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REVIEW
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Biophysical Tools and Concepts Enable Understanding of Asexual Blood Stage Malaria

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Forces and mechanical properties of cells and tissues set constraints on biological functions, and are key determinants of human physiology. Changes in cell mechanics may arise from disease, or directly contribute to pathogenesis. Malaria gives many striking examples. *Plasmodium* parasites, the causative agents of malaria, are single-celled organisms that cannot survive outside their hosts; thus, thost-pathogen interactions are fundamental for parasite's biological success and to the host response to infection. These interactions are often combinations of biochemical and mechanical factors, but most research focuses on the molecular side. However, *Plasmodium* infection of human red blood cells leads to changes in their mechanical properties, which has a crucial impact on disease pathogenesis because of the interaction of infected red blood cells with other human tissues through various adhesion mechanisms, which can be probed and modelled with biophysical techniques. Recently, natural polymorphisms affecting red blood cell biomechanics have also been shown to protect human populations, highlighting the potential of understanding biomechanical factors to inform future vaccines and drug development. Here we review biophysical techniques that have revealed new aspects of *Plasmodium falciparum* invasion of red blood cells and cytoadhesion of infected cells to the host vasculature. These mechanisms occur differently across *Plasmodium* species and are linked to malaria pathogenesis. We highlight promising techniques from the fields of bioengineering, immunomechanics, and soft matter physics that could be beneficial for studying malaria. Some approaches might also be applied to other phases of the malaria lifecycle and to apicomplexan infections with complex host-pathogen interactions.

Keywords: malaria, biophysics, imaging, *Plasmodium*, cytoadhesion, microfluidics, mechanobiology

INTRODUCTION

Malaria is an infectious disease caused by multiple species of *Plasmodium* protozoan parasites of the phylum Apicomplexa. Malaria killed more than half a million people in 2020 (World Malaria Report, 2021), with *Plasmodium falciparum* causing the overwhelming majority of deaths. Although *Plasmodium* research has typically focussed on the genetic and biochemical determinants of disease,

recent advances in imaging and mechanobiology offer the opportunity to explore how mechanics and the environment contribute to various aspects of the *Plasmodium* life cycle and pathogenesis, in particular the blood stage reported in **Figure 1**. Multiple biophysical tools are now being applied to understanding parasite-host interactions in malaria, particularly the mechanisms of highly dynamic processes such as parasite-red blood cell (RBC) invasion, and cytoadhesion, where the infected red blood cell (iRBC) interacts with the microvasculature and other human cells; both invasion and cytoadhesion play a critical role in severe malaria pathogenesis.

Progress in live imaging has been at the heart of recent discoveries: improvements on real-time and time-lapse microscopy speed and resolution have been crucial in imaging *Plasmodium* parasites in *in vitro* and *in vivo* experimental settings. Likewise, many biophysical methods have been applied to quantitatively characterise the mechanical properties and the adhesion force of healthy RBCs and *Plasmodium*-infected RBCs in bulk and at single-cell level [refer to (Depond et al., 2019)]: all terms and the relative technologies presented in this work are summarised in the Glossary **Table 1**. Techniques to probe and measure forces at the cell scale include micropipette aspiration (Mohandas et al., 1984), atomic force microscopy (Sinha et al., 2015), and optical tweezers (Crick et al., 2014). Recently, membrane flickering spectroscopy has been used as a powerful tool to elucidate the role of RBC mechanics in disease protection (Kariuki et al., 2020). To address the intricacies of parasite and human biology, sophisticated microfluidic devices have been engineered to tune physical and biological parameters in well-controlled reproducible assays while maintaining aspects of physiological relevance, and create *in vitro* models that more accurately reproduce *in vivo* tissue microenvironments.

These concepts fit within the broader efforts in "mechanobiology", i.e. the effort to generally understand the relationship between a cell and its surroundings. Here we use this term to specifically refer to how *Plasmodium* parasites respond to or cause changes in host cell mechanics, physical forces, and substrate structure, and if mechanical pathways influence the infected cells or host tissues.

In this review, we focus on disruptive technologies that have been used to investigate the physical traits of *P. falciparum*-human interactions during invasion (**Section 1**) and cytoadhesion (**Section 2**), where such technologies have generated paradigm shifts in our knowledge of malaria and important insights into potential new intervention strategies. **Section 3** will describe the biomechanical determinants of invasion and cytoadhesion in other species of *Plasmodium*. This review takes a problem-oriented approach, to show how specific research questions in malaria have been addressed using biophysical techniques, hoping to inform the reader in the importance of biophysics in infection, and inspire to adopt similar approaches to tackle their own research questions.

