1	19.4 – 14120	0 (0/552)	0	0	0	0
sTREM-1	31.4 – 22870	13.4 (74/552)	39	0	35	0
ELISA^b:						
CRP	15.6 – 1000	22.4 (121/541 ^c)	55	0	28	38

^a Biomarkers were processed using two separate Luminex panels. Panel 1 was performed at a 1:3 dilution and included Angpt-2, IL-6, IL-8, sFlt-1, sTNFR1, and sTREM-1. Panel 2 was performed at a 1:50 dilution and only included PCT.

^b Plasma samples were diluted at 1:250,000 for CRP.

^c 11 samples had insufficient volume for both assays, and Luminex was prioritized over ELISA.

Abbreviations: Angpt-2 (angiopoietin-2), CRP (C-reactive protein), ELISA (enzyme-linked immunosorbent assay), IL-6 (interleukin-6), IL-8 (interleukin-8), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

S2 Table. Biomarker concentrations in pneumonia cases by severity group

Biomarker^a	Pneumonia severe cases (n=220), median (IQR)	Pneumonia non-severe cases (n=252), median (IQR)	p-value^b
Angpt-2	4697.9 (2977.7, 7303.2)	4045.3 (2718.6, 6652.3)	0.101
CRP	64.2 (21.7, 149.1), n=216	84.2 (27.2, 167.7), n=245	0.076
IL-6	30.8 (10.9, 145.8)	35.2 (11.4, 83.0)	0.529
IL-8	18.5 (8.2, 49.2)	13.8 (7.5, 35.1)	0.046
PCT	1029.7 (325.2, 6457.8)	1349.2 (286.8, 6781.2)	0.973
sFlt-1	220.1 (157.4, 334.9)	202.2 (153.5, 287.4)	0.079
sTNFR1	2420.5 (1736.8, 3499.4)	2559.0 (1783.8, 3944.1)	0.402
sTREM-1	200.9 (136.2, 308.7)	196.7 (136.0, 279.2)	0.565

^aAll in pg/mL, except CRP in µg/mL.

^bp-values were computed using the Mann-Whitney U test.

Abbreviations: Angpt-2 (angiopoietin-2), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), IQR (interquartile range), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

S3 Table. Biomarker AUROCs for primary and secondary study outcomes

In-hospital mortality				
Biomarker	n = 432^a		n = 421^b	
	AUROC (95% CI)	p-value	AUROC (95% CI)	p-value
IL-8	0.877 (0.782, 0.972)	Reference ^c	0.868 (0.761, 0.975)	Reference ^c
sFlt-1	0.832 (0.729, 0.935)	0.473	0.855 (0.762, 0.948)	0.835
sTREM-1	0.822 (0.735, 0.908)	0.177	0.794 (0.702, 0.886)	0.106
sTNFR1	0.736 (0.573, 0.898)	0.004	0.703 (0.523, 0.883)	0.002
Angpt-2	0.598 (0.433, 0.762)	0.001	0.545 (0.371, 0.719)	0.001
PCT	0.590 (0.415, 0.764)	<0.001	0.555 (0.359, 0.751)	<0.001
IL-6	0.587 (0.410, 0.764)	<0.001	0.587 (0.385, 0.788)	0.001
CRP	-	-	0.402 (0.205, 0.600)	<0.001
28-day mortality				
Biomarker	n = 309^a		n = 303^b	
	AUROC (95% CI)	p-value	AUROC (95% CI)	p-value
IL-8	0.812 (0.716, 0.908)	Reference ^c	0.799 (0.696, 0.901)	Reference ^c
sFlt-1	0.811 (0.712, 0.910)	0.994	0.822 (0.723, 0.921)	0.693
sTREM-1	0.783 (0.684, 0.882)	0.519	0.760 (0.655, 0.865)	0.433
sTNFR1	0.687 (0.546, 0.828)	0.004	0.657 (0.507, 0.808)	0.002
Angpt-2	0.624 (0.490, 0.757)	0.009	0.592 (0.453, 0.731)	0.008
PCT	0.560 (0.425, 0.695)	<0.001	0.536 (0.391, 0.680)	<0.001
IL-6	0.550 (0.397, 0.703)	<0.001	0.541 (0.374, 0.708)	<0.001
CRP	-	-	0.412 (0.257, 0.567)	<0.001
90-day mortality				
Biomarker	n = 309^a		n = 303^b	
	AUROC (95% CI)	p-value	AUROC (95% CI)	p-value
IL-8	0.799 (0.717, 0.880)	Reference ^c	0.788 (0.703, 0.873)	Reference ^c
sTREM-1	0.757 (0.664, 0.850)	0.389	0.737 (0.640, 0.835)	0.329
sFlt-1	0.742 (0.645, 0.839)	0.293	0.744 (0.644, 0.844)	0.429
sTNFR1	0.645 (0.522, 0.768)	<0.001	0.619 (0.491, 0.747)	<0.001
Angpt-2	0.601 (0.487, 0.715)	0.003	0.575 (0.458, 0.691)	0.002
PCT	0.519 (0.403, 0.634)	<0.001	0.496 (0.375, 0.617)	<0.001
IL-6	0.514 (0.383, 0.645)	<0.001	0.503 (0.364, 0.643)	<0.001
CRP	-	-	0.380 (0.251, 0.509)	<0.001

^aFirst column AUROCs are derived excluding CRP from the analysis (11 individuals lacked CRP data, among whom 2 died during the hospital stay).

^bSecond column AUROCs are derived using the subset of individuals with complete 8 biomarker data.

^cAUROCs were compared to IL-8 AUROC (reference), and p-values were computed using the algorithm suggested by DeLong *et al.*

Abbreviations: Angpt-2 (angiopoietin-2), AUROC (area under the receiver operating characteristic curve), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

S4 Table. Biomarker concentrations in 28-day or 90-day deaths and survivors

Biomarker ^a	28-day mortality		p-value ^b	90-day mortality		p-value ^b
	28-day deaths (n=22), median (IQR)	28-day survivors (n=287), median (IQR)		90-day deaths (n=28), median (IQR)	90-day survivors (n=281), median (IQR)	
Angpt-2	6191.0 (3361.5, 9231.3)	4113.1 (2702.6, 6642.2)	0.053	5534.1 (3235.5, 8980.4)	4116.1 (2698.2, 6560.1)	0.077
CRP	29.0 (6.4, 157.2), n=20	64.9 (22.9, 150.2), n=283	0.189	29.0 (5.7, 115.7), n=26	66.6 (23.3, 153.3), n=277	0.043
IL-6	39.0 (6.3, 310.5)	25.9 (9.7, 93.3)	0.435	22.0 (7.1, 308.6)	26.6 (9.7, 93.3)	0.809
IL-8	86.8 (24.4, 284.2)	14.0 (6.8, 32.9)	<0.001	61.3 (23.4, 215.2)	13.6 (6.8, 32.2)	<0.001
PCT	1660.7 (274.5, 22537.9)	949.0 (266.2, 6495.0)	0.347	846.4 (272.8, 10839.5)	951.4 (267.3, 6570.6)	0.747
sFlt-1	452.7 (312.9, 848.3)	201.7 (153.1, 287.0)	<0.001	360.5 (210.2, 603.4)	202.2 (153.1, 291.7)	<0.001
sTNFR1	4535.4 (2574.8, 7062.3)	2316.4 (1657.0, 3480.9)	0.004	3031.8 (2181.3, 6686.2)	2314.7 (1657.0, 3494.0)	0.011
sTREM-1	344.8 (232.0, 512.5)	188.3 (129.5, 265.1)	<0.001	304.2 (223.5, 493.0)	187.5 (129.5, 261.4)	<0.001

^aAll in pg/mL, except CRP in µg/mL.^bp-values were computed using the Mann-Whitney U test.

Abbreviations: Angpt-2 (angiotensinogen-converting enzyme 2), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), IQR (interquartile range), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

S5 Table. Sensitivity analysis: Biomarker AUROCs for 28-day or 90-day deaths occurring only at post-discharge

28-day mortality (post-discharge only)				
Biomarker	n = 294^a		n = 290^b	
	AUROC (95% CI)	p-value	AUROC (95% CI)	p-value
sFlt-1	0.744 (0.523, 0.965)	Reference ^c	0.744 (0.523, 0.964)	Reference ^c
sTREM-1	0.686 (0.447, 0.925)	0.742	0.684 (0.445, 0.923)	0.738
IL-8	0.659 (0.473, 0.845)	0.506	0.660 (0.475, 0.844)	0.510
Angpt-2	0.656 (0.417, 0.894)	0.320	0.655 (0.417, 0.892)	0.317
sTNFR1	0.559 (0.290, 0.828)	0.269	0.557 (0.287, 0.827)	0.265
PCT	0.485 (0.228, 0.712)	0.064	0.482 (0.255, 0.709)	0.061
IL-6	0.423 (0.128, 0.718)	0.013	0.421 (0.125, 0.716)	0.013
CRP	-	-	0.409 (0.149, 0.669)	0.001
90-day mortality (post-discharge only)				
Biomarker	n = 294^a		n = 290^b	
	AUROC (95% CI)	p-value	AUROC (95% CI)	p-value
IL-8	0.700 (0.584, 0.816)	Reference ^c	0.701 (0.586, 0.816)	Reference ^c
sTREM-1	0.672 (0.512, 0.833)	0.759	0.671 (0.510, 0.832)	0.739
sFlt-1	0.628 (0.478, 0.777)	0.430	0.626 (0.476, 0.776)	0.415
Angpt-2	0.593 (0.437, 0.749)	0.259	0.592 (0.436, 0.748)	0.250
sTNFR1	0.529 (0.359, 0.698)	0.003	0.527 (0.356, 0.697)	0.003
PCT	0.433 (0.289, 0.577)	<0.001	0.429 (0.285, 0.573)	<0.001
IL-6	0.406 (0.224, 0.588)	<0.001	0.402 (0.220, 0.585)	<0.001
CRP	-	-	0.350 (0.183, 0.516)	<0.001

^aFirst column AUROCs are derived excluding CRP from the analysis (11 individuals lacked CRP data, among whom 2 died during the hospital stay).

^bSecond column AUROCs are derived using the subset of individuals with complete 8 biomarker data.

^cAUROCs were compared to IL-8 AUROC (reference), and p-values were computed using the algorithm suggested by DeLong *et al.*

Abbreviations: Angpt-2 (angiopoietin-2), AUROC (area under the receiver operating characteristic curve), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), PCT (procalcitonin), sFlt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

S6 Table. Demographic and clinical characteristics associated with in-hospital mortality in pneumonia cases

Variable ^a	Total pneumonia cases with a valid primary outcome (n=432)	In-hospital deaths (n=15)	OR (95% CI) ^b	p-value
Sex				
Male	221	9 (4.1)	1	0.484
Female	211	6 (2.8)	0.69 (0.24, 1.97)	
Age				
<2 months	23	1 (4.4)	1	0.606
2 to 11 months	162	7 (4.4)	0.99 (0.12, 8.46)	
12 to 59 months	214	5 (2.4)	0.53 (0.06, 4.71)	
5 to <10 years	33	2 (6.1)	1.42 (0.12, 16.64)	
Median (months)	14.8 [6.3, 27.5]	10.5 [3.0, 24.1]	1.00 (0.97, 1.02)	0.710
WAZ^c				
>-1	160	1 (0.6)	1	0.001
-1 to -3	198	6 (3.0)	4.97 (0.59, 41.70)	
<-3	74	8 (10.8)	19.27 (2.36, 157.16)	
Median	-1.5 [-2.6, -0.5]	-3.4 [-4.2, -1.6]	0.67 (0.53, 0.85)	0.002
MUAC (cm)	14.0 [13.0, 15.0], n=424	12.0 [10.0, 13.0]	0.53 (0.38, 0.73)	<0.001
HIV status				
Negative	353	8 (2.3)	1	0.010
Positive	79	7 (8.9)	4.19 (1.47, 11.93)	
Malaria parasitemia				
Negative	350	14 (4.0)	1	0.166
Positive	82	1 (1.2)	0.30 (0.04, 2.29)	
Invasive bacterial disease^d				
Negative	380	8 (2.1)	1	0.001
Positive	52	7 (13.5)	7.23 (2.50, 20.89)	
Viral respiratory infection^e, n=419				
Negative	143	7 (4.9)	1	0.214
Positive	276	7 (2.5)	0.51 (0.17, 1.47)	
Axillary temperature	38.1 [37.1, 39.1]	37.6 [36.5, 38.7]	0.69 (0.44, 1.07)	0.092
Fever ($\geq 37.5^{\circ}\text{C}$, axillary temperature)				
No	134	7 (5.2)	1	0.197
Yes	298	8 (2.7)	0.50 (0.18, 1.41)	
SpO₂ (units decreased from 100%)	3 [2, 4], n=412	3 [3, 9]	1.19 (1.02, 1.41)	0.049
Hypoxemia (SpO₂<90%), n=412				
No	403	14 (3.5)	1	0.324
Yes	9	1 (11.1)	3.47 (0.41, 29.70)	
Cyanosis				
No	417	14 (3.4)	1	0.538
Yes	15	1 (6.7)	2.06 (0.25, 16.75)	
Respiratory rate	56 [49, 62]	54 [44, 60]	0.98 (0.93, 1.03)	0.320
Low chest wall indrawing				
No	143	4 (2.8)	1	0.583
Yes	289	11 (3.8)	1.38 (0.43, 4.40)	
Nasal flaring				
No	227	8 (3.5)	1	0.950
Yes	205	7 (3.4)	0.97 (0.34, 2.72)	
Deep breathing				
No	385	12 (3.1)	1	0.292
Yes	47	3 (6.4)	2.12 (0.58, 7.80)	
Grunting				
No	384	12 (3.1)	1	0.307
Yes	48	3 (6.3)	2.07 (0.56, 7.60)	

Wheezing				
No	331	13 (3.9)	1	0.320
Yes	101	2 (2.0)	0.49 (0.11, 2.23)	
Crackles				
No	190	5 (2.6)	1	0.392
Yes	242	10 (4.1)	1.59 (0.54, 4.75)	
Ronchi				
No	281	11 (3.9)	1	0.484
Yes	151	4 (2.7)	0.67 (0.21, 2.13)	
Inspiratory stridor				
No	414	15 (3.6)	-	-
Yes	18	0 (0)	-	
Altered consciousness (BCS<5)				
No	416	14 (3.4)	1	0.575
Yes	16	1 (6.3)	1.91 (0.24, 15.53)	
Coma (BCS<3)				
No	431	15 (3.5)	-	-
Yes	1	0 (0)	-	
Prostration				
No	395	13 (3.3)	1	0.530
Yes	37	2 (5.4)	1.68 (0.36, 7.74)	
Convulsions, n=423				
No	398	14 (3.5)	1	0.901
Yes	25	1 (4.0)	1.14 (0.14, 9.06)	
Unable to drink/breastfeed, n=422				
No	402	13 (3.2)	1	0.183
Yes	20	2 (10.0)	3.32 (0.70, 15.85)	
Dehydration, n=423				
No	396	10 (2.5)	1	0.001
Yes	27	5 (18.5)	8.77 (2.76, 27.88)	
Radiological findings, n=375				
Normal	138	2 (1.5)	1	0.765
Other infiltrates	85	2 (2.4)	1.64 (0.23, 11.85)	
Endpoint pneumonia	152	4 (2.6)	1.84 (0.33, 10.19)	
Glucose (mmol/L)	6.1 [5.2, 7.0], n=390	5.9 [4.3, 7.7], n=13	1.12 (0.84, 1.50)	0.454
Hematocrit (%)	27.4 [23.1, 31.1], n=430	24.3 [22.9, 26.8]	0.96 (0.88, 1.04)	0.300
WBC count	14.2 [10.1, 20.4], n=409	14.6 [12.2, 21.1]	1.01 (0.95, 1.06)	0.814
WHO severity definition				
No	229	5 (2.2)	1	0.118
Yes	203	10 (4.9)	2.32 (0.78, 6.91)	
LODS	0 [0, 0]	0 [0, 1]	1.64 (0.70, 3.85)	0.295
RISC-Malawi score	1 [0, 4], n=405	5 [2, 8]	1.38 (1.18, 1.61)	<0.001
Previous admissions for pneumonia				
No	417	12 (2.9)	1	0.011
Yes	15	3 (20.0)	8.44 (2.10, 33.85)	

^a Data presented as median [interquartile range] or frequency (percent) as appropriate. Number of subjects indicated if presence of missing values.

^b ORs, 95% CIs, and p-values are from univariable logistic regression models.

^c Calculated using the LMS method and the 2000 US CDC Growth Reference data.

^d Defined as the detection of a non-contaminating bacterial organism in blood, cerebrospinal fluid, and/or pleural fluid through culture or PCR analysis of dried blood spots for *Streptococcus pneumoniae* and *Haemophilus influenzae* type B.

^e At least one virus detected in nasopharyngeal aspirate. PCR primers targeted adenovirus, bocavirus, coronavirus (229E and OC43), enterovirus, influenza virus (A, B, and C), metapneumovirus, parainfluenza virus (1, 2, 3, and 4), respiratory syncytial virus (A and B), and rhinovirus.

Abbreviations: BCS (Blantyre Coma Scale), CI (confidence interval), HIV (human immunodeficiency virus), LODS (Lambaréné Organ Dysfunction Score), MUAC (mid-upper arm circumference), OR (odds ratio), RISC-Malawi (Respiratory Index of Severity in Children-Malawi), WAZ (weight-for-age z-score), WBC (white blood cell), WHO (World Health Organization).

S7 Table. Biomarker performance estimators to identify in-hospital mortality using cut-offs based on the Younden Index

Biomarker	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)	PPV (95% CI)	NPV (95% CI)
IL-8	≥59.6 pg/mL	80.0 (51.9, 95.7)	86.8 (83.2, 89.9)	6.07 (4.26, 8.63)	0.23 (0.08, 0.63)	17.9 (9.6, 29.2)	99.2 (97.6, 99.8)
sFIt-1	≥312.9 pg/mL	80.0 (51.9, 95.7)	78.2 (73.9, 82.0)	3.67 (2.68, 5.01)	0.26 (0.09, 0.70)	11.7 (6.2, 19.5)	99.1 (97.4, 99.8)
sTREM-1	≥232.0 pg/mL	86.7 (59.5, 98.3)	66.9 (62.2, 71.4)	2.62 (2.06, 3.33)	0.20 (0.05, 0.73)	8.6 (4.7, 14.3)	99.3 (97.5, 99.9)
sTNFR1	≥4613.2 pg/mL	60.0 (32.3, 83.7)	86.1 (82.4, 89.3)	4.31 (2.68, 6.95)	0.46 (0.25, 0.86)	13.4 (6.3, 24.0)	98.4 (96.5, 99.4)
Angpt-2	≥4782.3 pg/mL	66.7 (38.4, 88.2)	57.1 (52.2, 61.9)	1.55 (1.07, 2.26)	0.58 (0.28, 1.20)	5.3 (2.6, 9.5)	97.9 (95.3, 99.3)
PCT	≥6420.6 pg/mL	53.3 (26.6, 78.7)	77.0 (72.6, 80.9)	2.32 (1.40, 3.84)	0.61 (0.35, 1.04)	7.7 (3.4, 14.6)	97.9 (95.7, 99.1)
IL-6	≥272.7 pg/mL	40.0 (16.3, 67.7)	87.8 (84.2, 90.8)	3.27 (1.67, 6.40)	0.68 (0.45, 1.03)	10.5 (4.0, 21.5)	97.6 (95.5, 98.9)
CRP	≥195.7 µg/mL	30.8 (9.1, 61.4)	82.8 (78.8, 86.4)	1.79 (0.77, 4.17)	0.84 (0.58, 1.20)	5.4 (1.5, 13.3)	97.4 (95.1, 98.8)

Abbreviations: Angpt-2 (angiopoietin-2), CRP (C-reactive protein), IL-6 (interleukin-6), IL-8 (interleukin-8), LR+ (positive likelihood ratio), LR- (negative likelihood ratio), NPV (negative predictive value), PCT (procalcitonin), PPV (positive predictive value), sFIt-1 (soluble fms-like tyrosine kinase-1), sTNFR1 (soluble tumor necrosis factor receptor-1), sTREM-1 (soluble triggering receptor expressed on myeloid cells 1).

ARTICLE



Soluble triggering receptor expressed on myeloid cells 1 (sTREM-1)
to risk-stratify paediatric and adult patients with febrile illness in southern Mozambique

Soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) to risk-stratify paediatric and adult patients with febrile illness in southern Mozambique

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ABSTRACT

Background: Fever is a leading reason for seeking healthcare globally. Early in the course of febrile illness, it is challenging to identify patients at risk of severe and fatal infections. We hypothesized that quantifying circulating biomarkers of immune and endothelial activation in febrile patients may indicate the risk of adverse outcomes.

Methods: From December 2018 to February 2021, we enrolled children ≥ 2 months and adults with fever visiting two Mozambican hospitals. Standard clinical and laboratory parameters, including lactate levels, were assessed at presentation. Plasma levels of Angpt-2, CHI3L1, CRP, IL-6, IL-8, PCT, sFlt-1, sTNFR1, sTREM-1, and suPAR at presentation were retrospectively determined using Luminex and ELISA. Clinical outcomes were evaluated up to 28 days. We determined the prognostic performance of biomarkers for 28-day mortality and other adverse outcomes.

Results: A total of 1,955 participants were enrolled and had biomarkers measured, with 93 deaths occurring within 28 days. All biomarker levels were elevated in inpatients compared to outpatients and were associated with 28-day mortality (all $p < 0.001$). sTREM-1 was the best biomarker predicting 28-day mortality with an AUROC of 0.82 (95% CI: 0.78-0.86), superior to that of PCT ($p < 0.001$), CRP ($p < 0.001$), and lactate ($p = 0.003$). Its prognostic performance was consistent across age and sex, but reduced in HIV-positive individuals (AUROC=0.73, 95% CI: 0.66-0.80). Adding sTREM-1 significantly improved the discrimination of clinical severity scores for 28-day mortality. Among inpatients discharged home, sTREM-1 was positively correlated with duration of hospitalisation ($p < 0.001$). Among outpatients, sTREM-1 levels were higher in those seeking further care ($p = 0.002$) or subsequently hospitalised ($p = 0.012$).

Conclusions: These findings confirm sTREM-1 as a promising biomarker for risk stratification of all-age, all-cause febrile illnesses in resource-limited settings.

KEYWORDS

Biomarkers, fever, prognosis, mortality, risk stratification, sepsis, severe infection, soluble triggering receptor expressed on myeloid cells 1 (sTREM-1)

INTRODUCTION

Febrile illnesses are a leading reason for clinical consultations globally, especially in low- and middle-income countries where the prevalence of infections is higher. Although fever can result from non-infectious causes, infections are a common trigger.¹ It is estimated that ~80% of individuals attending primary care facilities across Africa for an acute medical condition had self-reported or documented fever.¹ At primary care level, many febrile illnesses are uncomplicated and self-resolving. Only a small proportion involve a life-threatening infection requiring prompt and intensive intervention, including hospitalisation or referral for higher-level care.² However, early in the course of febrile illness, it is difficult to identify individuals at risk of severe and fatal infections among the majority who are not, since initial presentations of life-threatening infections may be subtle and non-specific.² In resource-limited settings, management of febrile illnesses is further complicated by the chronic shortage of clinicians and limited laboratory services and equipment.³ Clinical guidelines based on symptoms and signs are commonly used to diagnose and treat common infections, with the triage of clinical presentations based on a subset of danger signs that involve subjective recognition and lack sensitivity and specificity for adverse outcomes. It is therefore challenging to prioritize limited resources to those at highest risk of poor prognosis, and failure to identify severe disease results in unsafe discharge and increased mortality or long-term sequelae.⁴⁻⁷

Severe infections share common pathophysiological pathways. Endothelial and immune activation can contribute to endothelial destabilisation and organ dysfunction, being key determinants of progression to sepsis irrespective of aetiology. A growing body of evidence supports the hypothesis that measuring mediators of these pathways early in the disease course may help to improve risk stratification and management of febrile syndromes.^{8,9} Biomarkers have the advantage of being quantitative, objective indicators suitable for incorporation into potentially simple and inexpensive point-of-care rapid tests.⁹ Research conducted both in children and adults has shown that endothelial and immune activation biomarkers are promising prognostic tools across multiple infection aetiologies, including studies in sub-Saharan populations.¹⁰⁻¹⁶ However, most studies have been performed in patients admitted to hospital with a specific aetiology or syndrome. There is limited evidence available on the relationship between biomarker levels and adverse outcomes from patients of all ages, for all-cause fevers, and in non-hospitalised individuals. Moreover, it remains unclear whether the prognostic performance of biomarkers differs according to individual patient characteristics.