SECTION 1: MECHANICS OF RED BLOOD CELL INVASION

Malaria clinical symptoms arise during the *Plasmodium* blood stage, which starts when merozoites recognise and invade human

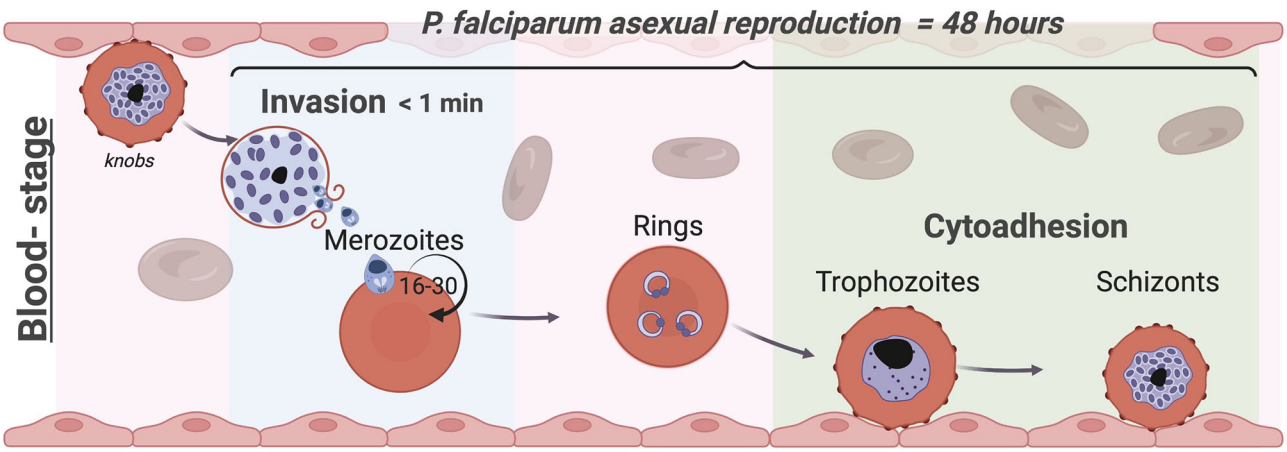

FIGURE 1 | The blood stage of *Plasmodium falciparum*. Life cycles of Apicomplexa involve asexual and sexual reproduction stages in different hosts. *Plasmodium*'s asexual replication in humans occurs in the liver and bloodstream while the sexual stages occur in the mosquito. Parasites are injected into the human skin through the bite of an infected female *Anopheles* mosquito (Frevert et al., 2005; Amino et al., 2006); they first travel to the liver (Menard et al., 2013), where they proliferate into merozoites, before being released into the blood stream. Merozoites invade circulating RBCs initiating the blood stage (Cowman et al., 2017). While invasion typically occurs in less than a minute, within the RBC the parasite undergoes maturation in approximately 48 hours from a ring-stage to a trophozoite by digesting haemoglobin before starting nuclear divisions into daughter merozoites as schizont. At this point, the iRBCs rupture releasing up to 30 daughter merozoites (Rudlaff et al., 2020) producing an exponential growth of parasites inside the host that causes all the symptoms of the disease. To avoid clearance by the spleen, trophozoites and schizonts of *P. falciparum* adhere to the endothelium and to other uninfected RBCs (rosettes) and sequester in the vasculature, leading to severe complications associated with this malaria parasite such as cerebral and placental malaria. In each cycle, 5-10% of parasites develop into the sexual forms and are transmitted to the mosquito vector to complete the life cycle (Frischknecht and Matuschewski, 2017). Image made using ©BioRender (https://biorender.com).

TABLE 1 | Red blood cell biophysical properties, techniques, and effects on malaria.