Here we evaluate the prognostic performance of immune and endothelial activation biomarkers measured at clinical presentation for 28-day mortality and other adverse clinical outcomes, in a large prospective cohort of febrile paediatric and adult patients in Mozambique presenting to hospital who were managed as outpatients or inpatients.

METHODS

Study population

This study was conducted in the Mozambican cohort of the Febrile Illness Evaluation in a Broad Range of Endemicities (FIEBRE) study, a multisite prospective observational study designed to identify the infectious causes of fever in Africa and Asia.¹⁷ Enrolment of participants in Mozambique was performed between December 2018 and February 2021. Individuals who sought care at Manhiça District Hospital (Manhiça, Mozambique) or General José Macamo Hospital (Maputo, Mozambique) were clinically evaluated. Decisions regarding whether patients were admitted to hospital or discharged home were made by non-study clinicians according to routine practice. Shortly after this decision, patients were assessed for study recruitment by trained clinical study staff and enrolled if selection criteria was fulfilled. Enrolment was stratified and aimed to achieve balanced samples of 600 child outpatients, 600 child inpatients, 600 adult outpatients, and 600 adult inpatients. This approach was in accordance to the sample size considerations made for the primary objective of the FIEBRE study. Children were defined as participants aged ≥ 2 months to < 15 years. In General José Macamo Hospital, only child and adult inpatients were recruited. Enrolled participants were aged ≥ 2 months, had tympanic or axillary temperature $\geq 37.5^{\circ}\text{C}$ at presentation, had not been hospitalised or undergone surgery in the preceding month, and were willing and able to provide demographic and clinical information and samples at enrolment and 28 days later. Outpatient selection criteria also included residence within the Manhiça district. All outpatients with symptoms of diarrheal diseases (≥ 3 loose stools within the preceding 24 hours) and adult outpatients with symptoms of lower respiratory infection (cough productive of green/yellow sputum or haemoptysis) were excluded. This was due to reasons related to the primary objective of the FIEBRE study, including pre-existing published evidence on the causes of fever from pneumonia and diarrhoea in Africa and Asia.^{18,19}

Study procedures

Upon enrolment, participants had a complete clinical history and physical exam systematically recorded. Venous blood and other clinical samples were taken for patient care and research purposes. Details on the methods and the standard operating procedures of FIEBRE study are available from the published study protocol.¹⁷ Besides specific diagnostic tests for determination of cause of fever, the standard care provided by health facility staff was unchanged. Human immunodeficiency virus (HIV) status was considered positive if self-reported by the participant/caregiver or confirmed through point-of-care testing (using antibody-detecting rapid tests) performed for all participants with unknown or negative self-reported HIV status. Lactate, considered a severity marker, was measured at clinical presentation on-site using venous blood and a point-of-care analyser (Lactate Scout, SensLab GmbH). Additional clinical data were collected for inpatients during admission and at discharge. A follow-up visit was set at day 28

after enrolment (acceptable range of 26–48 days, inclusive), during which vital status and clinical data related to the original illness episode were documented. Data on seeking further care and hospitalisation in outpatients were self-reported by participants or reported by parents/guardians. All data were collected using electronic case report forms in Open Data Kit (ODK).²⁰

Clinical severity scores calculation

Clinical signs collected at clinical presentation were used retrospectively to calculate disease severity scores. The Emergency Department Paediatric Early Warning Score (ED-PEWS), the Liverpool quick Sequential Organ Failure Assessment (LqSOFA) score, and the Lambaréné Organ Dysfunction Score (LODS) were used for children.^{21–23} For adults, the Modified Early Warning Score (MEWS), the Quick Sequential (Sepsis-Related) Organ Failure Assessment (qSOFA) score, and the Universal Vital Assessment (UVA) score were calculated (**Suppl. Table 1**).^{24–26}

Biomarker quantification

Host biomarkers with demonstrated prognostic value in acute infections were chosen for assessment, based on our prior research and review of the literature.^{10,11,16} C-reactive protein (CRP) and procalcitonin (PCT) were included as classic biomarkers commonly used in high-income settings. The ten selected biomarkers were angiopoietin-2 (Angpt-2), chitinase-3-like protein 1 (CHI3L1), CRP, interleukin-6 (IL-6), interleukin-8 (IL-8), PCT, soluble fms-like tyrosine kinase-1 (sFlt-1), soluble tumour necrosis factor receptor 1 (sTNFR1), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), and soluble urokinase-type plasminogen activator receptor (suPAR).

Plasma obtained from EDTA-anticoagulated venous blood was obtained for all study participants at clinical presentation and stored at -80°C in Manhica, Mozambique. After study completion, samples were shipped to University Health Network - Toronto General Hospital in Toronto, Canada, for biomarker measurement. Samples were stored at -80°C without freeze-thaw until batch analyte quantification. A single plasma sample from each participant was used to measure all biomarkers. Plasma concentrations of Angpt-2, CHI3L1, IL-6, IL-8, PCT, sFlt-1, sTNFR1, and sTREM-1 were quantified using the multiplex Luminex platform with custom-developed reagents from R&D Systems. Individual Luminex plates included 10% duplicates. CRP was quantified by DuoSet® enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) and all assays were run in duplicate. suPAR was quantified by suPARnostic® ELISA kits (ViroGates) and plates included 5% duplicates. Duplicate measurements were averaged to obtain the final concentration of biomarkers. Procedures were performed according to manufacturers' instructions and blinded to clinical data. Biomarker concentrations outside the dynamic range were assigned the highest limit of the standard curve or a value of one-third of the lowest limit (**Suppl. Table 2**).

Primary and secondary outcomes

We evaluated the prognostic performance of biomarkers for the primary and secondary outcomes. The primary outcome of this analysis was 28-day all-cause mortality. Deaths occurring after day 28, even if captured during follow-up visits, were not considered primary outcome events. This restriction was applied to strictly define the primary outcome within a 28-day period. We also conducted subgroup analyses on 28-day mortality by pre-specified patient characteristics (age, sex, and HIV status) and restricted to inpatients. Secondary outcomes included 7-day mortality in the entire cohort, length of hospital stay in inpatients discharged home, and seeking further care and hospitalisation for the same illness up until the follow-up visit in outpatients.

Statistical analysis

Demographic and clinical characteristics were presented as medians with interquartile ranges (IQRs) for non-normally distributed continuous variables, whilst counts and proportions were used to describe discrete variables. Biomarker concentrations in two groups were compared using Mann-Whitney U tests. Correlation of biomarker levels with continuous variables was assessed using Spearman's rank correlation coefficients. The ability of biomarkers to discriminate between two outcomes was evaluated using receiver operating characteristic (ROC) curves comparing the area under the ROC curves (AUROCs). The discriminatory power of the combination of two biomarkers or a biomarker and a clinical severity score was determined using multivariable logistic regression and using model predictions to compute the AUROCs. Logistic models were also used to estimate odds ratios (ORs). Biomarker data were log-transformed for inclusion in all regression models. AUROCs were compared pairwise using methods recommended by DeLong *et al.*²⁷ To stratify our study population into risk categories, we applied sTREM-1 cut-offs previously derived in hospitalised febrile children in Uganda for mortality prediction: low-risk (<239 pg/mL), intermediate-risk (239–628 pg/mL), and high-risk (≥629 pg/mL).¹⁰ Based on these risk categories, Kaplan-Meier curves were plotted and mortality hazard ratios calculated using Cox proportional-hazards models. Interactions in Cox models were assessed using likelihood ratio tests. Aside from analyses by these three risk categories, sTREM-1 was treated as a continuous variable. For length of hospital stay, we restricted the analysis to individuals discharged home and excluded in-hospital deaths, absconders, and transfers to a higher-level hospital. We performed complete case analyses, with missing values excluded. All tests were two-tailed and p-values <0.05 were considered statistically significant. Stata version 16 (Stata Corp, College Station, TX, USA) was used for analysis. Graphs were plotted with Stata version 16 and R version 4.2.2 (R Core Team).

Ethical approval

This study was approved by the CISM's Institutional Ethics Committee (Ref. CIBS-CISM/010/2018), the Mozambican National Bioethics Committee (Ref. 447/CNBS/18), the LSHTM Research Ethics Committee (Ref. 14538), and the University Health Network Institutional Review Board (Ref. UHN REB#18-6186). All research was conducted according to the principles expressed in the Declaration of Helsinki. After a detailed explanation of the study, written informed consent was obtained from all participants or from parents/guardians of paediatric participants. Children assented if aged >12 years.

RESULTS

Participant characteristics

A total of 2,182 participants were enrolled in the FIEBRE study in Mozambique. Of these, 1,955 (89.6%) had a plasma sample taken with enough volume available for biomarker measurement and were included in this analysis (**Suppl. Figure 1**). In 16/1,955 (0.8%) participants suPAR could not be assessed due to insufficient plasma volume for this biomarker. Among the 1,955 study participants presenting to hospital, there were 531 children and 509 adults subsequently managed as outpatients, and 509 children and 406 adults managed as inpatients (**Table 1**).

At day 28, vital status was known for 1,593 (81.5%) participants (**Table 2**). A total of 93 deaths occurred: 72 among adult inpatients, 19 among child inpatients, and two among adult outpatients. Most inpatients who died by day 28 did so in the hospital during their initial admission (39/91, 42.9%) or after they were transferred to a higher-level hospital (31/91, 34.1%), but some died after having been discharged home (19/91, 20.9%) or having absconded (1/91, 1.1%). For one deceased inpatient, this information was unavailable (1/91, 1.1%). Median time to death was 6 days (IQR: 1-12), with 28 (30.1%) deaths happening within the first 48 hours and 54 (58.1%) deaths within the first 7 days. Key clinical characteristics of paediatric and adult participants separately by vital status at day 28 are described in **Suppl. Tables 3-4**.

Study population biomarker levels

All biomarker levels at clinical presentation were significantly associated with the decision of hospital admission, being higher in inpatients compared to outpatients (all $p < 0.001$) (**Figure 1A**). Among participants with a known vital status at day 28, all biomarkers were significantly elevated at presentation in participants who subsequently died compared to those who remained alive at day 28 (all $p < 0.001$). Participants who died also had significantly higher biomarker levels than alive outpatients and alive inpatients separately, except for PCT levels in inpatients (**Figure 1B**).

Levels of biomarkers related to hospitalisation and vital status at day 28 are displayed separately for children and adult participants in **Suppl. Figure 2**.

Comparative performance of biomarkers to predict 28-day mortality

Among assessed biomarkers, sTREM-1 levels at clinical presentation best discriminated those who subsequently died within 28 days with an AUROC of 0.82 (95% confidence interval [CI]: 0.78-0.86) (**Figure 2A**). sTREM-1 was superior in terms of AUROCs when compared individually to PCT, CRP, IL-6, sFlt-1, and IL-8 (all $p < 0.001$), as well as to suPAR and sTNFR1 (both $p < 0.01$). With univariable logistic regression, the odds of 28-day mortality increased by 3.19 times (95% CI: 2.57-3.96) for every two-fold increase in sTREM-1 concentration (**Suppl. Table 5**). The best two-biomarker combination based on AUROCs for 28-day mortality was sTREM-1 and Angpt-2, with an AUROC of 0.85 (95% CI: 0.81-0.88) ($p = 0.054$ compared to that of sTREM-1 alone). Among inpatients only, sTREM-1 had the best discriminatory ability for 28-day mortality with an AUROC of 0.74 (95% CI: 0.68-0.79) (**Suppl. Figure 3**). When limiting the outcome to 7-day mortality, sTREM-1 remained the best prognostic biomarker with an AUROC of 0.82 (95% CI: 0.77-0.88) (**Suppl. Figure 4**).

We evaluated the discriminatory ability of all biomarkers for 28-day mortality across different patient subgroups (**Figure 2B-D**). sTREM-1 had the highest AUROC irrespective of age group and sex. By HIV status, sTREM-1 had the highest AUROC for HIV-negative individuals and second highest for HIV-positive individuals after suPAR. Comparisons of AUROCs for the same biomarker between subgroups showed no significant differences, except for sTREM-1 by HIV status. The AUROC for sTREM-1 was higher for HIV-negative participants (0.89, 95% CI: 0.84-0.94) compared to HIV-positive participants (0.73, 95% CI: 0.66-0.80) ($p < 0.001$). Among HIV-positive participants, the AUROC for sTREM-1 in those who reported taking antiretroviral therapy (ART) was 0.77 (95% CI: 0.69-0.84) and in those who did not or were newly diagnosed was 0.64 (95% CI: 0.49-0.78). sTREM-1 levels according to vital status at day 28 and HIV status are plotted in **Suppl. Figure 5**.

Performance of sTREM-1 to predict 28-day mortality using established risk categories

Using previously defined sTREM-1 cut-offs, 64.6% of participants were classified as low-risk (80.9% of outpatients vs. 46.0% of inpatients), 29.0% as intermediate-risk (18.0% of outpatients vs. 41.4% of inpatients), and 6.5% as high-risk (1.2% of outpatients vs. 12.6% of inpatients). This corresponded to a 28-day case fatality ratio of 34.0% (35/103) for the high-risk group, 7.9% (37/467) for the intermediate-risk group, and 2.1% (21/1,023) for the low-risk group (**Suppl. Table 6**).

Mortality hazard ratios were 19.5 (95% CI: 11.3-33.5) and 3.9 (95% CI: 2.3-6.6) in the high-risk and intermediate-risk categories compared to the low-risk category, respectively (**Figure 3A**). HIV status was an effect modifier of the association between sTREM-1 risk category and mortality ($p<0.001$) (**Figures 3B-3C**). Among the 21 deaths in the low-risk group, 15 (71.4%) were HIV-positive and 1 (4.8%) had an unknown HIV status. However, there was no effect modification by age group or sex (**Suppl. Figure 6**). Mortality hazard rates restricted to inpatients or by HIV-positive subgroups are plotted in **Suppl. Figure 6**.

Performance of sTREM-1 relative to lactate to predict 28-day mortality

A subsample of participants had documented lactate levels at clinical presentation and a known vital status at day 28 ($N=1,190$). Lactate was associated with 28-day mortality ($p<0.001$) and exhibited an AUROC of 0.72 (95% CI: 0.66-0.79) for 28-day mortality. Overall, this was outperformed by the sTREM-1 AUROC of 0.83 (95% CI: 0.78-0.88) ($p=0.003$). AUROCs of sTREM-1 and lactate by patient subgroups can be found in **Suppl. Figure 7**.

Performance of sTREM-1 relative to and combined with clinical severity scores to predict 28-day mortality

Different clinical severity scores were calculated for study participants. A one-unit increase in all scores was associated with increased odds of 28-day mortality (all $p<0.001$). However, sTREM-1 remained significantly associated with 28-day mortality when adjusted for each individual score (**Suppl. Table 7, Suppl. Figure 8**). ED-PEWS and UVA were the scores with the best discrimination for 28-day mortality in children and adults, respectively (**Figure 4**). sTREM-1 alone demonstrated comparable discrimination to clinical severity scores, with better discrimination than MEWS in adults ($p=0.016$). The combination of sTREM-1 with these scores significantly improved AUROCs for all models (all $p<0.01$) except for ED-PEWS in children ($p=0.188$) (**Figure 4**).

Comparative performance of biomarkers to predict non-mortality outcomes

The median duration of hospitalisation for inpatients discharged home was three days (IQR: 1-5). sTREM-1, as well as Angpt-2 and CHI3L1, were positively correlated with length of hospital stay (all $p<0.001$, $N=675$). Of these, sTREM-1 had the greatest positive Spearman's rank correlation coefficient (**Table 3**).

Among outpatients with biomarker and follow-up data at day ≥ 28 ($N=838$), 93.1% did not require additional care, 5.7% sought further care for the same illness but were not hospitalised, and 1.2% sought further care and required hospitalisation. sTREM-1, suPAR, PCT, and sTNFR1 levels at

presentation were significantly higher in outpatients who subsequently sought further care (**Table 3**). sTREM-1 levels, along with most assessed biomarkers, were significantly higher in outpatients who were later hospitalised (**Table 3**). sTREM-1 had an AUROC of 0.62 (95% CI: 0.55–0.69) for seeking further care and of 0.73 (95% CI: 0.60–0.87) for subsequent hospitalisation in outpatients (**Suppl. Table 8**).

DISCUSSION

In this cohort of Mozambican children and adults with febrile illness presenting to hospital, sTREM-1 was the best biomarker for predicting 28-day mortality. sTREM-1 was superior to other biomarkers of immune and endothelial activation and provided additional discriminatory ability when combined with clinical severity scores. Notably, sTREM-1 consistently demonstrated the best performance across different patient subgroups, though its performance was diminished in HIV-positive individuals. sTREM-1 remained the best biomarker if 7-day mortality was considered instead. sTREM-1 was associated with length of hospital stay and was also the best biomarker to predict mortality among inpatients. Outpatients had lower plasma concentrations of all studied biomarkers compared to inpatients, yet their sTREM-1 levels were associated with seeking further care and subsequent hospitalisation.

These results provide additional evidence supporting the use of sTREM-1 as a predictor of mortality in children and adults with all-cause febrile illness. In hospitalised febrile children in Uganda, sTREM-1 had the best prognostic performance for 7-day mortality among a similar biomarker panel, with an AUROC of around 0.90.¹⁰ Likewise, in Tanzanian febrile adults presenting to outpatient clinics, sTREM-1 was the best biomarker to predict 28-day mortality with an AUROC of 0.87 (95% CI: 0.81–0.92).¹¹ In diverse populations with specific syndromes or detected infectious agents (including malaria, pneumonia, COVID-19, or sepsis), high sTREM-1 levels have been consistently associated with subsequent mortality.^{12–15,28–31} When we used previously published sTREM-1 thresholds for risk stratification, we observed a stepwise increase in mortality between groups similar to other cohorts.^{10,13,15} Considering the challenges in determining the underlying cause of febrile illnesses, these data collectively suggest the broad applicability of sTREM-1 to risk-stratify febrile patients in a pathogen agnostic manner.

sTREM-1 significantly outperformed CRP and PCT in predicting 28-day mortality, consistent with earlier findings from similar populations.^{10,11} sTREM-1 also outperformed lactate. Although prior research on pneumonia, malaria, and sepsis has documented an association between lactate levels and fatal outcome, the prognostic performance of lactate was usually inferior to sTREM-1.^{12,14,32,33} In addition, we applied various relevant clinical severity scores to our study population, similarly to previous publications using data from the FIEBRE study,³⁴ and compared them to sTREM-1. Comprehensive clinical data collection occurred under our study conditions; however, it is worth noting that some variables may be measured with less accuracy or be unavailable in

routine clinical practice. sTREM-1 alone had similar discriminative ability for 28-day mortality when compared to clinical severity scores, and the addition of sTREM-1 added discriminatory ability to the scores. This result, coupled with similar previous findings, suggest that sTREM-1 could be integrated with relevant and context-specific clinical severity scores or decision algorithms to better risk-stratify patients with infections.^{11,13,15,28,35} CHI3L1 and Angpt-2 are other candidate biomarkers that had comparable performance to sTREM-1 overall, but not across all patient subgroups. Combining biomarkers could enhance mortality prediction, although this may limit feasibility and practicality for clinical use. sTREM-1 and Angpt-2 was the best two-biomarker combination to predict 28-day mortality in terms of AUROCs, but adding Angpt-2 only slightly increased the AUROC over sTREM-1 alone.

The prognostic performance of sTREM-1 for 28-day mortality differed by HIV status. While still discriminatory in HIV-positive participants, its performance was significantly inferior compared to HIV-negative participants. This was more notable in individuals who were not receiving ART or unaware of their HIV-positive status. Active HIV infection causes a combination of immunosuppression and chronic inflammation that can result in immune system exhaustion and altered host responses to pathogens.³⁶ Most of the deaths in the sTREM-1-based low-risk group were HIV-positive. Moreover, previous research has linked HIV infection with upregulation of TREM-1 in immune cells and increased plasma levels of sTREM-1, suggesting a possible contribution of TREM-1 to HIV-induced chronic inflammation, and hence potentially diminishing the prognostic performance of sTREM-1 in acute infections.^{37,38} One previous study in children with signs of severe infection explored sTREM-1 levels in relation to HIV status and mortality, but in this cohort sTREM-1 worked poorly regardless of HIV status.³⁹ Additional research is needed to elucidate the mechanistic impact of HIV infection on the TREM-1 pathway during febrile illnesses. Except sTREM-1 and HIV status, all biomarkers studied showed similar prognostic performance for 28-day mortality across patient subgroups. Nevertheless, almost all biomarkers tended to decrease their prognostic performance in HIV-positive participants. Existing limited evidence in febrile children found no sex-related differences in biomarker levels in relation to mortality.⁴⁰ However, in paediatric severe malaria, sTREM-1 and sFlt-1 predicted mortality better in males.¹⁵ PCT and IL-6 were inferior in identifying risk of poor prognosis in infants compared to older children with pneumonia in Bhutan.³⁵ Future studies are needed to examine potential differences in biomarker prognostic performance across patient characteristics.

Death is often the primary outcome in prognostic studies with inpatients. Yet associations with less severe outcomes are also important for patient management, particularly in lower mortality risk clinical settings. In this cohort, mortality among outpatients was very low, and other adverse outcomes were assessed within this subgroup. Along with other biomarkers, we demonstrated an association between high sTREM-1 levels and seeking additional care or subsequent hospitalisation in outpatients, suggesting that the studied biomarkers could play a future role in mitigating these adverse outcomes. Previous evidence on sTREM-1 and adverse outcomes in

patients attending outpatient clinics and emergency departments is scarce. In these contexts, sTREM-1 has been associated with hospital admission and with mortality, in line with our results.^{11,30} However, in Spanish outpatients aged over 50 years with mild-to-moderate COVID-19, sTREM-1 poorly predicted hospitalization by day 28. This contrasts with our study's findings, likely due to differences in demographics, disease severity, and healthcare settings.⁴¹ In addition, sTREM-1 has been reported to be less suitable for predicting supplementary oxygen requirement in respiratory diseases.⁴²⁻⁴⁴ Further research is necessary to better understand whether sTREM-1 is sufficiently altered early in the disease course and confirm its prognostic utility for other adverse outcomes beyond mortality.

sTREM-1 has a pathophysiologic link with sepsis. TREM-1 is a cell-surface receptor expressed mostly by myeloid immune cells, which amplifies inflammation in infections.⁴⁵ sTREM-1 acts as a decoy receptor and dampens TREM-1 activation.⁴⁵ It is thought that sTREM-1 release depends on TREM-1 pathway activation and that it counteracts excessive inflammatory reactions, but high levels may reflect an underlying immune dysfunction.^{46,47} Interestingly, efforts have been made to modulate TREM-1 amplification in severe infections.⁴⁷ Recent clinical trials in septic shock and COVID-19 have demonstrated that nangibotide, a TREM-1 specific inhibitor, was safe and holds potential to improve clinical status.^{48,49} Therefore, risk stratification strategies based on sTREM-1 could be coupled with targeted therapeutic strategies acting on the same pathways.

This study had several strengths, including its prospective enrolment of participants, a large and well-characterized cohort comprising both inpatients and outpatients, and a head-to-head comparison of several candidate biomarkers described in the literature. However, it also has limitations. The stratified enrolment strategy resulted in the inclusion of a high proportion of patients requiring hospitalisation, potentially limiting the representativeness with regard to all febrile cases presenting at the recruiting hospitals. There was a considerable loss to follow-up for the 28-day visit. Although this study had a significant sample size and number of primary outcome events overall, the statistical power for some secondary analyses was limited. Additionally, we lacked detailed information on barriers to seeking healthcare and when and where outpatients sought further care or were hospitalised, which could have provided further insights and allowed us to restrict events stringently to 28 days as we did with mortality. Furthermore, information on viral load or CD4 cells count for HIV-positive participants was unavailable, which would have better indicated participants with HIV-associated immunosuppression. Conversely, we relied on self-reported data regarding ART use and awareness of a previous HIV diagnosis. We also lacked data on HIV status confirmation by molecular methods for seropositive children aged <18 months and they were considered positive cases. All studied biomarkers, except lactate, were measured retrospectively on stored samples, which might have given slightly different results than if all the testing had been done at the point of care.

In conclusion, this study supports sTREM-1 as a robust predictor of mortality in both children and adults with all-cause fever and provides evidence of its association with other adverse outcomes. Moreover, it adds the novel finding that sTREM-1 prognostic performance may be diminished in HIV-positive individuals. While its application in different clinical scenarios warrants further investigation through interventional studies, measuring sTREM-1 in cases of suspected infection may facilitate timely risk stratification and management decisions with a positive impact on clinical outcomes.

FIGURES

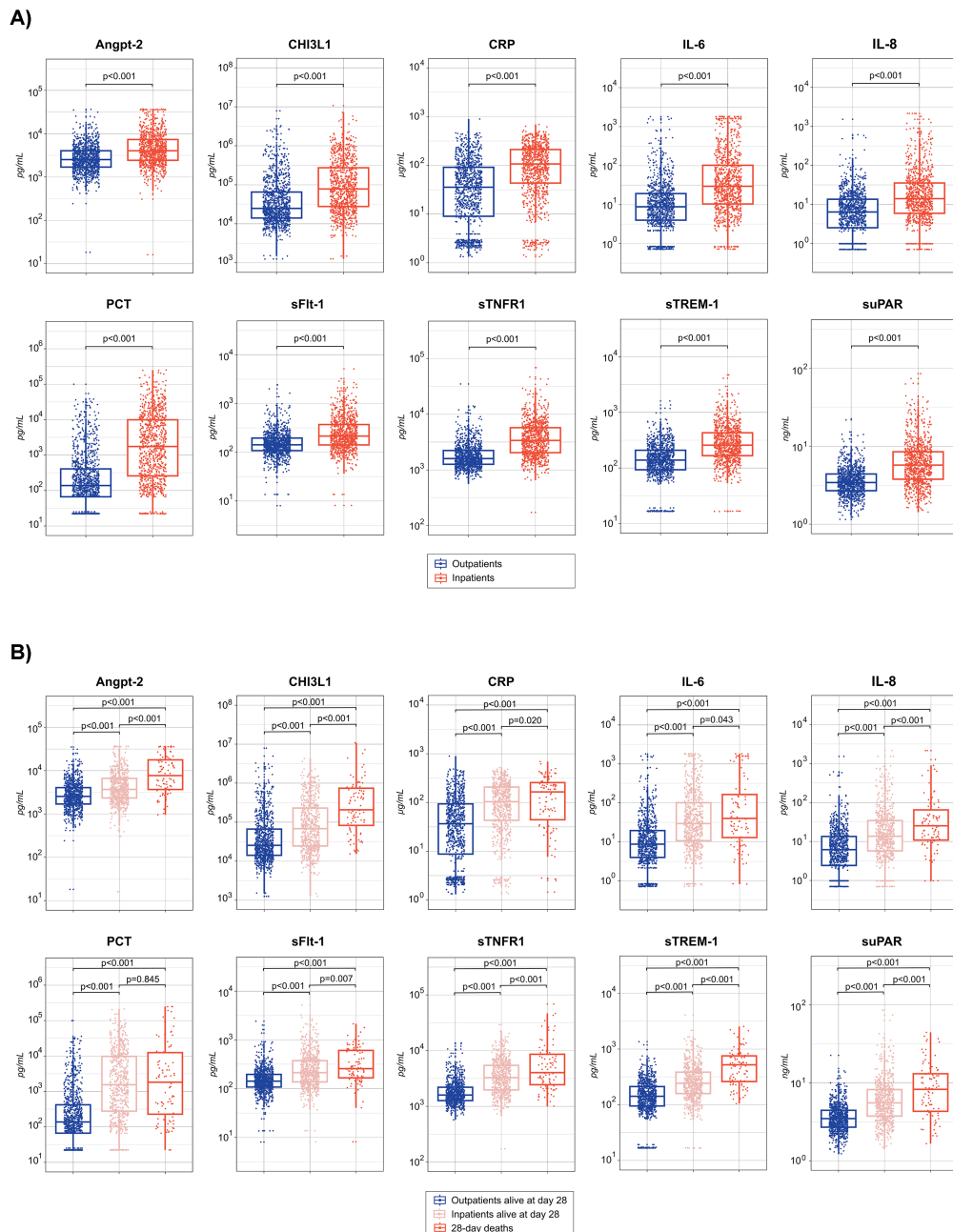


Figure 1. Plasma concentrations of host biomarkers at clinical presentation among enrolled paediatric and adult patients with febrile illness in southern Mozambique

Panel A) Concentrations in all outpatients and inpatients (N=1,955, except for suPAR N=1,939). Panel B) Concentrations in outpatients alive at day 28, inpatients alive at day 28, and deaths within 28 days (N=1,593, except for suPAR N=1,581). Boxplots with median and interquartile range. Whiskers represent one and a half times the interquartile range. Concentrations are in pg/mL, except CRP in $\mu\text{g/mL}$ and suPAR in ng/mL. All p-values were computed using Mann-Whitney U tests.

Abbreviations: Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFit-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

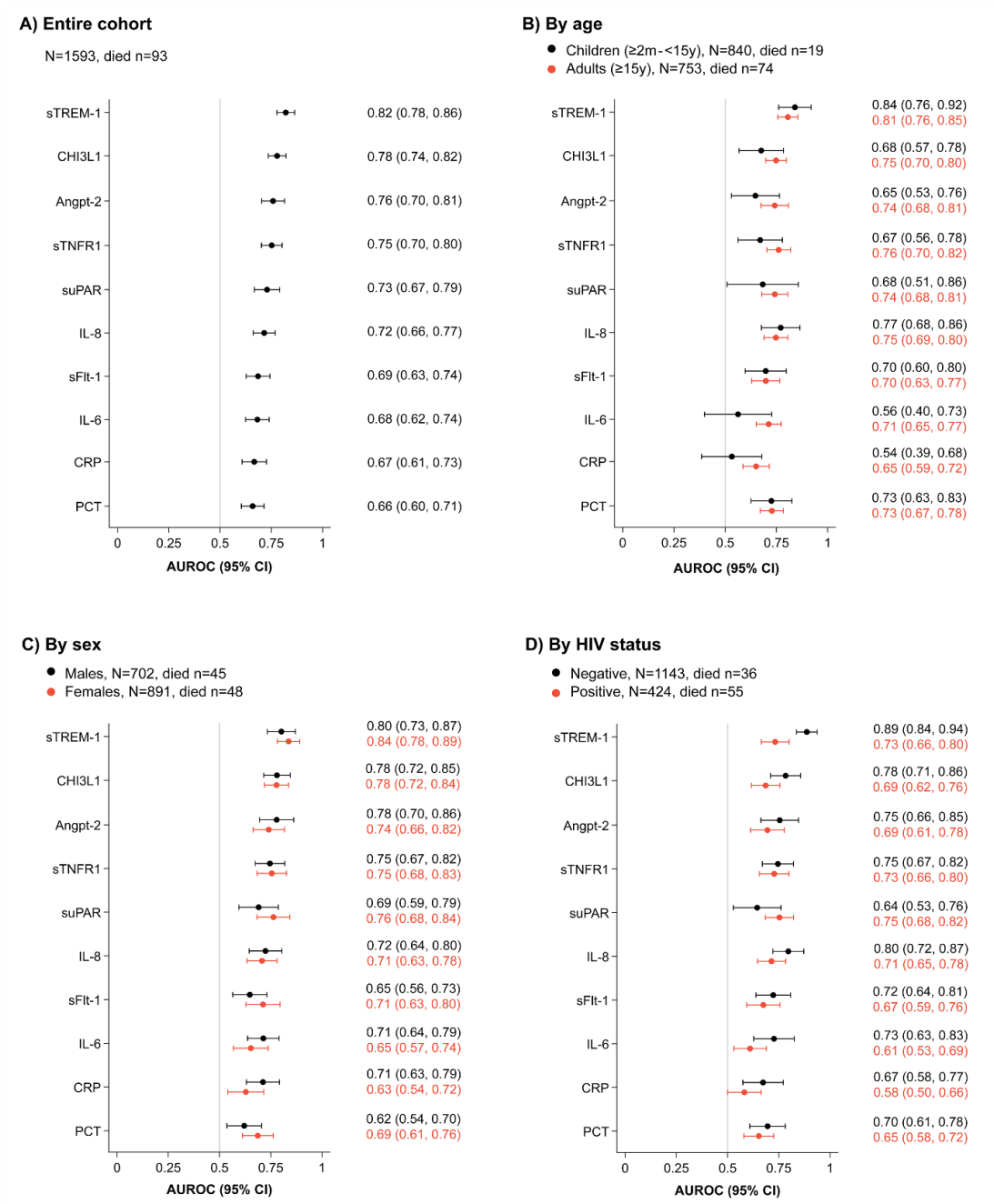


Figure 2. AUROC of each host biomarker for 28-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique

For suPAR, the total sample size is N=1,581 (91 deaths); analyses restricted to this population do not change the overall ranking of AUROCs or the sTREM-1 AUROCs.

Abbreviations: CI=confidence interval, Angpt-2=angiopoietin-2, AUROC=area under the receiver operating characteristic curve, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, HIV=human immunodeficiency virus, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFIt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

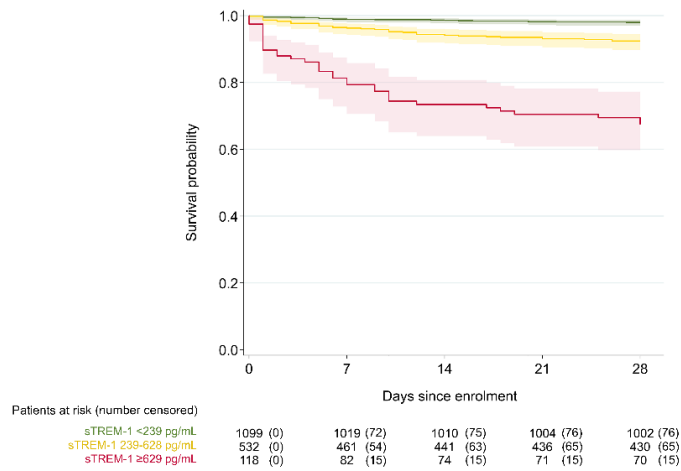
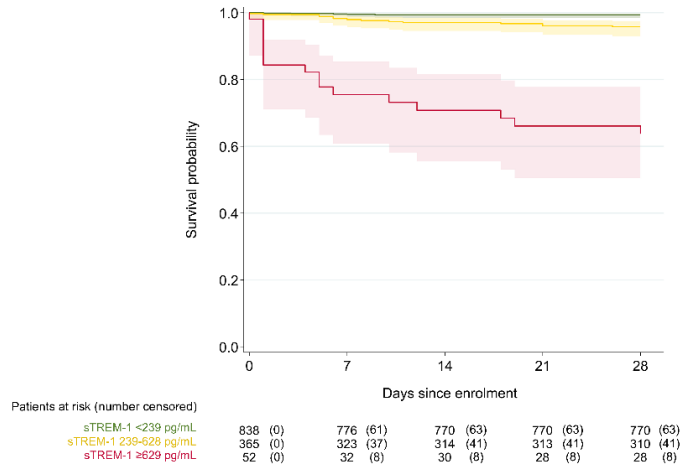
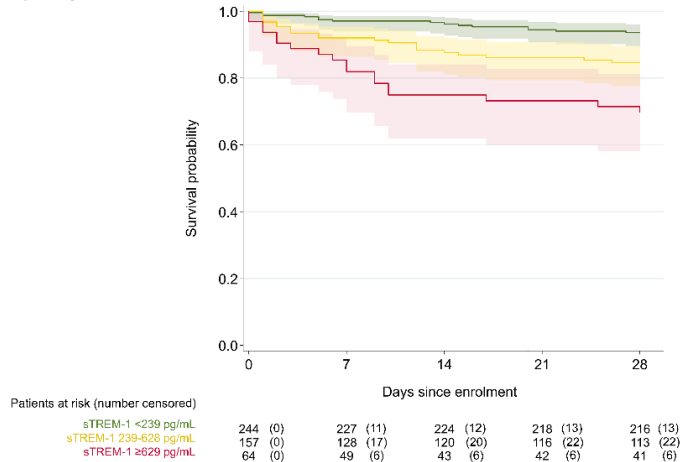
A) Entire cohort**B) HIV-negative status****C) HIV-positive status**

Figure 3. Kaplan-Meier curves for 28-day mortality by sTREM-1 categories among enrolled paediatric and adult patients with febrile illness in southern Mozambique

Inpatients with unknown vital status at day 28 were included in analyses and censored when discharged alive from hospital.

Abbreviations: HIV=human immunodeficiency virus, sTREM-1=soluble triggering receptor expressed on myeloid cells 1.

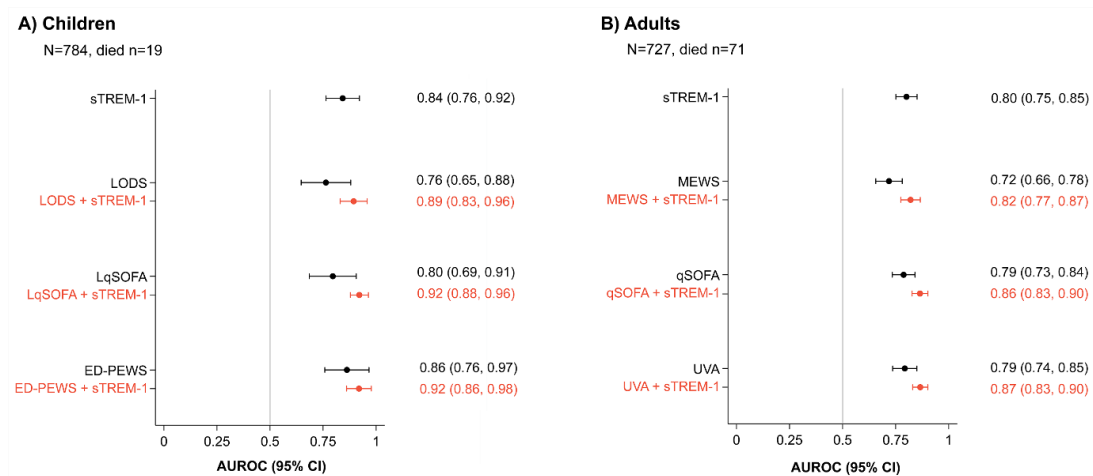


Figure 4. AUROC of each clinical severity score alone and combined with sTREM-1 for 28-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique

We included all participants with a known vital status at day 28 and clinical data for the calculation of all clinical severity scores.

Abbreviations: CI=confidence interval, AUROC=area under the receiver operating characteristic curve, ED-PEWS=Emergency Department Paediatric Early Warning Score, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MEWS=Modified Early Warning Score, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, UVA=and the Universal Vital Assessment.

TABLES

Table 1. Characteristics of enrolled paediatric and adult patients with febrile illness in southern Mozambique by age group and hospitalisation decision

	Entire cohort (N=1,955)	Child outpatients (N=531)	Child inpatients (N=509)	Adult outpatients (N=509)	Adult inpatients (N=406)
Demographics					
Female sex, n (%)	1,083 (55.4%)	260 (50.0%)	220 (43.2%)	384 (75.4%)	219 (53.9%)
Age in years, median (IQR)	11.2 (3.3, 33.0)	3.6 (1.8, 6.4)	3.4 (1.5, 7.0)	30.0 (23.0, 41.0)	38.0 (29.0, 53.0)
Site of enrolment, n (%)					
Manhiça District Hospital	1,706 (87.3%)	531 (100%)	412 (80.9%)	509 (100%)	254 (62.6%)
General José Macamo Hospital	249 (12.7%)	0 (0%)	97 (19.1%)	0 (0%)	152 (37.4%)
Days with fever before enrolment, median (IQR)	2 (1, 3)	1 (1, 2)	2 (1, 3)	2 (2, 3)	3 (2, 7)
Diagnostic tests at enrolment, n (%)					
HIV infection ^a	507/1,924 (26.4%)	14/528 (2.7%)	51/500 (10.2%)	214/496 (43.1%)	228/400 (57.0%)
Malaria RDT positivity ^b	309/1,939 (15.9%)	17/531 (3.2%)	221/501 (44.1%)	29/506 (5.7%)	42/401 (10.5%)
Clinical severity scores, median (IQR) ^c					
LODS	0 (0, 0)	0 (0, 0)	0 (0, 1)	-	-
LqSOFA	0 (0, 0)	0 (0, 0)	0 (0, 0)	-	-
ED-PEWS	8 (7, 11)	8 (7, 10)	9 (7, 16)	-	-
MEWS	3 (1, 5)	-	-	2 (1, 3)	4 (2, 5)
qSOFA	0 (0, 1)	-	-	0 (0, 0)	1 (0, 1)
UVA	2 (0, 2)	-	-	1 (0, 2)	2 (1, 3)

^a HIV status was considered positive if self-reported by the participant or confirmed through HIV point-of-care testing (using antibody-detecting rapid tests) performed to all participants with unknown or negative self-reported status.

^b Based on antigen-detecting lateral flow malaria RDT, which combines detection of histidine-rich protein 2 and *Plasmodium* lactate dehydrogenase.

^c LODS, LqSOFA, and ED-PEWS scores were calculated among children only. MEWS, qSOFA, and UVA scores were calculated among adults only.

Missing data: Days with fever before enrolment, n=88; HIV infection, n=31; malaria RDT positivity, n=16; LODS, n=56; LqSOFA, n=44; ED-PEWS, n=65; MEWS, n=4; qSOFA, n=4; UVA, n=29.

Abbreviations: ED-PEWS=Emergency Department Paediatric Early Warning Score, HIV=human immunodeficiency virus, IQR=interquartile range, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MEWS=Modified Early Warning Score, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, RDT=rapid diagnostic test, UVA=Universal Vital Assessment.

Table 2. Characteristics of enrolled paediatric and adult patients with febrile illness paediatric and adult patients with febrile illness in southern Mozambique by vital status at day 28

	Alive at day 28 (N=1,500)	Dead by day 28 (N=93)
Sex, n (%)		
Female	843 (94.6%)	48 (5.4%)
Male	657 (93.6%)	45 (6.4%)
Age in years		
Median (IQR)	10.4 (3.2, 31.0)	37.0 (24.0, 56.0)
Patient group, n (%)		
Child outpatients	426 (100%)	0 (0%)
Child inpatients	395 (95.4%)	19 (4.6%)
Adult outpatients	426 (95.5%)	2 (0.5%)
Adult inpatients	253 (77.9%)	72 (22.2%)
Site of enrolment, n (%)		
Manhiça District Hospital	1,366 (95.9%)	58 (4.1%)
General José Macamo Hospital	134 (79.3%)	35 (20.7%)
Days with fever before enrolment		
Median (IQR)	2 (1, 3)	3 (2, 7)
HIV status, n (%)^a		
Positive	369 (87.0%)	55 (13.0%)
Negative	1,107 (96.9%)	36 (3.2%)
Malaria status by RDT, n (%)^b		
Positive	246 (99.6%)	1 (0.4%)
Negative	1,245 (93.3%)	89 (6.7%)
LODS^c		
Median (IQR)	0 (0, 0)	1 (0, 2)
LqSOFA^c		
Median (IQR)	0 (0, 0)	1 (0, 1)
ED-PEWS^c		
Median (IQR)	8 (7, 11)	24 (16, 35)
MEWS^c		
Median (IQR)	3 (1, 4)	5 (3, 6)
qSOFA^c		
Median (IQR)	0 (0, 1)	1 (1, 2)
UVA^c		
Median (IQR)	2 (0, 2)	3 (2, 5)

^a HIV status was considered positive if self-reported by the participant or confirmed through HIV point-of-care testing (using antibody-detecting rapid tests) performed to all participants with unknown or negative self-reported status.

^b Based on antigen-detecting lateral flow malaria RDT, which combines detection of histidine-rich protein 2 and *Plasmodium* lactate dehydrogenase.

^c LODS, LqSOFA, and ED-PEWS scores were calculated among children only. MEWS, qSOFA, and UVA scores were calculated among adults only.

Missing data: Days with fever before enrolment, n=76; HIV status, n=26; malaria RDT status by RDT, n=12; LODS, n=42; LqSOFA, n=34; ED-PEWS, n=47; MEWS, n=3; qSOFA, n=3; UVA, n=26.

Abbreviations: ED-PEWS=Emergency Department Paediatric Early Warning Score, HIV=human immunodeficiency virus, IQR=interquartile range, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MEWS=Modified Early Warning Score, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, RDT=rapid diagnostic test, UVA=Universal Vital Assessment.

Table 3. Association of host biomarker levels in plasma at clinical presentation with non-mortality outcomes among enrolled paediatric and adult patients with febrile illness in southern Mozambique

	Length of hospital stay in inpatients discharged home (N=675)		Seeking further care for the same illness by the follow-up visit in outpatients (N=838)			Subsequent hospitalisation for the same illness by the follow-up visit in outpatients (N=838)		
Biomarker ^a	Spearman's rank correlation coefficient (p)	p-value	Did not seek care (N=780), median (IQR)	Sought care (N=58), median (IQR)	p-value	No hospitalisation (N=828), median (IQR)	Hospitalisation (N=10), median (IQR)	p-value
Angpt-2	0.18	<0.001	2552.2 (1680.9, 4023.1)	2579.6 (1928.4, 4529.8)	0.236	2537.5 (1703.2, 4037.1)	4243.1 (2522.3, 6634.0)	0.138
CHI3L1	0.24	<0.001	24280.0 (13879.7, 67348.4)	28446.8 (17469.3, 60298.5)	0.171	24739.2 (14070.8, 66283.2)	50972.3 (33465.4, 172075.8)	0.110
CRP	0.01	0.775	35.6 (8.7, 91.8)	52.8 (16.6, 121.5)	0.083	36.0 (8.7, 93.1)	79.0 (55.7, 189.6)	0.012
IL-6	-0.03	0.431	8.5 (4.0, 19.5)	11.6 (5.0, 26.9)	0.118	8.6 (4.0, 19.6)	17.6 (12.3, 33.3)	0.008
IL-8	0.05	0.168	6.2 (2.3, 13.5)	5.7 (3.3, 16.0)	0.447	6.1 (2.4, 13.5)	9.5 (6.8, 44.4)	0.080
PCT	-0.07	0.080	128.9 (45.7, 396.8)	234.3 (89.7, 1029.5)	0.006	131.7 (66.3, 405.2)	1011.6 (487.1, 3113.5)	0.001
sFit-1	-0.10	0.010	139.7 (106.7, 193.4)	155.3 (116.3, 221.6)	0.055	142.1 (107.7, 195.2)	175.4 (107.2, 221.6)	0.313
sTNFR1	0.00	0.940	1574.1 (1256.6, 2166.1)	1711.7 (1445.8, 2532.2)	0.011	1584.3 (1264.9, 2180.8)	2672.3 (1752.6, 4312.3)	0.001
sTREM-1	0.34	<0.001	138.9 (93.4, 208.2)	168.6 (123.5, 257.8)	0.002	140.4 (94.2, 210.2)	214.4 (155.8, 371.3)	0.012
suPAR	0.01	0.882	3.4 (2.7, 4.4)	4.1 (3.2, 5.3)	0.004	3.5 (2.7, 4.4)	5.4 (4.2, 8.9)	0.002

^a Concentrations are in pg/mL, except CRP in µg/mL and suPAR in ng/mL. All included patients had complete biomarker data.