RBC properties (Unit)	Definition	Techniques (References); Values	Influence on invasion	Influence on intracellular growth and cytoadherence
CELL DEFORMABILITY/RIGIDITY/ STIFFNESS (deformability or elongation index, dimensionless)	General term that describes the ability of a soft body to change its shape in response to an external force In RBCs it depends on: (i) surface area-to-volume ratio (ii) intracellular viscosity (haemoglobin) (iii) membrane viscoelasticity Depending on the experimental protocol, it correlates to a combination of RBC shape and size, cell viscosity, membrane bending and tension	• Ektacytometry (Bessis et al., 1980; Mohandas et al., 1984; Chasis et al., 1985; Schrier et al., 1989; Deplaine et al., 2011; Sisquella et al., 2017; Barber et al., 2018; Ebel et al., 2021) (osmotic gradient ektacytometry); deformability index 0-0.65 (shear stress from 0.3 to 30 Pa) • Microsphiltration (Deplaine et al., 2011) and filtration (Reid et al., 1976); retention or enrichment rates (from < 10 to 100%) • Microfluidics (Glodek et al., 2010) time of passage, shape deformation and recovery through restrictions	• Decrease in deformability reduces invasion in Malayan ovalocytes (Mohandas et al., 1984) • Deformability reduced in RBCs pretreated with glycophorin A-specific antibodies (Chasis et al., 1985), but on the contrary binding of *Pf* ligand EBA-175 RII recombinant protein to RBCs increases deformability (Sisquella et al., 2017) • CR1 ligation increases RBC membrane deformability (Glodek et al., 2010)	• Reduced elongation index in RBCs from *P. falciparum (Pf)* and *P. knowlesi (Pk)*-infected patients (Barber et al., 2018)
SURFACE AREA (μm^2)	Membrane surface area is maintained by cohesion between the lipid bilayer and the spectrin-based membrane skeleton	• Image flow cytometry (Safeukui et al., 2013) • 4D Lattice light-sheet microscopy (Geoghegan et al., 2021); before invasion 135-150 μm^2, after invasion 115-135 μm^2 • Microfluidics (Herricks et al., 2009); 140-160 μm^2 • Laminar shear flow (Suwanarusk et al., 2004); 50 μm^2 for *P. vivax* rings – 90 μm^2 for *P. vivax* schizonts • Micropipette aspiration (Nash et al., 1989);130-150 μm^2 • Confocal fluorescence microscopy and 3D reconstruction (Waldecker et al., 2017); normalised surface area	• Stepwise reduction in RBC surface area is related to the total sum of surface area due to the PVM of each parasite (Geoghegan et al., 2021)	• *Pf*-iRBCs have a smaller surface area: 9.6% (rings) and 14.2% (trophozoites) surface area loss (Safeukui et al., 2013; Waldecker et al., 2017) • Measure *Pf*-iRBC surface area over 48-hour time course (Herricks et al., 2009) • Decreased surface area to volume ratio (Nash et al., 1989) • Increase surface area for *P. vivax (Pv)*-iRBCs (Suwanarusk et al., 2004)
VOLUME (μm^3, fL)	The volume is determined by the water content and its homeostasis is regulated by various membrane-associated ion transporters and channels	• 4D Lattice light-sheet microscopy (Geoghegan et al., 2021); reduced volume increases after merozoite penetration • Microfluidics (Herricks et al., 2009); 60-120 μm^3 for uninfected RBCs - 80-150 μm^3 for iRBCs • Micropipette aspiration (Nash et al., 1989); 90 fL for uninfected RBCs -100 fL for iRBCs • Confocal fluorescence microscopy and 3D reconstruction (Esposito et al., 2010; Waldecker et al., 2017); reduced volume 0.5 to 0.9 after 48 hours	• Echinocytosis starts immediately after parasite penetration (Geoghegan et al., 2021)	• Volume of *Pf*-iRBCs remains almost constant during parasite maturation (Esposito et al., 2010), but Nash 1989 (Nash et al., 1989) found 11.3% increase volume in rings • Measure *Pf*-iRBC volume over 48-hour time course (Herricks et al., 2009; Waldecker et al., 2017)

(Continued)