Abbreviations: Angpt-2=angiotensinogen-converting enzyme 2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, IQR=interquartile range, PCT=procalcitonin, sFit-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

FUNDING

The FIEBRE study is funded by UK aid from the UK government; the views expressed, however, do not necessarily reflect the UK government's official policies. Biomarker analyses were supported by the Canadian Institutes of Health Research (CIHR) Foundation grant FDN-148439 (KCK), the Canada Research Chair Program (KCK), and donations from Kim Kertland and the Tesari Foundation. ISGlobal receives support from the grant CEX2023-0001290-S funded by MCIN/AEI/10.13039/501100011033, and support from the Generalitat de Catalunya through the CERCA Program. CISM is supported by the Government of Mozambique and the Spanish Agency for International Development (AECID). NB is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260). BB is a Beatriu de Pinós postdoctoral fellow granted by the Government of Catalonia's Secretariat for Universities and Research, and by Marie Skłodowska-Curie Actions COFUND Program (BP3, 801370). JB is supported by a career development grant from the EDCTP2 program supported by the European Union (TMA2019SFP-2836 TB lung-FACT2). The funders had no role in the study design; data collection, analysis, or interpretation; writing of the report; or decision to submit the paper for publication.

ACKNOWLEDGEMENTS

We are grateful to children and adults who participated in the study. We also thank all FIEBRE collaborators and staff from Manhíça District Hospital and General José Macamo Hospital who supported this study.

COMPETING INTERESTS

KCK is a named inventor on a patent "Biomarkers for early determination of a critical or life-threatening response to illness and/or treatment response" held by the University Health Network. The remaining authors declare no competing interests.

DATA AVAILABILITY

The de-identified dataset, along with the corresponding data dictionary that defines each field in the set, are freely available with no restrictions via LSHTM's Data Compass and can be accessed and downloaded at <http://doi.org/10.17037/DATA.00004583>

AUTHOR CONTRIBUTIONS

HH, DCWM, SY, and QB were part of the co-investigator group who conceived and designed the larger FIEBRE study on which this work was based. QB and KCK conceived this substudy and supervised it. NB, BB, SA, TM, KCK, and QB contributed to study design. SA, JB, and MV coordinated the study implementation in Mozambique. SA, ZB, JB, AC, and MV were involved in participant recruitment and data collection in Mozambique. NB, BB, HM, AMW, JKW, and KZ participated in biomarker quantification. NB, SA, EJAF, HH, SHK, SL, and CM were involved in data curation and management. NB led the analysis and interpretation of the data, and received input from all authors. NB and SA have access to and have verified all data used in the analysis, and EJAF and SHK have access to and have verified all FIEBRE clinical data. All authors had full access to all the data in the study and accept responsibility to submit for publication. NB prepared the first draft of the manuscript and all authors reviewed and edited it. All authors read and approved the final manuscript.

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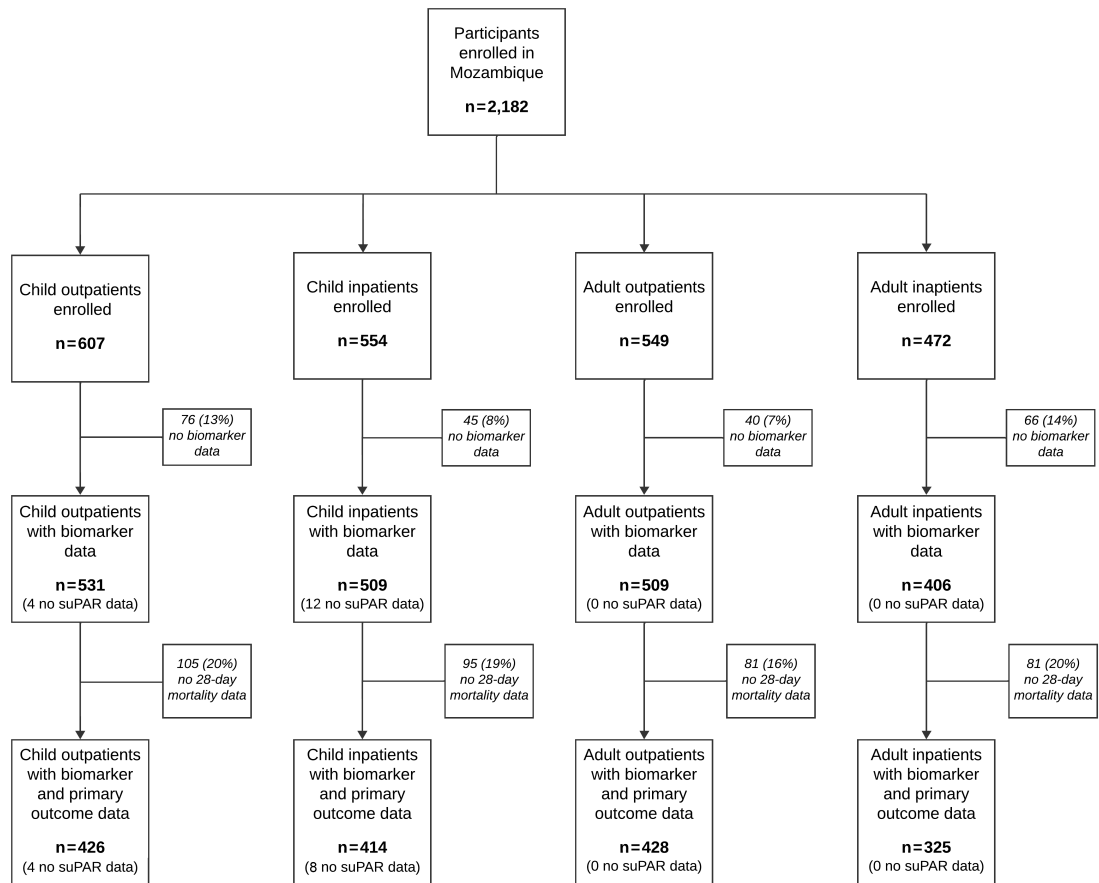
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SUPPLEMENTARY MATERIAL

Soluble triggering receptor expressed on myeloid cells 1 (sTREM-1)
to risk-stratify paediatric and adult patients with febrile illness in southern Mozambique

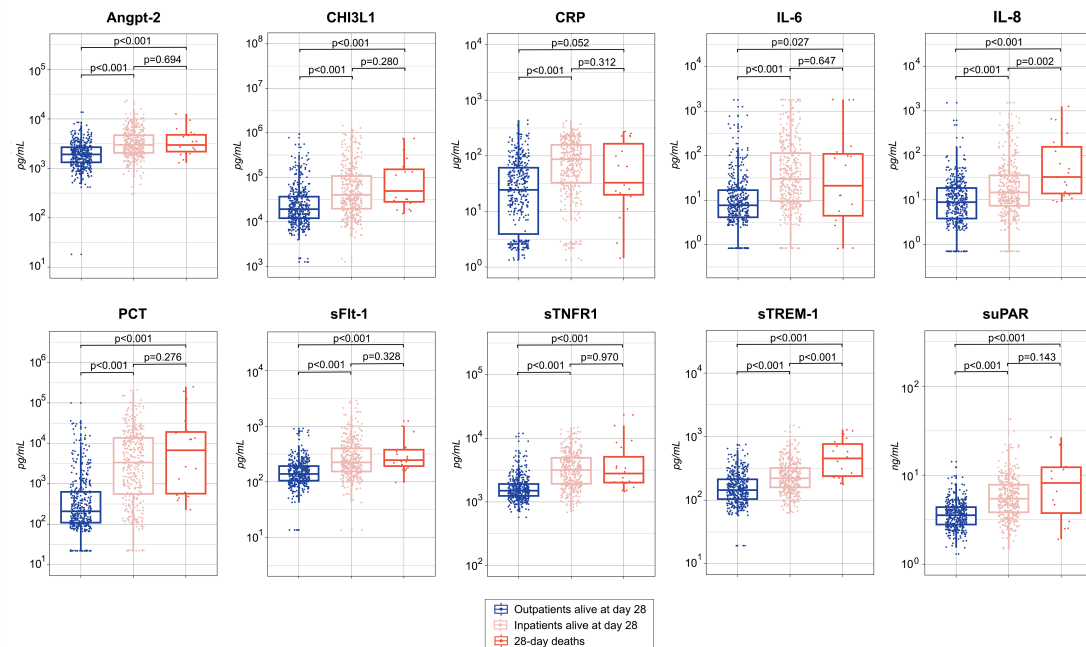
I. SUPPLEMENTARY FIGURES

Supplementary Figure 1. Flowchart of study participants

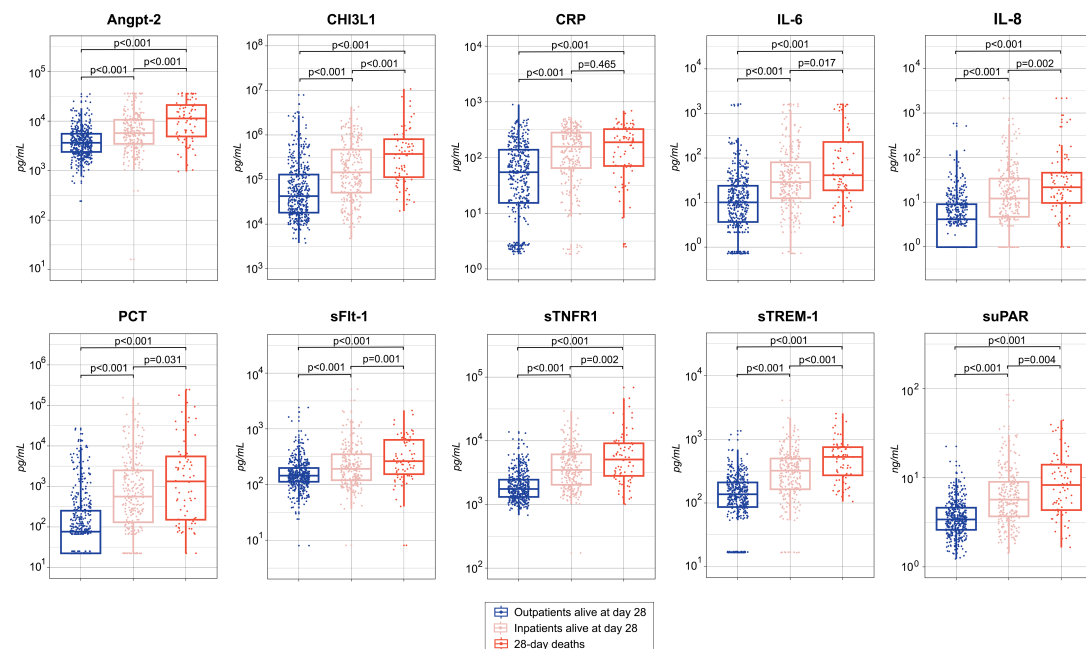


Supplementary Figure 2. Plasma concentrations of host biomarkers at clinical presentation among enrolled paediatric and adult patients with febrile illness in southern Mozambique in relation to hospitalisation decision and vital status at day 28, by age group

A) Children



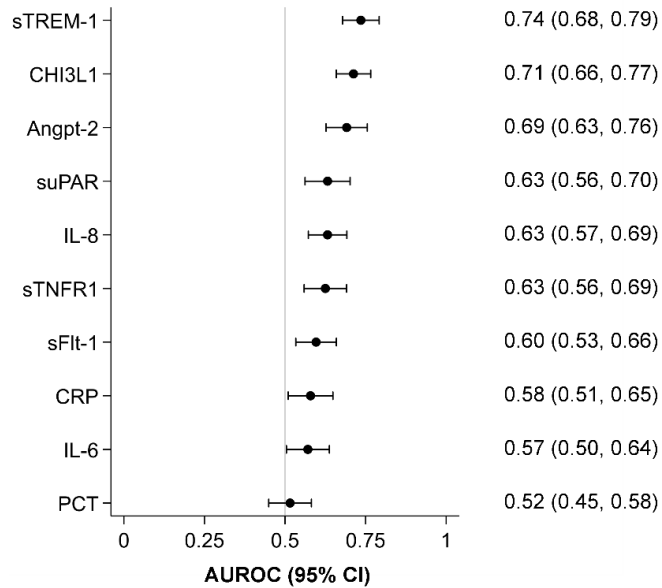
B) Adults



Concentrations in outpatients alive at day 28, inpatients alive at day 28, and deaths within 28 days in children (Panel A) (N=840, except for suPAR N=828) and in adults (Panel B) (N=753). Boxplots with median and interquartile range. Whiskers represent one and a half times the interquartile range. Concentrations are in pg/mL, except CRP in µg/mL and suPAR in ng/mL. All p-values were computed using Mann-Whitney U tests.

Abbreviations: Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

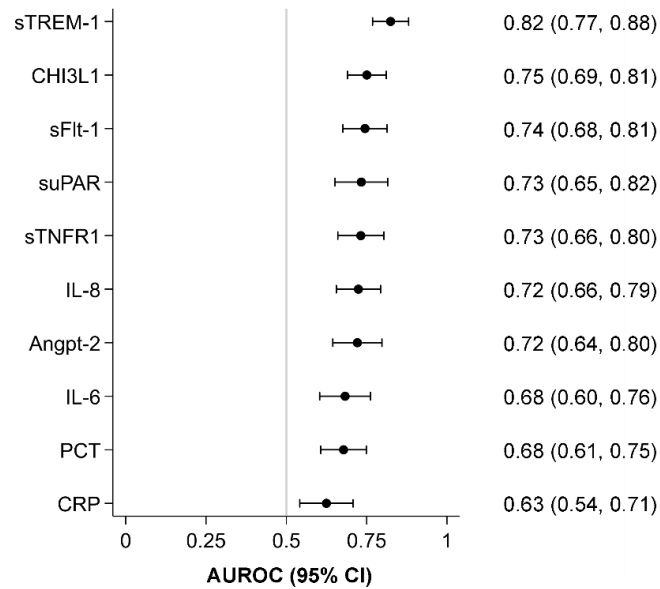
Supplementary Figure 3. AUROC of each host biomarker for 28-day mortality among enrolled paediatric and adult inpatients with febrile illness in southern Mozambique



The total sample size is N=739 participants (91 deaths). For suPAR, N=731 (89 deaths); analyses restricted to this population do not change the overall ranking of AUROCs and the sTREM-1 AUROC remains 0.74 (95% CI: 0.68-0.79).

Abbreviations: CI=confidence interval, Angpt-2=angiopoietin-2, AUROC=area under the receiver operating characteristic curve, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

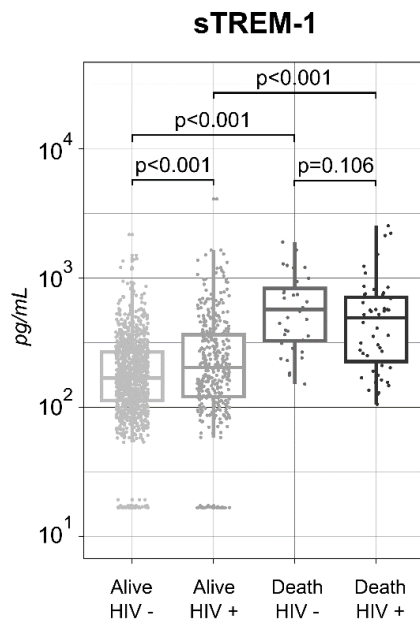
Supplementary Figure 4. AUROC of each host biomarker for 7-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique



The total sample size is N=1,608 participants (54 deaths). For suPAR, N=1,596 (53 deaths); analyses restricted to this population do not change the overall ranking of AUROCs and the sTREM-1 AUROC remains 0.82 (95% CI: 0.77-0.88).

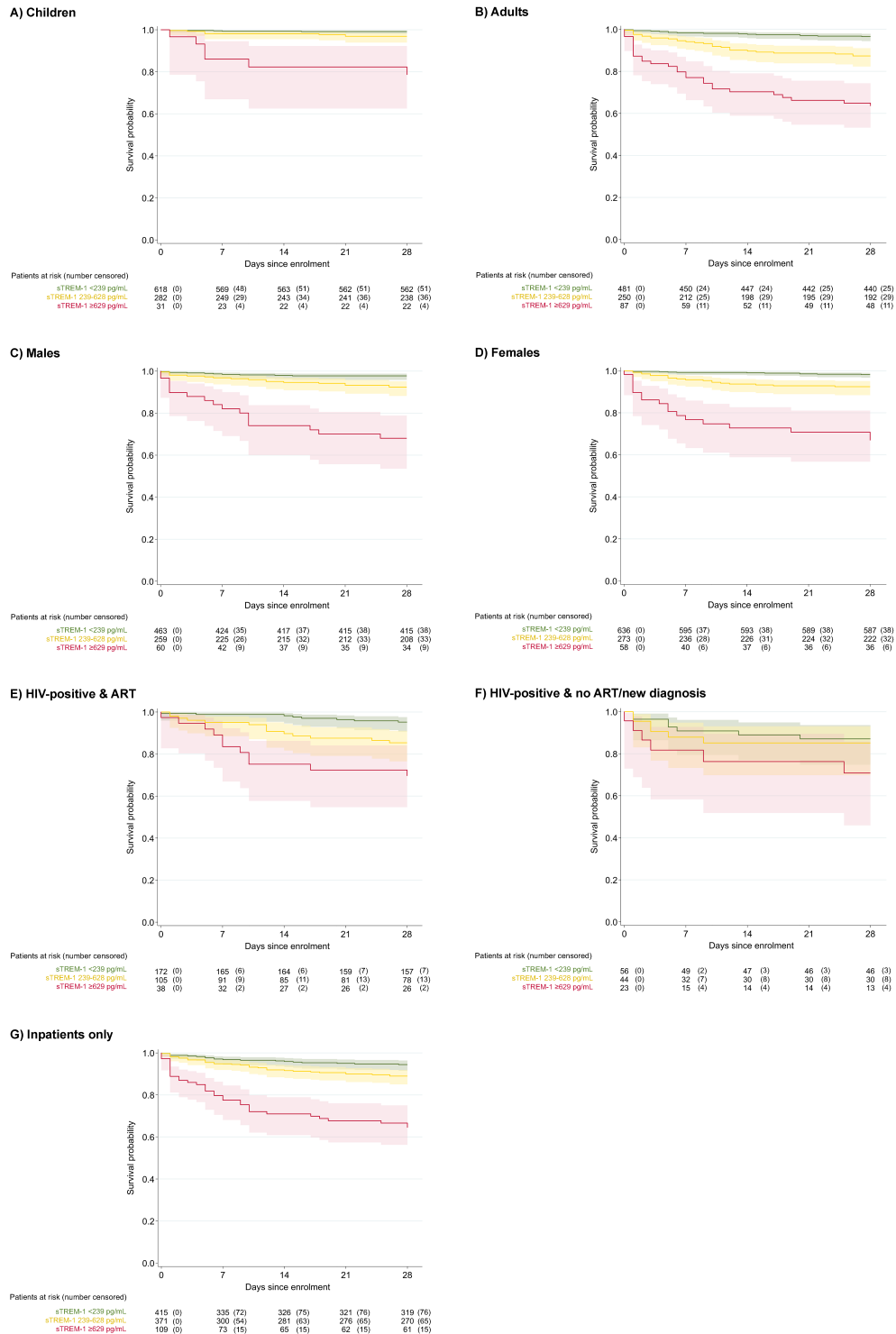
Abbreviations: CI=confidence interval, Angpt-2=angiopoietin-2, AUROC=area under the receiver operating characteristic curve, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

Supplementary Figure 5. sTREM-1 concentrations by vital status at day 28 and HIV status among enrolled paediatric and adult patients with febrile illness in southern Mozambique



Abbreviations: HIV=human immunodeficiency virus, sTREM-1=soluble triggering receptor expressed on myeloid cells 1.

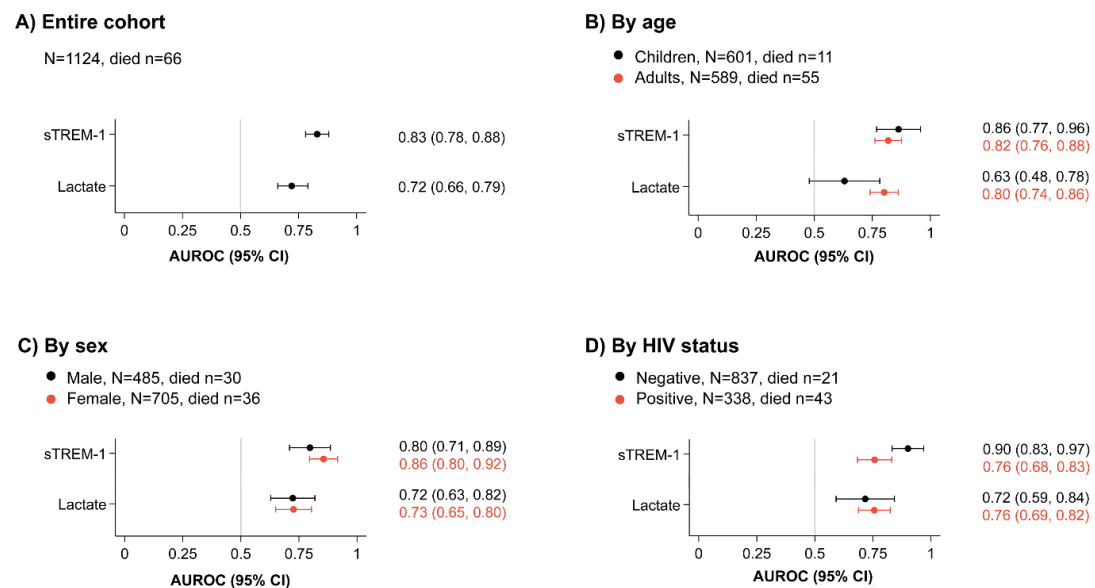
Supplementary Figure 6. Kaplan-Meier curves for 28-day mortality by sTREM-1 categories in different patient subgroups of enrolled paediatric and adult patients with febrile illness in southern Mozambique



Inpatients with unknown vital status at day 28 were included in analyses and censored when discharged alive from hospital.

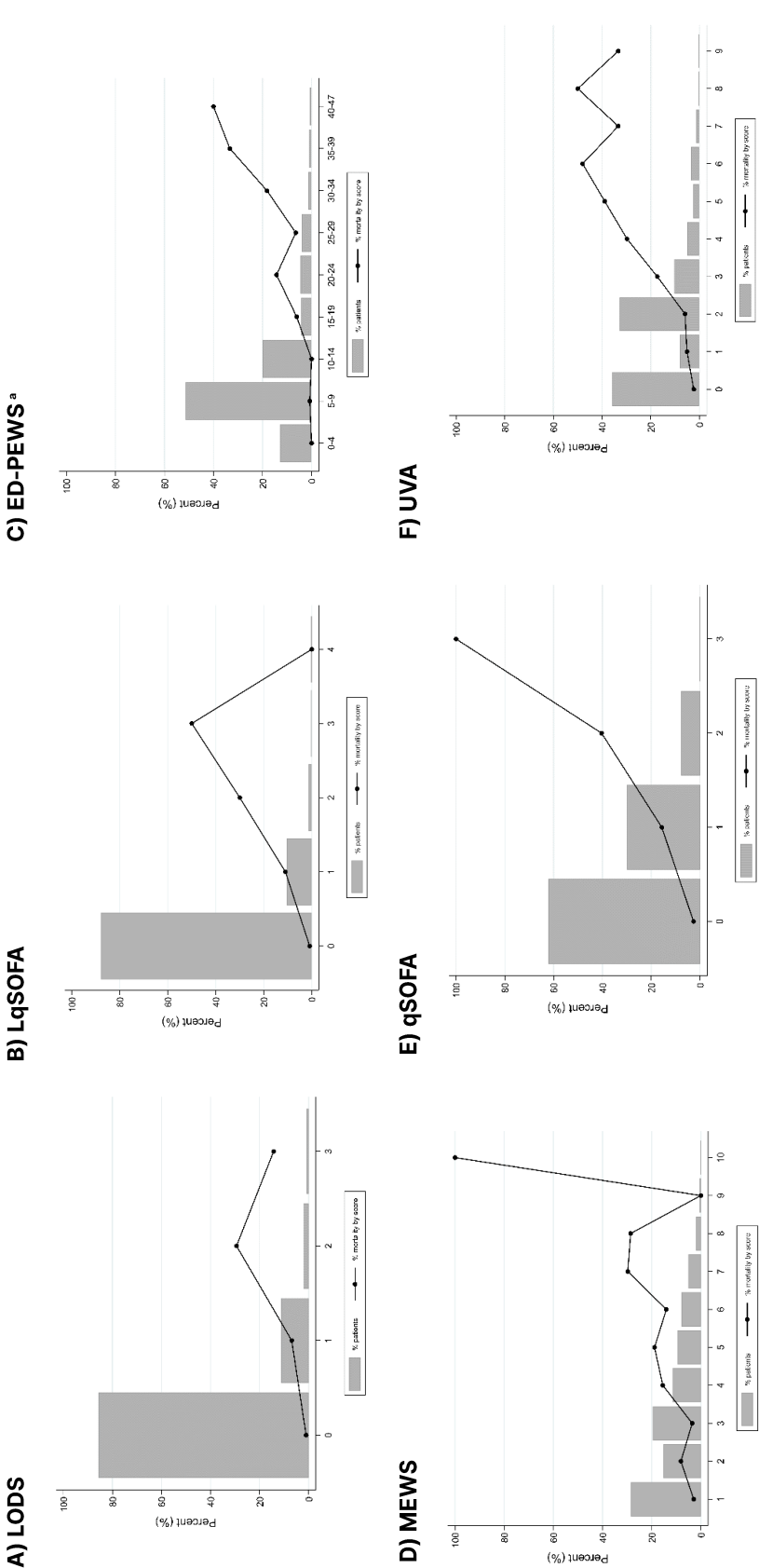
Abbreviations: ART=antiretroviral therapy, HIV=human immunodeficiency virus, sTREM-1=soluble triggering receptor expressed on myeloid cells 1.

Supplementary Figure 7. AUROC of sTREM-1 and lactate for 28-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique



Abbreviations: CI=confidence interval, AUROC=area under the receiver operating characteristic curve, HIV=human immunodeficiency virus, sTREM-1=soluble triggering receptor expressed on myeloid cells 1.

Supplementary Figure 8. The percentage distribution of each severity score and associated 28-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique



^a ED-PEWS scores were grouped in categories to improve visualization. We included all participants with a known vital status at day 28 and clinical data for the calculation of all clinical severity scores. For children the total sample size is N=784 (Panels A-C), and for adults the total sample size is N=727 (Panels D-F).

Abbreviations: ED-PEWS=Emergency Department Paediatric Early Warning Score, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MEWS=Modified Early Warning Score, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, UVA=Universal Vital Assessment.

II. SUPPLEMENTARY TABLES

Supplementary Table 1. Components of the different clinical severity scores used and their threshold values and points allocated

CHILDREN			ADULTS		
Lambaréné Organ Dysfunction Score (LODS)	Liverpool quick Sequential Organ Failure Assessment (LqSOFA)	Emergency Department Paediatric Early Warning Score (ED-PEWS)	Modified early warning score (MEWS)	Quick Sequential (Sepsis-Related) Organ Failure Assessment score (qSOFA)	Universal Vital Assessment (UVA)
Derived with data from 23,890 children ≤15 years old with severe <i>P. falciparum</i> malaria hospitalized in one of the 6 participating African hospitals located in the Gambia, Malawi, Kenya, Ghana, and Gabon. Primary outcome: in-hospital mortality. ¹	Derived with data from 1,121 febrile children <16 years old attending the emergency department and requiring a blood test at a specialist paediatric hospital in the United Kingdom. Primary outcome: critical care admission within 48 hours of emergency department attendance. Secondary outcome: sepsis-related mortality. ²	Derived with data from 19,209 children <16 years old attending five diverse emergency departments in Netherlands, United Kingdom, Austria, and Portugal. Primary outcome: a three-category reference standard (high, intermediate, low urgency) as a proxy for true patient urgency. Secondary outcomes: hospital admission and intensive care unit admission. ³	Based on a modified version of the early warning signs score (EWS). ⁴	Based on the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3) Task Force. It provides simple bedside criteria to identify adult patients with suspected infection who are likely to have poor outcomes. ⁵	Derived with data from 5,573 patients admitted to hospitals in Gabon, Malawi, Sierra Leone, Tanzania, Uganda, and Zambia. Primary outcome: in-hospital mortality. ⁶
Range: 0-3 points	Range: 0-4 points	Range: 0-68 points	Range: 0-14 points	Range: 0-3 points	Range: 0-12 points
Coma (BCS ≤2): • No: 0 points • Yes: 1 point	Consciousness (AVPU scale) ⁴ : • A: 0 points • V, P or U: 1 point	Age: • 0-4: 0 points • 5-11: 4 points • 12-16: 6 points	Temperature: • ≥38.5: 2 points • 35-38.4: 0 points • <35: 2 points	Respiratory rate (breaths/min): • ≥22: 1 point • <22: 0 points	Temperature • ≥36: 0 points • <36: 2 points
Prostration (not being able to breast-feed, sit, stand, or walk, depending on the age of the child): • No: 0 points • Yes: 1 point	Capillary refill time: • <3 s: 0 points • ≥3 s: 1 point	Consciousness: • Normal: 0 points • Decreased: 14 points	Heart rate (beats/min): • ≥130: 3 points • 111-129: 2 points • 101-110: 1 point • 51-100: 0 points • 41-50: 1 point • ≤40: 2 points	Systolic blood pressure (mmHg): • ≤100: 1 point • >100: 0 points	Heart rate (beats/min): • ≥120: 1 point • <120: 0 points
Deep breathing: • No: 0 points • Yes: 1 point	Respiratory rate: • ≤99 th centile Bonafide <i>et al.</i> age-specific thresholds: 0 points ⁷ • >99 th centile Bonafide <i>et al.</i> age-specific thresholds: 1 point ⁷	Work of breathing ⁸ : • Normal: 0 points • Increased: 12 points	Respiratory rate (breaths/min): • ≥30: 3 points • 21-29: 2 points • 15-20: 1 point • 9-14: 0 points • <9: 2 points	Consciousness (GCS): • <15: 1 point • 15: 0 points	Respiratory rate (breaths/min): • ≥30: 1 point • <30: 0 points
	Heart rate: • ≤99 th centile Bonafide <i>et al.</i> age-specific thresholds: 0 points ⁷ • >99 th centile Bonafide <i>et al.</i> age-specific thresholds: 1 point ⁷	Oxygen saturation (%): • ≥98: 0 points • 94-97: 4 points • 88-93: 9 points • <88: 15 points	Consciousness (AVPU) ⁴ : • U: 3 points • P: 2 points • V: 1 point • A: 0 points		Systolic blood pressure (mmHg): • ≥90: 0 points • <90: 1 point

		Respiratory rate (breaths/min): • <30: 0 points • 30-39: 3 points • 40-59: 5 points • ≥60: 9 points	Systolic blood pressure (mmHg): • ≥200: 2 points • 101-199: 0 points • 81-100: 1 point • 71-80: 2 points • ≤70: 3 points		Oxygen saturation (%): • ≥92: 0 points • <92: 2 points
		Heart rate (beats/min): • <100: 0 points • 100-139: 3 points • 140-179: 6 points • ≥180: 9 points			Consciousness (GCS): • <15: 4 points • 15: 0 points
		Capillary refill time: • Normal: 0 points • Increased: 3 points			HIV status: • Positive: 2 points • Negative/unknown: 0 points

^aConsciousness status was measured in Mozambique using the classical BCS in children and GCS in adults. BCS and GCS were converted to AVPU for LqSOFA and MEWS score calculation.

^bHere defined as difficult breathing, indrawing, deep breathing, or grunting.

Abbreviations: AVPU=(A [alert], V [response on verbal stimuli], P [response on pain stimuli], and U [unresponsive]), BCS=Blantyre Coma Scale, GCS=Glasgow Coma Scale, HIV=human immunodeficiency virus.

Supplementary Table 2. Percentage and distribution of biomarker values outside the dynamic range

Biomarker	Number of samples outside the dynamic range, n/N (%)	Number of samples below	Number of samples above
Luminex:			
Angpt-2	31/1,955 (1.6%)	2	29
CHI3L1	11/1,955 (0.6%)	11	0
IL-6	210/1,955 (10.7%)	170	40
IL-8	326/1,955 (16.7%)	316	10
PCT	309/1,955 (15.8%)	306	3
sFlt-1	15/1,955 (0.8%)	15	0
sTNFR1	1/1,955 (0.1%)	1	0
sTREM-1	90/1,955 (4.6%)	90	0
ELISA:			
CRP	301/1,955 (15.4%)	301	0
suPAR	0/1,939 (0%)	0	0

Abbreviations: Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, ELISA=enzyme-linked immunosorbent assay, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

Supplementary Table 3. Demographic and clinical characteristics of enrolled paediatric patients with febrile illness in southern Mozambique by vital status at day 28

Variable	Children alive at day 28 (N=821)	Children dead by day 28 (N=19)
Sex, n (%)		
Female	443 (98.0%)	9 (2.0%)
Male	378 (97.4%)	10 (2.6%)
Age (years)		
Median (IQR)	3.6 (1.8, 6.8)	1.4 (0.7, 4.2)
Age group, n (%)		
<1	96 (94.1%)	6 (5.9%)
1 - <5	416 (97.9%)	9 (2.1%)
5- <15	309 (98.7%)	4 (1.3%)
Patient group, n (%)		
Inpatient	395 (95.4%)	19 (4.6%)
Outpatient	426 (100%)	0 (0%)
Site of enrolment, n (%)		
Manhiça District Hospital	60 (83.3%)	12 (16.7%)
Geral José Macamo Hospital	761 (99.1%)	7 (0.9%)
Length of hospital stay among inpatients (days)		
Median (IQR)	2 (2, 4)	3 (1, 5)
Temperature (°C)		
Median (IQR)	38.5 (37.9, 39.1)	38.5 (37.6, 39.0)
Days with fever before enrolment		
Median (IQR)	2 (1, 2)	2 (2, 3)
HIV status, n (%)		
Positive	46 (86.8%)	7 (13.2%)
Negative	767 (98.6%)	11 (1.4%)
Malaria status by RDT, n (%)		
Positive	187 (100%)	0 (0%)
Negative	630 (97.4%)	17 (2.6%)
Severe acute malnutrition (WHZ/WLZ <-3 or MUAC <11.5 cm), n(%) ^a		
Yes	36 (81.8%)	8 (18.2%)
No	595 (99.2%)	5 (0.8%)
Increased respiratory rate, n (%) ^b		
Yes	164 (93.7%)	11 (6.3%)
No	655 (98.8%)	8 (1.2%)
Systolic blood pressure, n (%) ^c		
Hypotension	131 (97.0%)	4 (3.0%)
Normotension	501 (98.2%)	9 (1.8%)
Hypertension	103 (97.2%)	3 (2.8%)
Heart rate, n (%) ^d		
Bradycardia	25 (96.2%)	1 (3.9%)
Normocardia	619 (98.4%)	10 (1.6%)
Tachycardia	173 (95.6%)	8 (4.4%)
Capillary refill time (seconds)		
Median (IQR)	1 (1, 2)	2 (1, 2)
Work of breathing, n (%) ^e		
Increased	85 (88.5%)	11 (11.5%)
Normal	725 (98.9%)	8 (1.1%)
Deep breathing, n (%)		
Yes	25 (92.6%)	2 (7.4%)
No	790 (97.9%)	17 (2.1%)
Oxygen saturation		
Median (IQR)	98 (97, 99)	98 (94, 100)

Altered mental status (BCS <5), n (%)		
Yes	33 (76.7%)	10 (23.3%)
No	760 (98.8%)	9 (1.2%)
Coma (BCS ≤2), n (%)		
Yes	11 (68.8%)	5 (31.3%)
No	782 (98.2%)	14 (1.8%)
Prostration, n (%)		
Yes	92 (88.5%)	12 (11.5%)
No	713 (99.0%)	7 (1.0%)
LODS		
Median (IQR)	0 (0, 0)	1 (0, 2)
LqSOFA		
Median (IQR)	0 (0, 0)	1 (0, 1)
ED-PEWS		
Median (IQR)	8 (7, 11)	24 (16, 35)
Time to death (days)		
Median (IQR)	NA	5 (3, 16)
In-hospital outcomes, n (%)		
Death	NA	5 (100%)
Discharge home	362 (97.7%)	1 (0.3%)
Absconder	0 (0%)	0 (0%)
Transfer	15 (55.6%)	12 (44.4%)

*Calculated using the WHO Child Growth Standards and the WHO Reference 2007.

^aIncreased respiratory rate was defined as ≥50 breaths/min in children aged 2–11 months, ≥40 breaths/min in children aged 12–59 months, ≥30 breaths/min in children aged 5–12 years, and ≥20 breaths/min in children aged 13–<15 years.

^cHypertension and hypotension were defined if above or under the normal ranges of: 70–100 mmHg for children aged 2–11 months, 75–105 for children aged 1–2 years, 80–110 mmHg for children aged 3–5 years, 85–115 for children aged 6–9 years, and 90–120 mmHg for children aged 10–<15 years.

^dTachycardia and bradycardia were defined if above or under the normal ranges of: 100–160 beats/min for children aged 2–11 months, 90–150 beats/min for children aged 1–2 years, 80–140 beats/min for children aged 3–5 years, 70–120 beats/min for 6–12 years, and 60–100 beats/min for children aged 13–<15 years.

^eDefined as difficult breathing, indrawing, deep breathing, or grunting.

Missing data: Length of hospital stay among inpatients, n=32 (out of 414 inpatients); days with fever before enrolment, n=15; HIV status, n=9; malaria status by RDT, n=6; severe acute malnutrition, n=196; increased respiratory rate, n=2; systolic blood pressure, n=89; heart rate, n=4; capillary refill time, n=5; work of breathing, n=6; deep breathing, n=6; oxygen saturation, n=14; altered mental status, n=28; coma, n=28; prostration, n=16; LODS, n=42; LqSOFA, n=34; ED-PEWS, n=47; in-hospital outcome, n=19 (out of 414 inpatients).

Abbreviations: BCS=Blantyre Coma Scale, ED-PEWS=Emergency Department Paediatric Early Warning Score, HIV=human immunodeficiency virus, IQR=interquartile range, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MUAC=mid-upper arm circumference, RDT=rapid diagnostic test, WHZ/WLZ=weight-for-height/length z-score.

Supplementary Table 4. Demographic and clinical characteristics of enrolled adult patients with febrile illness in southern Mozambique by vital status at day 28

Variable	Adults alive at day 28 (N=679)	Adults dead by day 28 (N=74)
Sex, n (%)		
Female	214 (85.6%)	36 (12.4%)
Male	465 (92.4%)	38 (7.6%)
Age (years)		
Median (IQR)	34.0 (25.0, 44.0)	42.5 (33.0, 60.5)
Age group, n (%)		
15 - <25	166 (97.1%)	5 (2.9%)
25 - <35	188 (92.2%)	16 (7.8%)
35 - <45	157 (88.2%)	21 (11.8%)
45 - <55	73 (91.3%)	7 (8.8%)
55 - <65	60 (78.9%)	16 (21.1%)
65+	35 (79.5%)	9 (20.5%)
Patient group, n (%)		
Inpatient	253 (77.8%)	72 (22.2%)
Outpatient	426 (99.5%)	2 (0.5%)
Site of enrolment, n (%)		
Manhiça District Hospital	605 (92.2%)	51 (7.8%)
Geral José Macamo Hospital	74 (76.3%)	23 (23.7%)
Length of hospital stay among inpatients (days)		
Median (IQR)	3.5 (1, 7)	3 (1, 7)
Temperature (°C)		
Median (IQR)	38.0 (37.7, 38.6)	38.2 (37.9, 38.9)
Days with fever before enrolment		
Median (IQR)	3 (2, 4)	4 (2, 7)
HIV status, n (%)		
Positive	323 (87.1%)	48 (12.9%)
Negative	340 (93.2%)	25 (6.8%)
Malaria status by RDT, n (%)		
Positive	59 (98.3%)	1 (1.7%)
Negative	615 (89.5%)	72 (10.5%)
BMI		
Median (IQR)	22.5 (20.1, 25.7)	22.1 (18.8, 25.4)
Respiratory rate (breaths/min)		
Median (IQR)	19 (18, 20)	22 (20, 28)
Systolic blood pressure (mmHg)		
Median (IQR)	117 (106, 130)	115 (96, 131)
Heart rate (beats/min)		
Median (IQR)	102 (90, 116)	111 (94, 125)
Capillary refill time (seconds)		
Median (IQR)	1 (1, 2)	2 (1, 3)
Oxygen saturation		
Median (IQR)	98 (96, 99)	95 (90, 97)
Altered mental status (GCS <15), n (%)		
Yes	27 (52.2%)	19 (41.3%)
No	652 (92.2%)	55 (7.8%)
Coma (GCS ≤8), n (%)		
Yes	3 (27.3%)	8 (72.7%)
No	676 (91.1%)	66 (8.9%)
MEWS		
Median (IQR)	3 (1, 4)	5 (3, 6)
qSOFA		
Median (IQR)	0 (0, 1)	1 (1, 2)

UVA		
Median (IQR)	2 (0, 2)	3 (2, 5)
Time to death (days)		
Median (IQR)	NA	6 (1, 12)
In-hospital outcomes, n (%)		
Death	NA	34 (100%)
Discharge home	203 (91.9%)	18 (8.1%)
Absconder	5 (83.3%)	1 (16.7%)
Transfer	30 (61.2%)	19 (38.8%)

Missing data: Length of hospital stay among inpatients, n=47 (out of 325 inpatients); days with fever before enrolment, n=61; HIV status, n=17; malaria status by RDT, n=6; BMI, n=15; respiratory rate, n=1; systolic blood pressure, n=2; oxygen saturation, n=7; MEWS, n=3; qSOFA, n=3; UVA, n=26; in-hospital outcome, n=15 (out of 325 inpatients).

Abbreviations: BMI=body mass index, GCS=Glasgow Coma Scale, HIV=human immunodeficiency virus, IQR=interquartile range, MEWS=Modified Early Warning Score, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, RDT=rapid diagnostic test, UVA=Universal Vital Assessment.

Supplementary Table 5. Associations of host biomarkers with 28-day mortality using univariable logistic regression among enrolled paediatric and adult patients with febrile illness in southern Mozambique

Biomarker	OR (95% CI)	p-value
Angpt-2	2.35 (1.98, 2.79)	<0.001
CHI3L1	1.55 (1.41, 1.71)	<0.001
CRP	1.35 (1.19, 1.52)	<0.001
IL-6	1.30 (1.20, 1.40)	<0.001
IL-8	1.40 (1.28, 1.53)	<0.001
PCT	1.19 (1.12, 1.27)	<0.001
sFlt-1	1.70 (1.42, 2.02)	<0.001
sTNFR1	2.44 (2.02, 2.95)	<0.001
sTREM-1	3.19 (2.57, 3.96)	<0.001
suPAR, N=1,581	2.51 (2.04, 3.10)	<0.001

The total sample size is N=1,593, except for suPAR that is N=1,581. ORs, 95% CIs, and p-values are from univariable logistic regression models. ORs presented indicate the increase in odds of 28-day mortality for every two-fold increase in each biomarker concentration.

Abbreviations: CI=confidence interval, Angpt-2=angiotensinogen-converting enzyme 2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, OR=odds ratio, PCT=procalcitonin, sFlt-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

Supplementary Table 6. Performance metrics associated with sTREM-1 cut-offs to predict 28-day mortality among enrolled paediatric and adult patients with febrile illness in southern Mozambique

Biomarker	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)	PPV (95% CI)	NPV (95% CI)
sTREM-1	<239 pg/mL	77.4 (67.6-85.4)	66.8 (64.4-69.2)	2.33 (2.05-2.66)	0.34 (0.23-0.49)	12.6 (10.0-15.6)	97.9 (96.9-98.7)
	≥629 pg/mL	37.6 (27.8-48.3)	95.5 (94.3-96.5)	8.30 (5.85-11.78)	0.65 (0.56-0.77)	34.0 (24.9-44.0)	96.1 (95.0-97.0)

Abbreviations: CI=confidence interval, LR+=positive likelihood ratio, LR-=negative likelihood ratio, NPV=negative predictive value, PPV=positive predictive value, sTREM-1=soluble triggering receptor expressed on myeloid cells 1.

Supplementary Table 7. Associations of sTREM-1 and clinical severity scores with 28-day mortality using univariable and multivariable logistic regression among enrolled paediatric and adult patients with febrile illness in southern Mozambique

CHILDREN (N=784)		
Variable	OR (95% CI)	p-value
sTREM-1	4.67 (2.76, 7.90)	<0.001
LODS	3.85 (2.41, 6.16)	<0.001
LqSOFA	5.20 (2.90, 9.31)	<0.001
ED-PEWS	1.15 (1.11, 1.20)	<0.001
sTREM-1 adjusted for LODS	3.29 (1.88, 5.75)	<0.001
sTREM-1 adjusted for LqSOFA	3.35 (1.93, 5.82)	<0.001
sTREM-1 adjusted for ED-PEWS	2.65 (1.48, 4.74)	0.001
ADULTS (N=727)		
Variable	OR (95% CI)	p-value
sTREM-1	2.48 (1.97, 3.13)	<0.001
MEWS	1.44 (1.29, 1.62)	<0.001
qSOFA	5.08 (3.52, 7.32)	<0.001
UVA	1.72 (1.51, 1.96)	<0.001
sTREM-1 adjusted for MEWS	2.23 (1.76, 2.82)	<0.001
sTREM-1 adjusted for qSOFA	2.02 (1.58, 2.58)	<0.001
sTREM-1 adjusted for UVA	2.10 (1.64, 2.68)	<0.001

We included all participants with a known vital status at day 28 and clinical data for the calculation of all clinical severity scores. ORs, 95% CIs, and p-values are from univariable or multivariable logistic regression models. For sTREM-1, ORs presented indicate the increase in odds of 28-day mortality for every two-fold increase in sTREM-1 concentration. For clinical severity scores, ORs presented indicate the increase in odds of 28-day mortality for each one-unit increase in the score.