TABLE 1 | Continued

RBC properties (Unit)	Definition	Techniques (References); Values	Influence on invasion	Influence on intracellular growth and cytoadherence
OSMOTIC STRESS (Pa) (Osmotic fragility: the propensity of a red cell to lyse while absorbing water under hypotonic stress - reflects biophysical membrane properties)	Pressure due to imbalance of solutes across a semipermeable membrane A sudden change in the solute concentration around a cell will cause a rapid movement of water across its cell membrane through osmosis: change in cell size, shape, hydration, viscosity, haemoglobin concentration, crowding May correlate to a combination of RBC shape and size, cell viscosity, membrane bending and tension	• Osmotic fragility tests (Ebel et al., 2021); % haemolysis/haemoglobin concentration (Tiffert et al., 2005)	• Dehydrated RBCs become denser and less susceptible to *Pf* invasion (Tiffert et al., 2005)	• Higher red cell osmotic fragility sustains slower *Pf* growth *in vitro* (Ebel et al., 2021)
VISCOSITY (η, Pa s) (Dissipation in dynamical response. Note that in principle each of elastic shear modulus, Young's modulus, and bending modulus has a dynamical dissipative counterpart, but they have not been measured individually in RBCs)	Resistance to deformation at a given rate Viscosity = plasma membrane viscosity + cytoplasm viscosity (mainly haemoglobin) May correlate to a combination of RBC shape and size, membrane bending and tension	• Flickering spectroscopy (Evans et al., 2008; Betz et al., 2009; Yoon et al., 2009; Froühlich et al., 2019; Kariuki et al., 2020; Davies et al., 2020); 0.01-10 Pa s • Micropipette aspiration (Evans et al., 1984) • AFM (Dulinska et al., 2006) • High-frequency electric field (Engelhardt et al., 1984)	• Higher viscosity in dehydrated RBCs correlates to reduced *Pf* invasion (Tiffert et al., 2005)	• Viscosity increases during *Pf* intraerythrocytic development, no difference in the kinase FIKK 4.1 knock-out lines (Froühlich et al., 2019; Davies et al., 2020)
MEMBRANE SHEAR MODULUS (μ, N/m^2, mDynes/cm^2 or N/m and mDynes/cm for 2D)	A material property that describes the ability of a soft body to change its shape in response to an external force It is well defined for a homogeneous material (3D block or 2D sheet) but in the context of a red blood cell it can correlate to a combination of RBC shape and size, membrane bending and tension	• Optical tweezers (Suresh et al., 2005; Mills et al., 2007); from 5 μN/m (uninfected RBCs) to 53 μN/m (schizonts) • Micropipette aspiration (Nash et al., 1989; Glenister et al., 2002; Rug et al., 2006); 5-20 mDynes/cm^2 - up to 50 pN/μm for *Pk*-iRBCs (Barber et al., 2018) • Laminar shear flow (Suwanarusk et al., 2004); 3-5 mDynes/cm^2 • Diffraction phase microscopy (Park et al., 2008); from 6 μN/m (uninfected RBCs) to 100 μN/m (schizonts) • Holographic microscopy (Byeon et al., 2015); 5-25 μN/m from healthy to trophozoites • High-frequency electric field (Engelhardt et al., 1984); 7x10^{-6} N/m • AFM (Sinha et al., 2015); RBCs treated with different compounds 5-15 μN/m	• Decrease in deformability reduces invasion in Malayan ovalocytes (Mohandas et al., 1984) • RBCs with stiffened membranes do not support merozoite penetration (Sinha et al., 2015)	• Shear modulus increases during *Pf* parasite development (Suresh et al., 2005; Park et al., 2008; Byeon et al., 2015) -supported by simulations (Hosseini and Feng, 2012; Zhang et al., 2015) and change with temperature (Mills et al., 2007; Park et al., 2008) • Knobs are responsible for increasing *Pf*-iRBC rigidity (Glenister et al., 2002) • Decrease in *Pv*-iRBCs (Suwanarusk et al., 2004), increase in both human and macaque *Pk*-iRBCs (Barber et al., 2018)

(Continued)