Abbreviations: CI=confidence interval, ED-PEWS=Emergency Department Paediatric Early Warning Score, LODS=the Lambaréné Organ Dysfunction Score, LqSOFA=Liverpool quick Sequential Organ Failure Assessment, MEWS=Modified Early Warning Score, OR=odds ratio, qSOFA=Quick Sequential (Sepsis-Related) Organ Failure Assessment, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, UVA=Universal Vital Assessment.

Supplementary Table 8. AUROC of each host biomarker for non-mortality outcomes among enrolled paediatric and adult outpatients with febrile illness in southern Mozambique

	Seeking further care for the same illness by the follow-up visit in outpatients, AUROC + 95% CI (N=838)	Subsequent hospitalisation for the same illness by the follow-up visit in outpatients, AUROC + 95% CI (N=838)
Angpt-2	0.55 (0.47, 0.62)	0.64 (0.42, 0.85)
CHI3L1	0.55 (0.49, 0.62)	0.65 (0.48, 0.81)
CRP	0.57 (0.49, 0.65)	0.73 (0.63, 0.83)
IL-6	0.56 (0.48, 0.64)	0.74 (0.65, 0.84)
IL-8	0.53 (0.46, 0.60)	0.66 (0.48, 0.85)
PCT	0.61 (0.53, 0.68)	0.80 (0.68, 0.92)
sFit-1	0.58 (0.50, 0.65)	0.59 (0.41, 0.77)
sTNFR1	0.60 (0.53, 0.67)	0.79 (0.68, 0.90)
sTREM-1	0.62 (0.54, 0.69)	0.73 (0.60, 0.87)
suPAR	0.61 (0.54, 0.69)	0.79 (0.62, 0.96)

Abbreviations: Angpt-2=angiopoietin-2, CHI3L1=chitinase-3-like protein 1, CRP=C-reactive protein, IL-6=interleukin-6, IL-8=interleukin-8, PCT=procalcitonin, sFit-1=soluble fms-like tyrosine kinase-1, sTNFR1=soluble tumour necrosis factor receptor 1, sTREM-1=soluble triggering receptor expressed on myeloid cells 1, suPAR=soluble urokinase-type plasminogen activator receptor.

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ARTICLE



Neurofilament light chain as a biomarker of neuronal damage
in children with malaria

Neurofilament Light Chain as a Biomarker of Neuronal Damage in Children With Malaria

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Malaria can cause brain injury. Neurofilament light chain (NfL) is a biomarker of neuronal damage. Here we examined longitudinal plasma NfL levels in children aged 1–12 years with uncomplicated and severe malaria from Mozambique. NfL levels were similar in all malaria cases at hospital admission. However, levels increased over time and the increment was significantly higher in severe malaria cases with neurological manifestations (ie, coma, impaired consciousness, or repeated seizures). NfL may be useful to identify and quantify brain injury in malaria.

Keywords. biomarker; brain injury; malaria; neurofilament light chain; neuronal damage.

Malaria is a leading cause of neurodisability in African children. Previous studies have linked malaria, and especially cerebral malaria, to long-term neurological deficits, cognitive impairments, and behavioral alterations in surviving children [1]. While the underlying mechanisms are incompletely understood, it has been proposed that malaria-induced injury to neurons and glial cells results in neurological sequelae [2]. Postmortem immunohistochemistry studies in fatal malaria have reported neuropathological features including axonal and myelin damage [3]. Nevertheless, current tools to evaluate

brain injury or sequelae (ie, neuroimaging, electroencephalograms, or neurocognitive tests) are limited in malaria-endemic areas, hindering efforts to understand its pathobiology, manage patients accordingly, and evaluate putative neuroprotective interventions.

Neurofilament light chain (NfL) is a neuron-specific protein. It is located in the neuronal cytoplasm and is highly expressed in large, myelinated axons. Low levels of NfL are constantly released from neurons into the extracellular space and ultimately reach the cerebrospinal fluid (CSF) and blood. However, circulating NfL levels increase when axonal injury occurs due to inflammatory, neurodegenerative, traumatic, or vascular injury [4, 5]. NfL has been extensively studied in neurodegenerative diseases and traumatic brain injury [4, 5]. More recently, it has been measured in different infectious diseases (eg, sepsis, pneumonia, or meningitis), where NfL was associated with neurological signs and symptoms, brain damage, neurological sequelae, and unfavorable outcomes [6–10]. Here we tested the hypothesis that plasma NfL levels function as a biomarker of neuronal damage in children with malaria.

METHODS

Study Design

This study was conducted at the Manhiça District Hospital, in southern Mozambique. Children aged 1–12 years with malaria were enrolled in a randomized controlled trial of adjunctive therapy with rosiglitazone. The safety and tolerability of rosiglitazone were first assessed in uncomplicated malaria (UM) cases between February and March 2016 [11]. From March 2016 to December 2019, efficacy was studied in severe malaria (SM) cases (ClinicalTrials.gov identifier: NCT02694874). Children were included in both study phases if they had a positive malaria diagnostic test (blood smear or histidine-rich protein 2/*Plasmodium* lactate dehydrogenase rapid diagnostic test) and confirmation of >2500 parasites/μL on thick smears. SM was defined as having repeated seizures (≥2 in the preceding 24 hours), prostration, impaired consciousness (Blantyre Coma Scale score <5 or Glasgow Coma Scale score <15), respiratory distress (sustained nasal flaring, deep breathing, or subcostal retractions), hypoglycemia (glucose <2.5 mmol/L), hyperlactatemia (lactate >5 mmol/L), or requiring hospitalization and parenteral treatment based on physician assessment. Children with known underlying illness (ie, neurological or neurodegenerative disorders; cardiac, renal, or hepatic disease; diabetes; epilepsy; or cerebral palsy), presenting solely with severe anemia (considered neither UM nor SM cases), or human immunodeficiency virus type 1 positive were excluded. Apart from the

Received 16 May 2023; editorial decision 24 August 2023; accepted 29 August 2023; published online 30 August 2023

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The Journal of Infectious Diseases®

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intervention, all children received the Mozambican standard of care for UM (artemether-lumefantrine) or SM (intravenous artesunate).

Plasma NfL Quantification

Venous blood was collected in ethylenediaminetetraacetic acid-coated tubes and centrifuged, and the derived plasma was isolated and stored at -80°C until shipment to the University Health Network, Canada. NfL concentrations were determined using a commercial Simple Plex NfL assay kit (ProteinSimple, San Jose, California) on the ELLA microfluidic platform. NfL was quantified in samples from all participants obtained at admission and at 12, 36, 60, and 84 hours. For SM cases, samples from the follow-up visits at 96 hours and day 14 were also included. Plasma was diluted at 1:2. ELLA provides triplicate data for each sample and those with a coefficient of variation $>20\%$ were excluded. Concentrations under the dynamic range were assigned a value of half of the lowest detection limit. Procedures were performed according to the manufacturer's instructions and blinded to clinical data.

Statistical Methods

Data were analyzed using Stata version 16.0 (StataCorp, College Station, Texas) and R version 4.0.3 (R Core Team, Vienna, Austria) software. The kinetics of NfL were assessed using linear mixed-effects (LME) models with intercepts modeled for each subject as random effects. An interaction term between each variable of interest (severity group, coma, impaired consciousness, or repeated seizures) and time postadmission was included in LME models. All models also incorporated child age, child sex, and treatment arm (placebo vs rosiglitazone) as fixed effects. Biomarker data were log-transformed for inclusion in LME models. Wald tests were used to assess NfL differences at hospital admission by severity group, interaction effects, and NfL slopes in each group. A 2-sided P value of $<.05$ was considered statistically significant.

Ethical Approval

This study was reviewed and approved by the Mozambican National Bioethics Committee (reference number 230/CNBS/15), the pharmaceutical department of the Mozambican Ministry of Health (reference number 374/380/DF2016), the Clinical Research Ethics Committee of the Hospital Clinic, Barcelona, Spain (reference number HCB/2015/0981), and the University Health Network Research Ethics Committee, Toronto, Canada (reference number 15-9013-AE). All research was conducted according to the principles expressed in the Declaration of Helsinki. After a detailed explanation of the study, parents or legal guardians provided written informed consent and children aged >8 years gave verbal assent.

RESULTS

A total of 167 malaria-positive children (30 with UM and 137 with SM) had at least 1 sample available for NfL quantification and were included in this study. This represents 100% (30/30) of recruited UM cases and 76.1% (137/180) of recruited SM cases. However, the distribution of demographic and clinical features was similar between SM children with and without NfL assessment. Table 1 presents the characteristics of the study cohort. At hospital admission, 59.9% (82/137) of children with SM displayed neurological manifestations: 15 had coma, 40 had impaired consciousness, and 69 had a history of ≥ 2 seizures in the preceding 24 hours. The overlap between these neurological manifestations is depicted in Supplementary Figure 1.

After adjusting for age, sex, and treatment arm, NfL levels at hospital admission were not significantly different between UM cases, SM cases without neurological manifestations, and SM cases with neurological manifestations (all $P > .050$). NfL concentrations increased significantly in all children over 84 hours ($P = .001$ for UM, $P < .001$ for both SM groups). The adjusted increase in NfL over 84 hours was higher in SM cases with neurological manifestations compared to the other 2 groups ($P = .010$, $P = .001$), but NfL longitudinal dynamics did not differ between SM without neurological manifestations and UM ($P = .789$) (Figure 1A). Among SM cases, the adjusted increase in NfL over 14 days was higher in children presenting each of the neurological manifestations. There was a significant interaction between time postadmission and coma ($P < .001$), impaired consciousness ($P < .001$), and repeated seizures ($P = .001$) (Figure 1B–D). In all models, NfL levels were negatively associated with age, but NfL levels were not associated with sex or treatment received from the clinical trial.

DISCUSSION

In this study we longitudinally evaluated NfL, a biomarker of neuronal damage, in a cohort of malaria patients. The only previous report of NfL quantification in malaria was a case report of a traveler presenting with postmalaria acute motor axonal neuropathy [12]. Another study determined NfL levels in a mouse model of experimental cerebral malaria, where levels were elevated in mice with acute disease, but did not distinguish fatal from reversible brain edema [13]. Here we report that NfL levels at hospital admission were similar in all malaria cases irrespective of severity or clinical neurological traits. NfL levels increased over time in all children with malaria infection, suggesting some degree of subclinical neuronal damage in both UM cases and SM cases without overt neurological symptomatology [1]. However, the increase was more pronounced in SM cases being admitted with neurological manifestations. Thus, there was a delayed increase in NfL and it continued to rise through day 14.

When neuronal damage occurs at a specific and known time-point, such as in traumatic brain injury, NfL levels in blood and

Table 1. Study Population Characteristics

Characteristic	UM (n = 30)	SM Without Neurological Manifestations ^a (n = 55)	SM With Neurological Manifestations ^a (n = 82)
Demographics			
Female sex	18 (60.0)	25 (45.5)	41 (50.0)
Age, y, median (IQR)	7.1 (4.7–9.2)	3.2 (1.9–5.1)	3.0 (2.2–4.1)
Baseline clinical characteristics			
Temperature, °C, median (IQR)	36.9 (36.3–38.1)	38.0 (37.2–39.1)	38.5 (37.7–39.5)
Respiratory distress ^b	0 (0)	11 (20.0)	18 (22.0)
History of ≥2 seizures in the preceding 24 h	0 (0)	0 (0)	69 (84.1)
Prostration	0 (0)	40 (72.7)	67 (81.7)
Impaired consciousness (BCS <5 or GCS <15)	0 (0)	0 (0)	40 (48.8)
Coma (BCS <3 or GCS <9)	0 (0)	0 (0)	15 (18.3)
Weight-for-age z score ^c , mean (SD)	−0.9 (1.0)	−0.8 (0.9)	−0.9 (1.2)
Baseline laboratory findings			
Hypoglycemia (glucose <2.5 mmol/L)	0 (0)	1 (1.8)	6 (7.3)
Hyperlactatemia (lactate >5 mmol/L)	0 (0)	14 (25.5)	17 (20.7)
Severe anemia (hemoglobin ≤5 g/dL)	0 (0)	5 (9.1)	4 (4.9)
Treatment arm			
Placebo	10 (33.3)	43 (78.2)	44 (53.7)
Rosiglitazone	20 (66.7)	12 (21.8)	38 (46.3)
Outcome			
Discharged alive	30 (100)	55 (100)	79 (96.3)
Transferred	0 (0)	0 (0)	1 (1.2)
Death	0 (0)	0 (0)	2 (2.4)

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: BCS, Blantyre Coma Scale score; GCS, Glasgow Coma Scale score; IQR, interquartile range; SD, standard deviation; SM, severe malaria; UM, uncomplicated malaria.

^aAt admission, having coma, impaired consciousness, or a history of ≥2 seizures in the preceding 24 hours.

^bSustained nasal flaring, deep breathing, or subcostal retractions.

^cCalculated using the LMS method and the 2000 US Centers for Disease Control and Prevention Growth Reference data.

CSF increase over a few days [4]. A progressive increase in NfL levels has also been reported in infectious diseases with neurological involvement, similar to the trend observed in our study [8–10]. The dynamics of blood NfL in pediatric malaria seem to differ from those of other brain injury biomarkers such as tau [14]. In fact, differences between these biomarkers were previously noted by authors working on traumatic brain injury [15]. Future studies are necessary to compare NfL to other proposed biomarkers of neuronal damage in malaria, with respect to kinetics and potential clinical utility.

The limited data available on NfL in healthy populations describe variations in levels according to age. Younger children have higher NfL levels and these decrease with age until reaching the lowest level at 10–15 years old [5]. NfL then increases up to late adulthood and rises steeply afterward, probably due to structural and metabolic changes that occur with aging [4, 5]. Consequently, it is important to consider age with special caution when studying NfL changes in pathological conditions. Here we noticed that age was an important confounder and adjusted all results for age. Nevertheless, we lacked NfL data in age-matched healthy community controls to define population-specific normal ranges for comparison.

This study benefits from the inclusion of multiple sample timepoints collected from a well-characterized cohort.

However, it has limitations including a limited number of UM cases and a follow-up period of 84 hours (UM) or 14 days (SM) postenrollment. Further testing of later times is required to better understand NfL kinetics and to determine when levels plateau or normalize. Moreover, a larger sample size would be necessary to analyze possible interactions between neurological manifestations impacting NfL longitudinal dynamics. We did not associate NfL changes with neuroimaging, electroencephalographic results, or long-term neurocognitive testing. Children were participants in a randomized controlled trial, but there were no significant differences in NfL levels between treatment arms and results were adjusted for treatment received. We cannot exclude the possibility that other factors besides age and sex could affect NfL levels [5, 6]. Larger studies are needed to elucidate how additional factors might influence NfL levels, including underlying illnesses that were excluded as per study inclusion criteria, and how this impacts the interpretation of NfL levels as indicative of malaria-associated neuronal damage. Last, we lacked detailed information on previous episodes of SM or other infections that could contribute to cumulative neuronal damage and affect NfL levels during a given malaria episode.

Additional studies in malaria with larger cohorts and long-term outcomes are required. NfL could be useful to predict

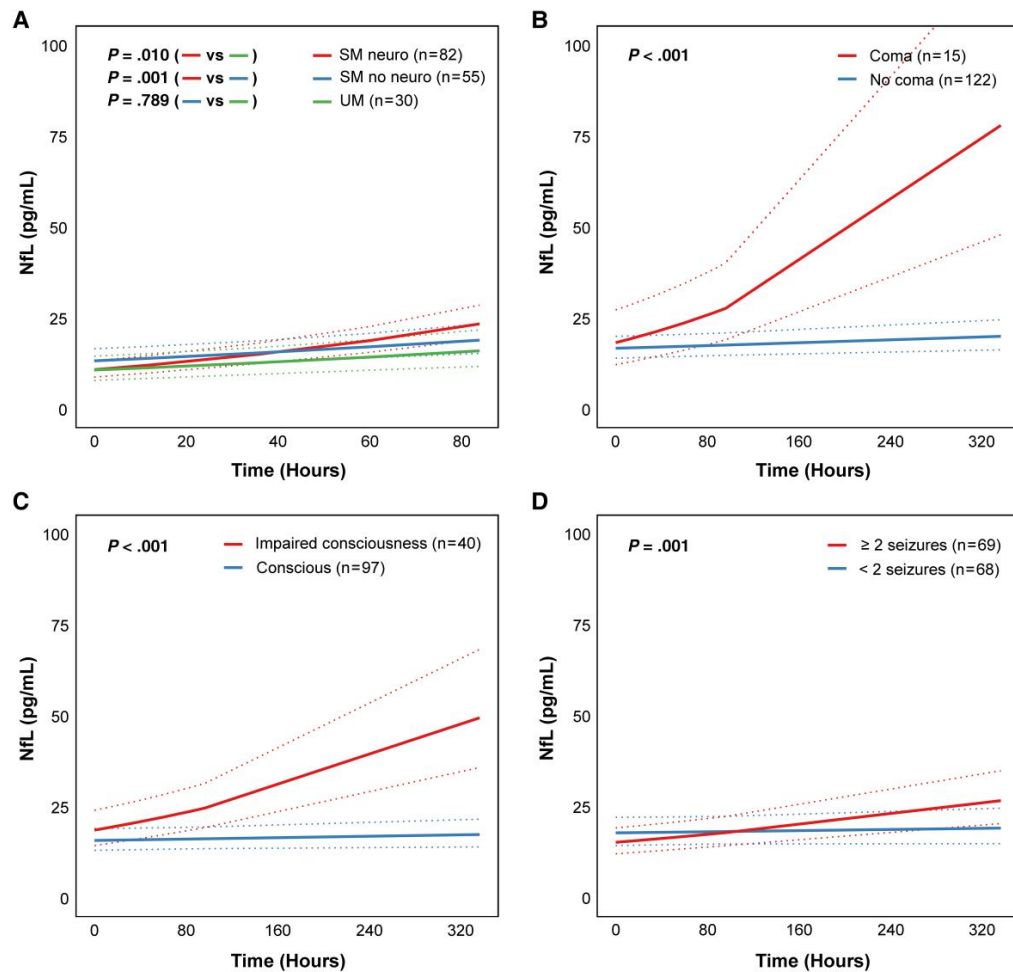


Figure 1. Plasma neurofilament light chain (NfL) concentrations in children with malaria. Longitudinal differences in concentrations of plasma NfL from admission to 84 hours by severity group (uncomplicated malaria [UM], severe malaria [SM] without neurological manifestations, or SM with neurological manifestations) (A) or from admission to day 14 by presence of each clinical neurological trait in children with SM (B–D). Longitudinal regression lines depict linear mixed-effects (LME) model–predicted, back-transformed values and 95% confidence intervals of NfL over time stratified by severity group (A) or clinical trait (B–D), while holding other fixed effects constant. All LME models included time postadmission, child sex, child age, and treatment arm (rosiglitazone vs placebo) as fixed effects, and a by-participant intercept as a random effect. An interaction term between severity group (A) or each clinical trait (B–D) and time postadmission was included in each model to account for variation in NfL over time. *P* values overlaid on graphs indicate the statistical significance of the interaction term assessed using a Wald test. A total of 30 children had UM, 55 had SM without neurological manifestations, and 82 had SM with neurological manifestations (15 with coma, 40 with impaired consciousness, and 69 with a history of ≥ 2 seizures in the preceding 24 hours). For the selected timepoints, 71.1% (788/1109) of samples were available for NfL quantification.

persistent neurocognitive dysfunction, allowing targeted follow-up and cognitive rehabilitation to children with the highest risk, as well as to serve as a surrogate marker for interventions designed to reduce neurocognitive impairment. The new immunoassays Simoa and ELLA have increased sensitivity and allow NfL quantification in blood, avoiding the need for invasive lumbar punctures to obtain CSF (where NfL levels are estimated to be ~ 40 times higher) and opening the door to easily incorporate NfL quantification in studies conducted in malaria-endemic settings [4].

Our results support plasma NfL as a potential biomarker of neuronal damage in pediatric malaria. However, these findings indicate that NfL is not an early indicator, given its gradual increase after neurological manifestations appear. Measurement of NfL levels during malaria infection may enhance the identification and quantification of brain injury.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to

benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We are grateful to the patients and their families for participation in this study. We thank the nurses, field assistants, laboratory technicians, and other hospital staff who cared for the patients and contributed to study data collection. We also thank Dan Ouchi for statistical advice during this study.

Author contributions. K. C. K. and V. M. C. conceived the study. N. B., C. K. F., V. M. C., A. M. W., B. B., R. V., Q. B., and K. C. K. contributed to study design. R. V. and V. M. C. were involved in study implementation. R. V. acquired clinical data. C. K. F., V. M. C., and K. Z. acquired the biomarker data. N. B., V. M. C., and R. V. were involved in data management. N. B. and A. M. W. conducted the statistical analysis. N. B. and C. K. F. drafted the manuscript. N. B., C. K. F., V. M. C., A. M. W., B. B., R. V., Q. B., and K. C. K. interpreted the data and critically revised the manuscript. All authors read and approved the final manuscript.

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Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclaimer. The funding bodies played no role in study design; collection, analysis, or interpretation of the data; writing of the report; or the decision to submit the manuscript for publication.

Financial support. This work was supported by the Canadian Institutes of Health Research Foundation grant FDN-148439 (to K. C. K.); a Canada Research Chair in Molecular Parasitology (to K. C. K.); and the Thomas Mather Fund (to K. C. K.). ISGlobal receives support from the grant CEX2018-000806-S funded by MCIN/AEI/10.13039/501100011033, and support from the Generalitat de Catalunya through the CERCA program. This research is part of ISGlobal's Program on the Molecular Mechanisms of Malaria, which is partially supported by the Fundación Ramón Areces. Centro de Investigação em Saúde de Manhiça is supported by the Government of Mozambique and the Spanish Agency for International Development. N. B. is supported by an FPU predoctoral fellowship from the Spanish Ministry of Universities (FPU18/04260). B. B. is a Beatriu de Pinos postdoctoral fellow granted by the Government of Catalonia's Secretariat for Universities and Research and by the Marie Skłodowska-Curie Actions COFUND Program

(BP3, 801370). R. V. had a fellowship from the program Río Hortega of the Instituto de Salud Carlos III (CM16/00024) during the conduct of the study. Funding to pay the Open Access publication charges for this article was provided by the grant CEX2018-000806-S funded by MCIN/AEI/10.13039/501100011033.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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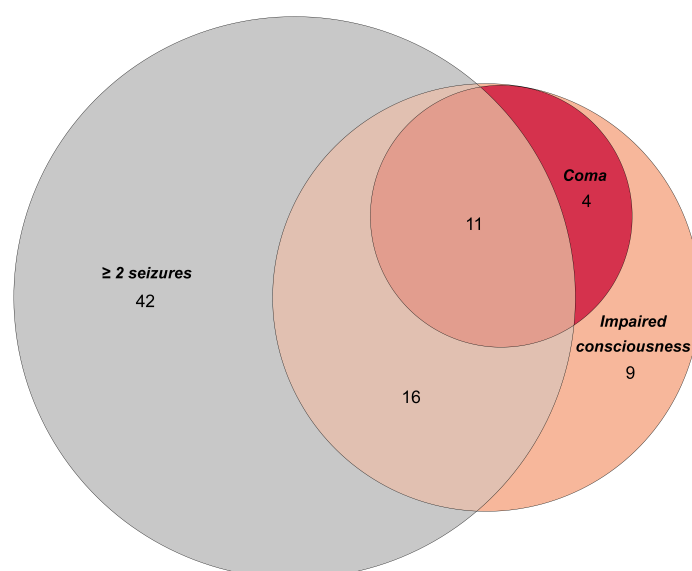
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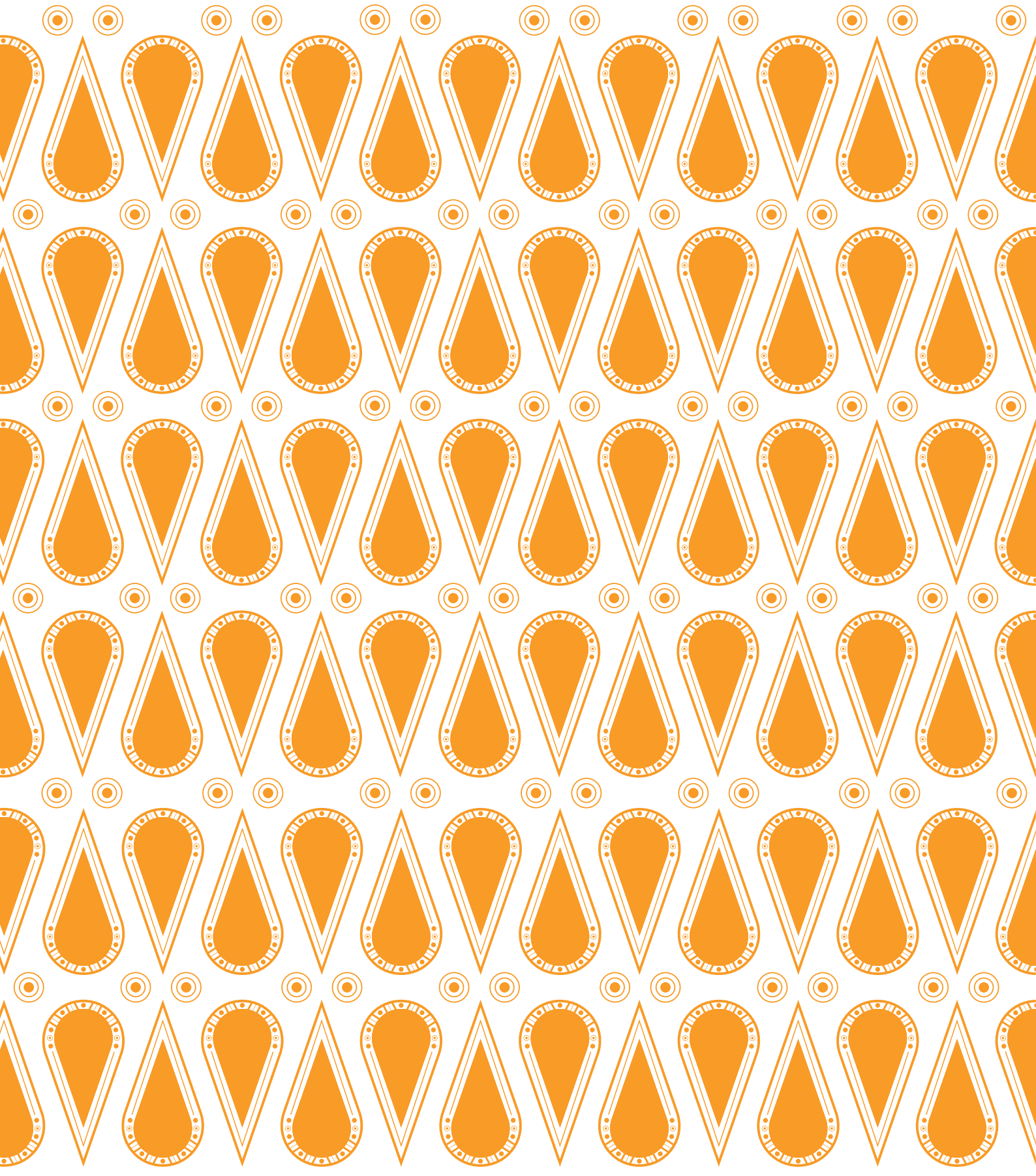
SUPPLEMENTARY MATERIAL

Neurofilament light chain as a biomarker of neuronal damage
in children with malaria

Supplementary Figure 1. Euler diagram showing the overlap between neurological manifestations in children with severe malaria



8. DISCUSSION



8. DISCUSSION

8.1. OVERALL DISCUSSION OF THESIS RESULTS

The results of this thesis contribute to global efforts to improve management strategies in patients with acute infections in resource-limited settings. The different biomarker studies included, conducted in Mozambican populations, add significant knowledge towards the development of novel host biomarker-based tools to improve patient triage and risk stratification. Moreover, this thesis also contributes to describing pediatric healthcare in southern Mozambique through a gender lens, where these host biomarker-based tools could significantly impact the management of patients and help mitigate potential gender-based disparities.

The first article of this thesis presents a retrospective analysis of data from the Manhiça HDSS [202,203], examining various indicators of healthcare access and provision among children under 15 years of age in Manhiça district. This region, which also serves as the setting for the other studies included in this thesis, is marked by a high burden of infectious diseases [202,203,205,206]. Leveraging extensive morbidity and demographic surveillance data from the Manhiça HDSS, we were able to gain a comprehensive understanding of 17 years of pediatric healthcare in the area. For instance, our findings revealed that caregivers of children sought care after experiencing symptoms for a median duration of one day (interquartile range [IQR]: 1-2), referrals from peripheral outpatient clinics to hospital occur in less than 2% of children, 4% of children revisited an outpatient clinic within seven days after being discharged home or absconding, the hospital case fatality ratio was 4%, and the 28-day post-discharge mortality stood at 2%. Importantly, by reporting all results separately for girls and boys, and comparing them, we explored potential differences between these groups. Our analysis showed that girls had fewer referrals and admissions to hospital. Girls had lower odds of being referred or admitted to hospital after seeking care (girls-to-boys odds ratio [OR]=0.82 [95% CI: 0.79-0.86] and OR=0.85 [95% CI: 0.84-0.87], respectively) and the girls-to-boys incidence rate ratio (IRR) for hospitalizations at the population level was 0.81 (95% CI: 0.79-0.84). However, girls were also less likely to have severe manifestations at clinical presentation. Other studied indicators of healthcare access and provision were overall similar for boys and girls. Despite these generally encouraging findings, these results are only representative of a single district in southern Mozambique and further research is necessary. Studies on this topic that include data from Mozambique or sub-Saharan African countries are very scarce, with varying results by study and country [212–214]. Differences observed in Manhiça district in referrals and admissions to hospital may be a result of gender-based inequities in clinical evaluation and decision-making, but could also reflect the need for care and be explained by differences in signs and symptoms at presentation. In any case, the introduction of an objective, biomarker-based tool could always help reduce subjectivity in evaluation and decision-making by healthcare providers. To a lesser extent, it could also encourage families to follow the

recommendations of healthcare providers by showing the result of the test. Such a test could promote greater gender-based equity in healthcare and, more broadly, potentially address other dimensions of inequity beyond gender. Nevertheless, changing initial healthcare-seeking behaviors cannot be achieved through a test deployed at the healthcare facility, and modifying deeply ingrained sociocultural norms and roles remains a complex challenge.

The second and third articles in this thesis present findings from ancillary studies of two prospective observational studies: the RAPID (*“Diagnostic biomarkers for respiratory infection and malaria”*) study [215–217], and the FIEBRE (*“The Febrile Illness Evaluation in a Broad Range of Endemicities”*) study [218]. In this thesis, we only used the Mozambican cohort of the FIEBRE study. We evaluated the prognostic performance of different biomarkers of immune and endothelial activation in these two large, well-characterized, and prospectively recruited cohorts of Mozambican patients with varying demographics, clinical presentation, and disease severity. The RAPID study included children aged <10 years hospitalized with WHO-defined clinical pneumonia, and the FIEBRE study included children aged ≥2 months and adults with all-cause febrile illness presenting to hospital and managed as outpatients or inpatients. Biomarkers studied were selected based on review of the literature on promising candidates for prognosis in acute infections. In both studies, plasma levels of Angpt-2, CRP, IL-6, IL-8, PCT, sFlt-1, sTNFR1, and sTREM-1 were retrospectively quantified head-to-head from samples taken at clinical presentation. Additionally, CHI3L1, suPAR, and lactate were measured in the third study. The same methodology and laboratory facilities (University Health Network – Toronto General Hospital, Toronto, Canada) were used in both studies for measuring biomarkers.

The second article included children hospitalized with WHO-defined clinical pneumonia, a leading cause of child illness and mortality worldwide [2,219], and a common cause of hospitalization and death among children in Mozambique [205]. Of the 472 pneumonia cases with host biomarkers measured, 15 (3.2%) children died during the hospital stay. IL-8, sFlt-1, and sTREM-1 levels at clinical presentation were associated with in-hospital mortality and showed the best discrimination with AUROCs of 0.88 (95% CI: 0.78–0.97), 0.83 (95% CI: 0.73–0.94) and 0.82 (95% CI: 0.74–0.91), respectively. These results align with findings from two similar studies, published shortly before ours, which also examined a similar panel of biomarkers in hospitalized children meeting WHO-defined clinical pneumonia criteria. In 805 Ugandan children, sTREM-1, followed by IL-8 and sFlt-1, were also the best prognostic biomarkers for 48-hour in-hospital mortality [128]. In 118 Bhutanese children, sTREM-1 was the best prognostic biomarker for a composite outcome of poor prognosis that included death [220]. The consistency of findings across studies conducted in diverse settings strengthens the validity of our results, particularly given the limited number of events for our primary outcome. Furthermore, our study benefited from the addition of 80 healthy community controls within the same age group and geographic area as the pneumonia cases, which allowed us to demonstrate that concentrations of all studied biomarkers were significantly higher in pneumonia cases as compared to healthy controls. Biomarker levels were also compared between non-severe and severe cases at presentation as defined by WHO 2013 Pocket Book [36], with IL-8 being

significantly higher in the latter. Among participants living in the Manhiça HDSS study area, seven and 13 additional deaths were identified to occur within 28 days and 90 days of hospital admission using the Manhiça HDSS databases. The availability of post-discharge mortality data in this cohort provided the opportunity to confirm that IL-8, sFlt-1, and sTREM-1 remained associated with both 28-day and 90-day mortality, with AUROCs ranging from 0.74 (95% CI: 0.65-0.84) to 0.81 (95% CI: 0.72-0.91).

In the third article, host biomarkers were studied in pediatric and adult patients presenting to hospital with all-cause febrile illness. As mentioned previously, fever is a leading reason for medical consultations globally and a frequent symptom at clinical presentation associated with infectious diseases [22]. A total of 1,955 pediatric and adult participants had host biomarkers measured. These were managed as either outpatients or inpatients. By day 28, there were 93 deaths (5.8%) among the 1,593 participants with known vital status. Levels of all studied biomarkers at clinical presentation were elevated in inpatients compared to outpatients and were associated with 28-day mortality. sTREM-1 was the best biomarker predicting 28-day mortality with an AUROC of 0.82 (95% CI: 0.78-0.86). CHI3L1 and Angpt-2 had comparable performance to sTREM-1 overall but not across different patient subgroups. sTREM-1 also emerged as the best biomarker in predicting death when considering 7-day mortality, or when focusing specifically on the inpatient population where most fatalities occurred. This finding corroborates recent reports in febrile cohorts highlighting sTREM-1 as an excellent biomarker of risk of death. In 2,502 hospitalized febrile children in Uganda, sTREM-1 had the best prognostic performance for 7-day mortality among a similar biomarker panel, with an AUROC of around 0.90 [169]. In 507 Tanzanian febrile adults presenting to outpatient clinics, sTREM-1 was the best biomarker to predict 28-day mortality with an AUROC of 0.87 (95% CI: 0.81-0.92) [172].

Based on the findings of the second and third studies of this thesis, along with the aforementioned studies on pneumonia and febrile illness, and other studies on other syndromes or etiologies (e.g., in malaria, COVID-19, or sepsis) in LMICs and HICs with similar results [129,161,170,171,221,222], there is now compelling growing evidence linking high sTREM-1 levels with subsequent mortality in acute infections. In fact, sTREM-1 thresholds have been derived to stratify patients with febrile illness into three categories in terms of mortality risk: low-risk, intermediate-risk, and high-risk [169]. This proposes a “traffic-light” system of risk stratification that could be coupled with appropriate management decisions, similar to the one proposed in IMCI guidelines. Interestingly, when these thresholds were applied to our two study cohorts in Mozambique, similar mortality risks were observed across categories. Another study applied these thresholds to a cohort of pediatric inpatients with malaria and found similar results [170]. Nevertheless, further studies are necessary to determine if these sTREM-1 thresholds are useful in different populations and use-cases, or if they require adaptation. Furthermore, thresholds were derived and applied in populations where sTREM-1 was measured retrospectively, using Luminex multiplex assays, on plasma samples stored at -80°C and thawed before their use. Using other quantification techniques or fresh blood at the point of care could give slightly different results [223].

In both studies, the prognostic performance of biomarkers for primary outcomes was compared to that of clinical signs and severity scores. A few relevant indicators of severity and prognosis were pre-specified, supported by evidence in similar populations. In the second study, oxygen saturation, the WHO Pocket Book definition of severe pneumonia, the LODS, and the Respiratory Index of Severity in Children-Malawi (RISC-Malawi) were selected [36,54,224]. In the third study, the Emergency Department Pediatric Early Warning Score (ED-PEWS), the Liverpool quick Sequential Organ Failure Assessment (LqSOFA), and the LODS were used for children; and the Modified Early Warning Score (MEWS), the Quick Sequential (Sepsis-Related) Organ Failure Assessment (qSOFA), and the UVA were used for adults [5,54,55,225–227]. In each study, the best prognostic biomarkers demonstrated similar or improved discrimination ability compared to these clinical variables. Moreover, combining biomarkers with clinical parameters increased the discrimination ability beyond that of clinical parameters alone. These findings, along with similar ones from studies doing analogous analyses [129,162,170–172,174,220], suggest that integrating sTREM-1 or other host biomarkers with appropriate clinical scoring systems or decision algorithms could provide useful additional information and improve triage and risk stratification of patients with acute infections.

Many studies on prognostic biomarkers are conducted on inpatients and use death as the primary outcome. Yet research on non-hospitalized individuals and prediction of other less severe outcomes is also important for patient management. In the third study, febrile patients who were managed as outpatients were included. Among them, 28-day mortality was very low, with only two deaths (0.2%) occurring in 854 participants with a known vital status at day 28. However, a higher percentage of outpatients reported seeking further care for the same illness (7.0%) or requiring subsequent hospitalization (1.2%) up until the study follow-up visit at around day 28. Levels of different biomarkers of immune and endothelial activation, including sTREM-1, were associated with these two adverse outcomes. Previous evidence on sTREM-1 prognostic performance in patients attending outpatient clinics and emergency departments is scarce. In these contexts, sTREM-1 has been associated with hospitalization, as we also observed in this study, but it has been reported to be less suitable for predicting other outcomes such as supplementary oxygen requirement in respiratory diseases [162,173,222,228]. In a recent study by our research group, involving outpatients aged over 50 years who presented with mild-to-moderate COVID-19 in Spain, sTREM-1 was not a good predictor of subsequent hospitalization by day 28 [229]. This result differs from the one obtained in this thesis, but the demographics, severity profiles, and healthcare contexts of the two cohorts differ greatly. Additional studies are needed to better understand whether sTREM-1 is sufficiently altered early in the disease course or if other biomarkers might be better predictors of other adverse outcomes beyond mortality, depending on the specific context.

CRP and PCT were measured in the second and third studies. Both biomarkers showed poor discrimination ability for subsequent mortality outcomes. These results align with other reports from sub-Saharan Africa, where the value of CRP or PCT to predict mortality was limited and they were outperformed by other host biomarkers of immune and endothelial activation [116,117,128,169,170,172].

However, it is noteworthy that in the third study, CRP and PCT levels were higher in patients requiring hospitalization, as well as in patients seeking further care or hospitalized after being discharged as outpatients. Additionally, CRP and PCT have been proposed for uses beyond severity estimation and prognosis, such as distinguishing non-bacterial from bacterial infections or monitoring antibiotic therapy, applications that were not investigated in this thesis [112–114]. Another classic biomarker that was studied in the third study of this thesis was lactate, measured on-site using a POC analyzer. In accordance with previous evidence, lactate was associated with 28-day mortality. However, sTREM-1 outperformed lactate in terms of discrimination ability, replicating what was seen in prior research on pneumonia, malaria, and sepsis [128,129,230,231]. In conclusion, this body of evidence collectively suggests that novel biomarkers offer promising alternatives to classic host response biomarkers for predicting death and poor prognosis in acute infections in populations in LMICs.

Measuring several host biomarkers simultaneously could improve prognostic performance in acute infections, especially if biomarkers reflect different pathophysiological pathways. Some studies have shown that combining biomarkers enhances the prediction of adverse outcomes compared to relying on a single biomarker [232,233], while other studies have not found significant improvements [128,171,172]. Panels of various host biomarkers are available on certain analytical platforms and conceived to be used in conjunction [222]. In the second and third studies of this thesis, two-biomarker combinations were explored for predicting mortality. In these Mozambican cohorts, adding a second biomarker had little impact on the discrimination ability, as it only slightly increased the AUROCs compared to that of the best biomarkers alone. While using larger sample sizes and exploring other host biomarker combinations could yield different results and show meaningful improvements in prognostic performance, it is crucial to bear in mind that simplicity is essential for developing practical tools for resource-limited environments. A combination biomarker approach may compromise feasibility and practicality for clinical use if this complicates biomarker measurement, while also incurring higher costs.

A biomarker may demonstrate good prognostic performance in a cohort overall, yet its accuracy in predicting adverse outcomes may vary depending on individual patient characteristics. In many studies, subgroup analyses are not possible due to the low number of outcomes. This was the case in the second study of this thesis. However, in the third study, we had enough outcomes to explore the discrimination ability of host biomarkers for 28-day mortality by age group (adults and children), sex (males and females), and HIV status (positive and negative). The only biomarker with an AUROC that varied between subgroups was sTREM-1 based on HIV status. While sTREM-1 remained a good predictor of mortality in HIV-positive participants (AUROC=0.73, 95% CI: 0.66–0.80), its performance was significantly inferior in this group compared to HIV-negative participants (AUROC=0.89, 95% CI: 0.84–0.94). Furthermore, in HIV-positive participants, the AUROC for sTREM-1 was 0.77 (95% CI: 0.69–0.84) in those reporting antiretroviral treatment use, and 0.64 (95% CI: 0.49–0.78) in participants who were either not on treatment or newly diagnosed. Although differences by HIV status were non-significant for other biomarkers, almost all biomarkers showed a trend towards poorer prognostic performance

in HIV-positive participants. Active HIV infection causes a combination of immunosuppression and chronic inflammation, which can result in immune system exhaustion and altered host responses to pathogens [234]. However, reports on the mechanistic impact of HIV infection on the TREM-1 pathway are limited [235,236]. As HIV infection is prevalent in many resource-limited settings, future studies need to carefully consider potential differences in the prognostic performance of sTREM-1 and other host biomarkers based on HIV status and HIV-related immunosuppression.

The absence of sex-specific differences in biomarker prognostic performance among our large population of febrile patients suggests that biomarkers studied work similarly in males and females. A previous study in febrile children in Uganda also found no significant differences in biomarker levels by sex in relation to mortality, using a similar panel of host biomarkers [214]. Combined with the objective and quantitative nature of biomarkers, these findings reinforce their possible utility in mitigating potential gender bias in the assessment of disease severity and patient management [86–88]. However, further research is needed to validate these results, especially considering a study on pediatric severe malaria in Uganda that reported sex differences in biomarker prognostic performance, with sFlt-1 and sTREM-1 predicting in-hospital mortality more accurately in males than females [170]. Another study that investigated the prognostic performance of similar biomarkers by patient characteristics was conducted in children with pneumonia in Bhutan. Although authors did not analyze differences by sex, they found that PCT and IL-6 were less accurate predictors of poor prognosis in infants compared to older children [220]. In our study we selected age, sex, and HIV status *a priori* as relevant variables that may influence biomarker levels and their prognostic performance, but exploring other factors is necessary in future biomarker discovery and validation studies. It would be valuable to understand if potential differences exist by further age groups (e.g., neonates, infants, and elderly), malnutrition status, other causes of immunosuppression apart from HIV (e.g., malignant neutropenia), specific infection etiologies or clinical syndromes, co-infections, relevant comorbidities, and duration of previous symptomatology. While an ideal biomarker would offer robust prognostic performance across all patients with suspected acute infections for the sake of simplicity, it is possible that different biomarkers will perform better in specific patient subgroups, or that biomarker thresholds may need to be adjusted accordingly to optimize prognostic value.

In the second and third studies, biomarkers were measured only in samples collected at clinical presentation. At this moment, information provided by biomarkers on the severity and risk of patients is particularly valuable, as key decisions need to be made. However, serial biomarker measurements, though beyond the scope of these studies, could have provided additional insights into biomarker kinetics. Biomarkers can exhibit varying longitudinal dynamics, taking more or less time to elevate (or decrease) and return to normal levels following recovery. Dynamic monitoring of biomarkers could help identify their optimal sampling windows to maximize biomarker prognostic performance and to understand when a value may lack utility. Moreover, in inpatients, looking at changes in biomarker levels between two measurements over time may reveal associations with future adverse outcomes, be useful for monitoring disease progression, and provide insights into

treatment response [237]. To date, there is limited published data on the dynamics of the novel biomarkers included in this thesis. For sTREM-1, trends in plasma concentration were reported in a population of Ugandan children with severe malaria. This study found a rapid decrease in sTREM-1 levels in patients who recovered and were discharged from hospital [129]. Similar trends have been found in studies in populations from HICs [238]. This suggests that sTREM-1 levels do not remain elevated for extended time periods and that serial sTREM-1 measurements during hospitalization could be used to evaluate patient evolution. However, confirmation of these results is needed.

Conversely, in the fourth article, we presented longitudinal levels of the biomarker NfL. This study used serial plasma samples collected within the context of the ROSI ("*Rosiglitazone Adjunctive Therapy for Severe Malaria in Children*") study, a randomized controlled trial in Mozambican children aged one to 12 years with malaria. Malaria poses a significant burden on Mozambique, with over 10 million cases reported in the country in 2022 alone [19]. The main aim of this trial was to evaluate the efficacy of rosiglitazone as adjunctive therapy in severe malaria [237,239]. However, in this thesis we addressed a secondary objective of this study, and we measured plasma NfL levels at different timepoints during the disease course. Different studies have linked malaria, and especially cerebral malaria, with long-term neurological, cognitive, and behavioral *sequelae* [16]. Although the underlying mechanisms are not fully understood, it has been suggested that malaria causes injury to neurons [240,241]. Since NfL is a neuron-specific protein and a biomarker of neuronal damage, which had not been previously studied in cohorts of malaria patients, it was chosen for investigation in this study cohort and measured in 167 children with malaria. NfL levels at hospital admission were found to be similar in uncomplicated malaria, severe malaria without neurological manifestations (i.e., coma, impaired consciousness, or repeated seizures), and severe malaria with neurological manifestations. NfL levels increased over time in all children, which suggests some degree of subclinical neuronal damage in children without overt neurological manifestations. However, this increase was significantly higher in severe malaria cases with neurological manifestations, in whom levels continued to rise for up to 14 days. These results support plasma NfL as a potential biomarker of neuronal damage in pediatric malaria that could help identify and quantify brain injury, but indicate that it is not an early indicator of such damage. This observed progressive increase in NfL over time aligns with findings from studies on traumatic brain injury and other infectious diseases [182,242–245]. If confirmed by further research, this would indicate that NfL may have limited utility for guiding clinical decisions at initial presentation but could be useful as a later biomarker.

Following the publication of these results, two additional studies reported NfL data in two cohorts of malaria patients; however, both used single timepoint samples and could not explore longitudinal dynamics. One study found no differences in plasma NfL levels between symptomatic *Plasmodium knowlesi*-infected Malaysian adults and community age-matched uninfected controls [246]. In contrast, the other study reported significantly higher plasma NfL levels in Ugandan children with *P. falciparum* cerebral malaria and severe malarial anemia compared to asymptomatic community children. However, and similarly to our study, levels did not differ between cerebral malaria and severe

malarial anemia at hospitalization. Notably, in patients with cerebral malaria, elevated NfL levels were associated with neurologic deficits at discharge (but not at any follow-up timepoint), worse attention Z-scores over 24-months follow-up, and mortality [178]. Nonetheless, despite these recent findings, research on NfL in malaria remains limited. Similarly, studies on NfL levels in other acute infections have provided promising insights into its potential as a biomarker of brain injury, but available data remain limited [182,244,245,247,248]. Further research should include large cohorts, use several timepoints for NfL measurement, consider carefully factors that could impact levels (such as age, as shown by our findings and previous studies [242,249]), and define thresholds of abnormality for different use-cases. Comparing NfL levels with long-term neurocognitive outcomes, as well as with neuroimaging and electroencephalographic findings, is crucial. However, this was beyond the scope of our study. Other biomarkers of brain injury have been investigated in infectious diseases, though evidence remains scarce. In malaria, Tau levels, in plasma or CSF, have demonstrated considerable promise for clinical application [178–180,246,250]. Measuring biomarkers indicative of brain injury in infectious diseases, particularly in blood samples, presents exciting new avenues to objectively and accurately identify individuals at high risk of long-term neurocognitive dysfunction and other adverse outcomes, which could facilitate timely and appropriate treatment or rehabilitation interventions.

Studying biomarkers of disease severity, specific organ injury, and prognosis in infectious diseases deepens our understanding of the mechanisms underlying severe infections. These insights can guide the development of novel therapeutic strategies targeting the same pathophysiological pathways, with the objective of mitigating disease progression and improving patient outcomes [101,251–255]. For example, IL-6 inhibitors, commonly used for autoimmune and chronic inflammatory conditions like rheumatologic disorders, have recently been studied in COVID-19. IL-6 inhibition has been associated with reduced 28-day mortality in hospitalized patients with COVID-19 [256,257]. Consequently, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) authorized tocilizumab, a monoclonal antibody against the IL-6 receptor, for treating hospitalized adults with COVID-19 who are receiving systemic corticosteroids and require respiratory support [258,259]. The TREM-1 pathway has also been studied as a druggable pathway for pharmacological intervention. TREM-1 inhibitory peptides have been developed to attenuate the amplification of the inflammatory response triggered by TREM-1 [254]. In cell cultures and animal models, different TREM-1 inhibitors have been shown to reduce the release of inflammatory cytokines, prevent poor outcomes, and decrease mortality [254]. Of these, the LR12 peptide, known as nangibotide, is the only one that has recently reached the clinical stage [260–262]. In phase 2 trials involving adults with septic shock and COVID-19 in HICs, nangibotide was safe and well-tolerated. Additionally, nangibotide was demonstrated to be effective in improving clinical status or showed potential to do so in adults with initially high sTREM-1 levels [261,262]. Thus, a strategy that combines measuring sTREM-1 with treating patients who have high sTREM-1 levels with nangibotide may be particularly effective.

This thesis is focused on resource-limited settings, where host biomarker-based tools could have the greatest impact on reducing morbidity and mortality from infectious diseases. However, the

COVID-19 pandemic overwhelmed healthcare systems globally, including those in HICs. During the first waves, limited resources made it essential to identify high-risk individuals and prioritize their care. This underscored the need for improved triage and risk stratification tools for acute infections and spurred research on biomarkers of host response and organ-specific injury in COVID-19. Such host biomarker-based tools could bring benefits in different settings and scenarios and, especially in HICs, offer economic savings by optimizing resource allocation. Clinical trials aimed to enhance risk stratification strategies using novel biomarkers in COVID-19 patients yielded exciting results. In a randomized controlled trial conducted in Greece and Italy, adults with COVID-19 and high suPAR levels received either a placebo or anakinra, an IL-1 receptor antagonist. Anakinra reduced the odds of worse clinical outcomes at day 28, but it was also demonstrated that suPAR effectively guided patient risk stratification. Patients with low suPAR levels, who were excluded from the study, had better outcomes than those with high levels regardless of the intervention received [166]. These results validated findings from a similar previous open-label non-randomized trial [167]. An additional example of a randomized control trial with a similar strategy was conducted on Greek adults admitted to the emergency department with clinical suspicion of infection. In this study, the qSOFA score and suPAR levels were used to select patients at high risk of poor outcomes and they were treated with either a placebo or the antibiotic meropenem. Although this study stopped prematurely due to reasons related to the COVID-19 pandemic, it showed that suPAR could effectively guide patient risk stratification and treatment decisions in this scenario, with a positive impact in preventing early deterioration [168].

8.2. RESEARCH STRENGTHS AND LIMITATIONS

The studies included in this thesis have several strengths worth commenting on. The first study used very large databases from 17 years of continuous morbidity and demographic surveillance. Data available allowed us to assess multiple healthcare aspects separately for boys and girls and expand the analysis beyond the healthcare facility level to encompass post-discharge outcomes and provide additional insights at the community level. Moreover, another strength was being able to stratify all results into four distinct age groups to explore age-related differences. All biomarker studies of this thesis were conducted in the same geographic area as the first study, each focusing on different populations and specific use-cases. The second and third studies used the same methodology to quantify head-to-head several promising prognostic biomarkers of immune and endothelial activation described in the literature. All measurements were performed in the same laboratory facilities at the University Health Network – Toronto General Hospital, Toronto, Canada. This consistent approach enabled a better comparison of the prognostic performance of biomarkers between the two cohorts. All biomarker studies of this thesis benefited from prospective recruitment of participants and comprehensive clinical data collection. An additional key strength of the second

study is the inclusion of a healthy community control group and access to post-discharge mortality data, unavailable in other cohorts of children hospitalized with pneumonia, but available in our study through the linkage with the ongoing demographic surveillance in Manhiça district. The third study also benefited from an active follow-up of study participants until day 28 after presenting to hospital, a substantial number of primary outcomes allowing subgroup analysis, as well as the inclusion of outpatient participants (for whom published data on disease severity and prognostic biomarkers is scarce). Lastly, the fourth study benefited from having multiple sample timepoints.

Nevertheless, these studies also have certain limitations. The main limitations of the first study are its retrospective nature and exploratory approach, using routine surveillance data collected by evolving surveillance systems, being able to assess some outcomes only for residents with an HDSS permanent identification number, and lacking information on all healthcare facilities and informal or traditional medicine providers in the Manhiça district. The remaining studies, which involved biomarker measurements, used stored samples from studies designed for a different main objective, introducing some limitations. In the second study, there were a limited number of primary outcomes and this prevented the exploration of biomarker prognostic performance across participant subgroups. Additionally, post-discharge mortality data were not originally collected as part of the study and were available only for a subset of participants. While the third study had a larger number of primary outcomes, there was considerable loss to follow-up at the 28-day visit, limited statistical power for some secondary analyses, lack of granular details on subsequent care-seeking and hospitalization for outpatients, and imperfect data on immunosuppression in HIV-positive participants. Moreover, the stratified enrolment strategy may limit the representativeness with regard to all febrile cases presenting at the recruiting hospitals. Finally, the fourth study had a limited sample size, sampling times, and follow-up period. This constrained the exploration of additional factors that might affect NfL levels. Although NfL levels could be studied in relation to disease severity and neurological manifestations, we lacked data on neuroimaging, electroencephalographic results, or long-term neurocognitive testing, which would have provided more robust insights into brain injury and potential NfL clinical utility.

8.3. THE WAY FORWARD

Data on host biomarkers in resource-limited settings have substantially increased in recent years and collaborative efforts between research groups are crucial to move things forwards. Datasets from various large studies investigating similar host biomarkers should be combined, standardized, and jointly analyzed to yield more robust conclusions and address further questions with greater statistical power [263]. While this pooling effort of individual-level data requires careful consideration of methodological variations across studies and specific characteristics of each study population, it would enable the use of the same outcome definitions and statistical

methods in analyses and obtained pooled estimates. Furthermore, this would allow doing subgroup analyses based on participant characteristics with more statistical power compared to individual studies. The influence of HIV on prognostic biomarker performance, identified in this thesis, could be further investigated using this methodology, given that HIV infection remains –unfortunately– still highly prevalent among sick individuals in LMICs and that HIV status is a variable commonly collected in studies. The absence of sex-specific differences in biomarker prognostic performance could also be confirmed in this way. Lastly, this methodology could also help redefine or establish actionable biomarker thresholds for different clinical scenarios. Building on the findings of this thesis and aligning with this approach, our group is currently conducting a systematic review and individual participant data (IPD) meta-analysis to enhance our understanding of the performance of sTREM-1 to assess and predict disease severity in children with acute infection (PROSPERO registration number CRD42024616666).

Despite this increase in host biomarker data from patients in resource-limited settings, further studies on specific areas discussed throughout this thesis would be beneficial. First, additional data on biomarkers of immune and endothelial activation are needed from non-hospitalized patients. The best biomarkers for guiding management decisions in hospitalized patients may differ from those best suited for clinical settings with lower severity. Therefore, more studies should include populations visiting outpatient facilities or emergency departments and focus on outcomes that are specifically relevant to them. An ongoing large study, named SPOT-SEPSIS (*"Prediction of Disease Severity in Young Children Presenting With Acute Febrile Illness in Resource-limited Settings"*), plans to measure several of the host biomarkers studied in this thesis in pediatric inpatients and outpatients presenting with acute febrile illness across Asia. This will contribute to shedding light on the existing knowledge gap in the pediatric outpatient population [264]. Second, data on longitudinal dynamics of biomarkers of immune and endothelial activation would provide a deeper understanding of their levels during disease progression. Third, novel biomarkers of brain injury in the context of infections, including NfL, remain scarce. Future studies are needed to generate high-quality data across diverse populations and clinical scenarios.

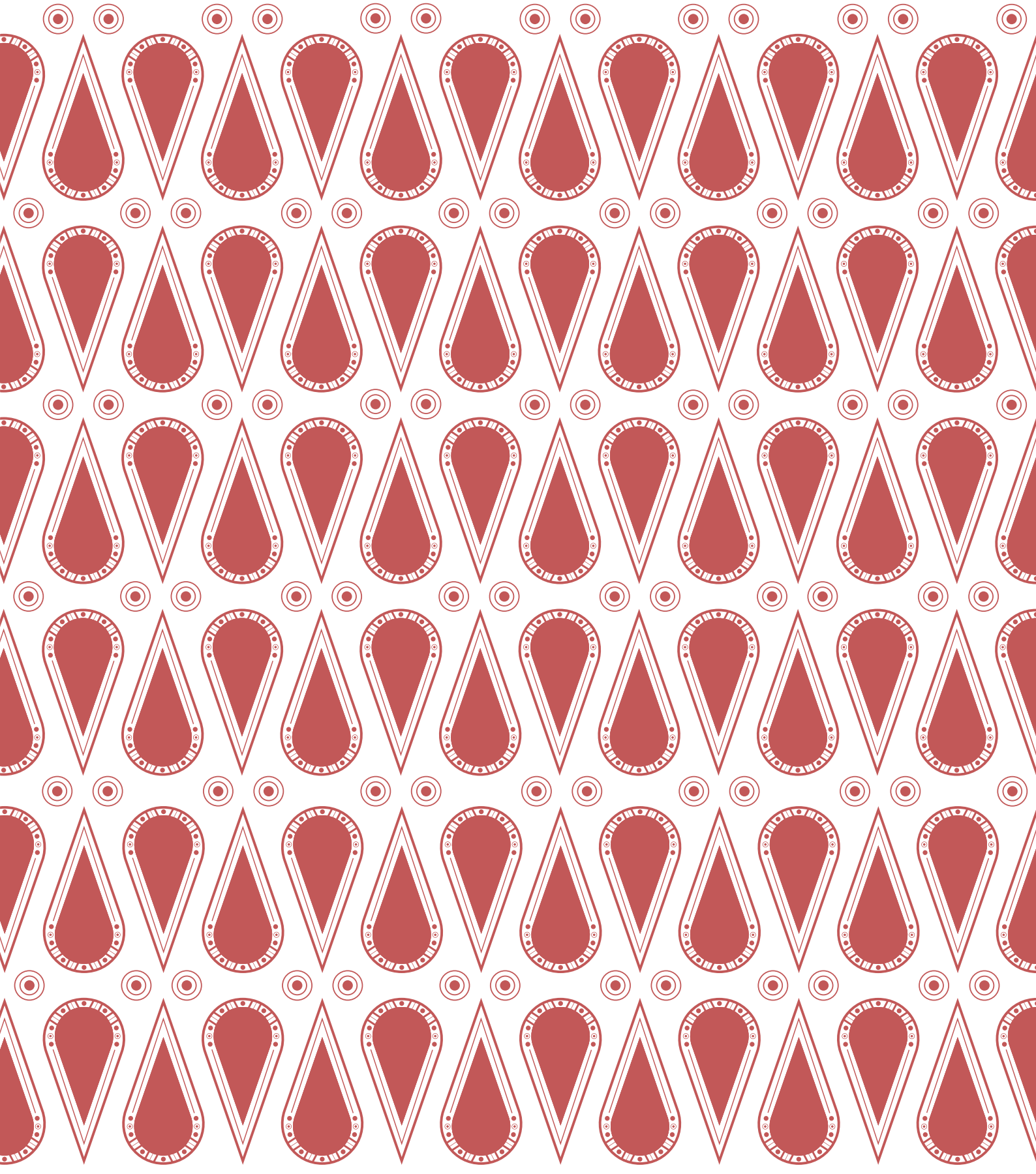
In the short term, another crucial step is the development of improved tools to measure novel host biomarkers. To be useful in clinical practice in resource-limited settings, such tests need to be designed to address their specific needs and conditions, minimizing costs and avoiding extensive laboratory processing. Currently, novel biomarkers can be measured using immunoassays performed in benchtop laboratory equipment, such as enzyme-linked immunosorbent assay (ELISA), Luminex, or ELLA. New host biomarker-based tools must be developed for practical use in clinical settings and, specifically for community and primary care levels, should be in a POC format. As listed by the ASSURED criteria of WHO, desired features include being affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users [265]. Malaria RDTs and glucometers provide precedents for similar hypothetical devices, widely implemented worldwide with sustained use and culturally accepted by communities [99].

Rigorous and large interventional studies are the final step needed to evaluate the clinical utility and impact of adding host biomarker measurements into the standard of care in different scenarios. Assessing statistical measures of associations or prognostic performance of biomarkers, relative to and combined with clinical signs and severity scores, provides insights into which biomarkers could be more useful in clinical practice. The potential benefits of using a biomarker (alone or as part of a score in combination with clinical parameters) can be modeled through decision-analytic measures, but ultimately, interventional studies are necessary to generate evidence on changing clinical practices [266,267]. Trials in HICs that used risk stratification strategies based on suPAR serve as potential examples to build upon [166–168].

The results presented in this thesis on biomarkers of immune and endothelial activation, and in particular the confirmation of sTREM-1 as an accurate indicator of mortality risk during acute infections in populations from Mozambique, have contributed to establishing a solid scientific evidence base from which the EChLiBRiST (*“Enhancing Children’s Lives with Biomarkers for Risk Stratification and Triage”*) Horizon-Europe funded project has emerged. This project aims to develop a quantitative and POC test for measuring sTREM-1 and to clinically validate this tool in two multi-country randomized controlled trials in Mozambique, Gabon, and Ethiopia with pediatric participants aged one to 59 months. One trial will assess if adding sTREM-1 measurement in febrile children at initial clinical assessment impacts the appropriateness of discharge decisions and patient outcomes. A second trial will enroll hospitalized febrile children at high risk of adverse outcomes, identified by high sTREM-1 levels, and evaluate the efficacy of supplementation with oral L-citrulline in improving short- and long-term outcomes. As part of the EChLiBRiST project, health economics, health impact assessment, and socio-behavioral science studies will also be conducted. This will include qualitative research on potential gender-based inequities in healthcare access and provision among children, conducted through engagement with healthcare providers, caregivers, and key informants. This aligns with our recommendation to continue assessing potential gender biases and sex differences in pediatric healthcare in Mozambique, as well as to address evidence gaps in other sub-Saharan countries where current data on this topic remains scarce. A second study, named ACROBAT-newborns (*“Appraising the Critical Role of prognostic Biomarkers in the Assessment and Triage of sick African newborns”*), has also been funded to complement the efforts of EChLiBRiST. This observational study aims to generate evidence on the prognostic performance of host biomarkers specifically in newborns (a particularly understudied population) from Mozambique, Gabon, Ethiopia, and Uganda. Thus, the EChLiBRiST and ACROBAT-newborn projects are expected to significantly complement and expand the efforts undertaken in this thesis.

Finally, it is important to note that while developing new host biomarker-based tools and other innovations holds promise for addressing management challenges of patients with acute infections in resource-limited settings, parallel efforts must continue to strengthen clinical expertise and laboratory capacities. A comprehensive approach that combines novel technology with robust healthcare systems is essential for achieving significant improvements in healthcare delivery and enhancing patient outcomes in resource-limited settings.

9. CONCLUSIONS

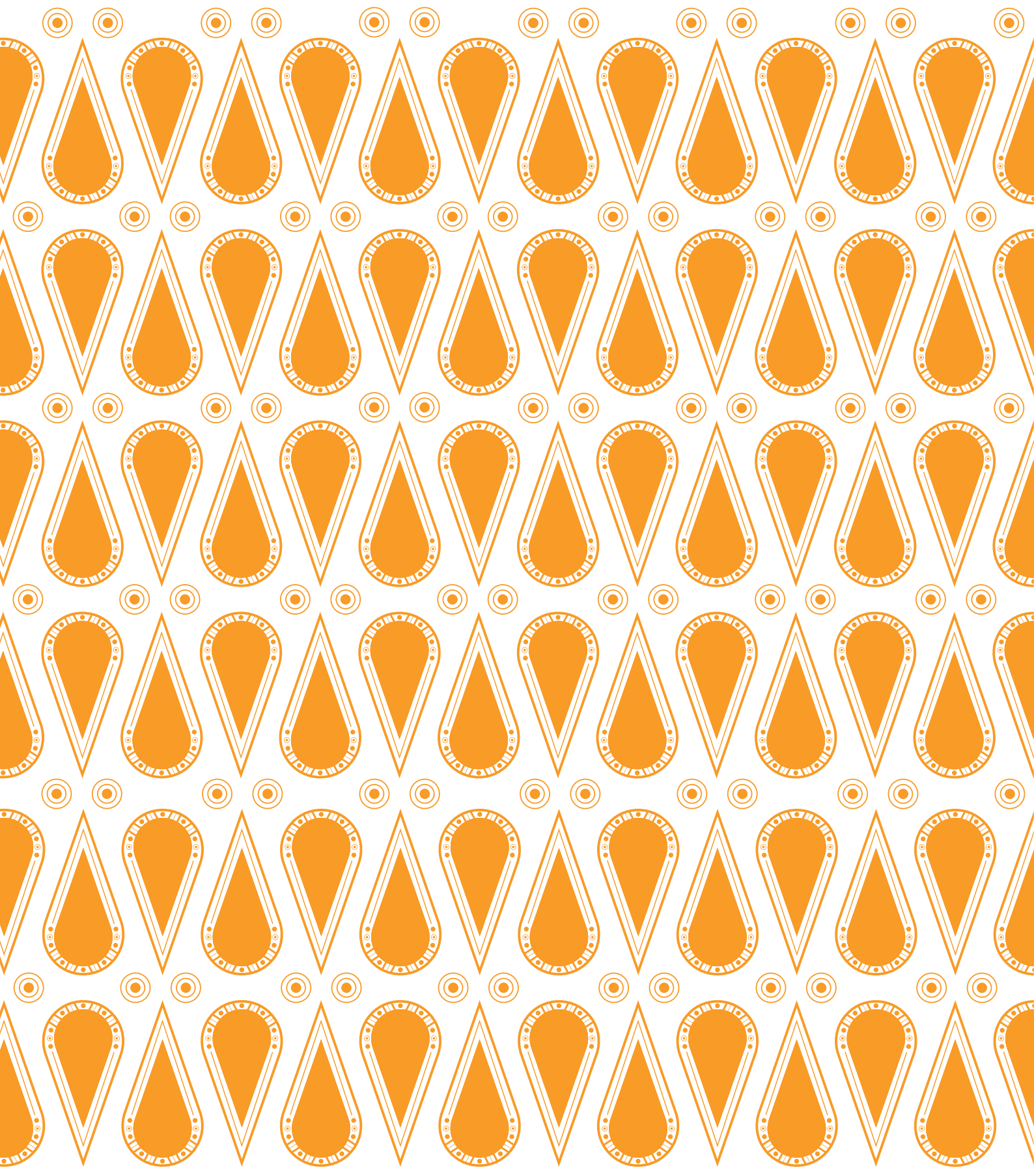


9. CONCLUSIONS

- 1 The observed lower referrals and admissions to hospital in girls compared to boys, along with the finding that severe manifestations were less frequently observed in girls at clinical presentation in Manhiça district, southern Mozambique, warrant further investigation. Apart from this, no major differences were found in other indicators of healthcare access and provision between boys and girls.
- 2 sTREM-1 is a robust predictor of mortality in patients with suspected acute infections. sTREM-1 levels in plasma at clinical presentation showed good prognostic performance for subsequent mortality in children hospitalized with clinical pneumonia and in children and adults with all-cause febrile illness presenting to hospital in Mozambique.
- 3 The prognostic performance of sTREM-1 for mortality was consistent across age and sex in patients with suspected acute infections in Mozambique. sTREM-1 performed worse in HIV-positive patients, but it remained a valuable prognostic biomarker. Future studies need to consider potential differences based on HIV status and HIV-related immunosuppression.
- 4 Other biomarkers of immune and endothelial activation were also good predictors of mortality, but their performance varied across populations. IL-8 and sFlt-1 were strong predictors in children hospitalized with clinical pneumonia, whereas CHI3L1 and Angpt-2 performed well in children and adults with all-cause febrile illness presenting to hospital in Mozambique. However, biomarker combination approaches did not substantially improve prognostic performance.
- 5 Novel biomarkers of immune and endothelial activation outperformed classic biomarkers (i.e., CRP, PCT, and lactate) in predicting mortality among patients with suspected acute infections in Mozambique, offering promising alternatives for this aim. They also added prognostic value to existing clinical severity scores, suggesting that they could be integrated with clinical parameters and provide additional insights.

- 6** Levels of sTREM-1 and other best-performing biomarkers of immune and endothelial activation were associated with disease (compared to healthy controls), hospitalization, and other adverse outcomes beyond mortality in Mozambican cohorts of patients with suspected acute infections. Further research is needed in clinical settings with low mortality risk, using relevant outcomes.
- 7** Plasma NfL is a potential biomarker of brain injury in pediatric patients with malaria, as levels increase over time particularly in cases with neurological manifestations. However, results suggest that NfL is a late indicator of brain injury, and other biomarkers with different kinetics could be more useful for early patient interventions.
- 8** Host biomarkers are a promising tool to improve current patient triage and risk stratification practices. Future research efforts should focus on conducting pooled analyses of existing biomarker databases, performing additional studies to address knowledge gaps in specific populations and use-cases, developing simple and inexpensive POC tests, refining or establishing actionable biomarker thresholds, and conducting interventional studies to evaluate clinical utility and impact (including mitigation of gender-based disparities) of measuring novel host biomarkers in different scenarios involving acute infections in resource-limited settings.

10. BIBLIOGRAPHY



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DOCTORAL THESIS

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