TABLE 1 | Continued

RBC properties (Unit)	Definition	Techniques (References); Values	Influence on invasion	Influence on intracellular growth and cytoadherence
YOUNG'S MODULUS (E, N/m^2 or N/m for 2D)	Given by stress/strain in a specific deformation geometry - Measures the resistance of a material to elastic deformations It is proportional to the elastic shear modulus for a given geometry of deformation	• AFM (Dulinska et al., 2006; Sisquella et al., 2017; Aniweh et al., 2017); 19-33 kPa	• Binding of *Pf* ligand EBA-175 RII recombinant protein shows a dramatic reduction of RBC Young's modulus, a decrease is also seen for Rh4-CR1 and Rh5-Basigin binding (Sisquella et al., 2017; Aniweh et al., 2017)	• Increase during *Pf*-iRBC development (Ademiloye et al., 2017)
BENDING MODULUS (or bending rigidity, κ, J)	Energy required to bend a membrane, by changing its curvature Depends on membrane asymmetry, thickness (including cytoskeleton and membrane proteins), lipid composition, lipid packing and order	• Flickering spectroscopy (Popescu et al., 2006; Evans et al., 2008; Betz et al., 2009; Yoon et al., 2009; Koch et al., 2017; Froühlich et al., 2019; Kariuki et al., 2020; Davies et al., 2020) • 10^{-20} - $9x10^{-19}$ J • Micropipette aspiration (Evans et al., 1984);	• Decrease in membrane bending increases invasion efficiency (Koch et al., 2017)	• No significant change in *Pf* rings, higher for trophozoites (Froühlich et al., 2019; Davies et al., 2020)
TENSION (or membrane extensional rigidity, σ, N/m)	Force needed to stretch the membrane Different regimes depending on the 'excess surface area' of the cell membrane and on membrane-cytoskeleton adhesion (Lim et al., 2002). Once the membrane is taught, then depends on lipid composition	• Flickering analysis (Popescu et al., 2006; Evans et al., 2008; Betz et al., 2009; Yoon et al., 2009; Koch et al., 2017; Froühlich et al., 2019; Kariuki et al., 2020; Davies et al., 2020); • 10^{-8} - 10^{-5} N/m • Optical tweezers (Yoon et al., 2008); stiffness = 10-30 pN/μm depending on the strain rate	• Correlation between increase tension and reduce *Pf* invasion (Kariuki et al., 2020) (Modelling Hillringhaus et al., 2009)	• Increase during *Pf* development (Froühlich et al., 2019; Davies et al., 2020) • Slightly reduced tension in knobless trophozoites (Froühlich et al., 2019)
ELECTRIC SURFACE CHARGE (or membrane potential mV)	Sialylated glycoproteins of the RBC membrane are responsible for a negatively charged surface	• Surface potential microscope (Aikawa, 1997; Akaki et al., 2002); charge of *Pf* knobs: +20 mV, free merozoite apex: +65.10 mV • Optical tweezer (Fontes et al., 2008); zeta potential RBC: -12.5 mV • Transmembrane distribution of radiolabelled anions (Mikkelsen et al., 1982); uninfected RBCs: -10 mV, rings: -16 mV, schizonts: -35 mV (Mikkelsen et al., 1986). *Pf* schizonts free of host cell membrane: -90 mV	• Merozoite apical end is positively charged while the body is negatively charged (Aikawa, 1997) - the apex binds to the negatively charged RBCs during invasion	• Knobs of *Pf*-iRBCs are positively charged, thus, facilitating adhesion to the endothelial cells which have a negative surface charge (Hillringhaus et al., 2009) • Membrane potential is -16 mV for rings and -35 mV for late stage iRBCs (Fontes et al., 2008)

RBCs. The invasion process is essential for parasite survival and replication, and consists of a sequence of mechanical and biochemical events that are precisely timed and tightly regulated. During invasion parasites are extracellular and hence exposed to the antibody mediated immune system, representing a potential target for vaccine and drug development. Advanced microscopy techniques such as cryo-electron tomography (Hanssen et al., 2013), cryo-X-ray tomography (Hanssen et al., 2011), electron microscopy (Kumar et al., 2017), and super-resolution structured illumination microscopy (Riglar et al., 2011) have been crucial to unveil the structure of merozoite organelles involved in invasion, and the molecular mechanisms that determine the irreversible attachment between merozoites and RBCs (Mauritz et al., 2010; Cho et al., 2012). However, these techniques operate with fixed samples providing static snapshots, which is not ideal for a highly dynamic and rapid process such as invasion, which is usually complete in less than a minute. Therefore, a main challenge in the field has been to follow the egress-invasion sequence in real time, and this is typically achieved with live imaging in brightfield, phase contrast, differential interference contrast (DIC), and epifluorescence (Glushakova et al., 2005; Gilson and Crabb, 2009; Crick et al., 2013; Introini et al., 2018a).

Subsection 1.1. Live Microscopy Reveals the Egress-Invasion Sequence and Intraerythrocytic Cycle

Over the last two decades, progress on the video temporal and spatial resolution permitted the recording of the kinetics of merozoite egress and subsequent invasion of RBCs up to hundreds of frames per second with sub-μm resolution, and has been used to assess the effect of antibodies, enzyme inhibitors, and ion signalling on the invasion process (Glushakova et al., 2005; Gilson and Crabb, 2009; Crick et al., 2013; Weiss et al., 2015; Bustamante et al., 2017). The egress was initially described in the literature as an "explosive" phenomenon (Lew, 2005; Mauritz et al., 2010), but Abkarian et al. (Abkarian et al., 2011) revealed a detailed dynamic morphology of the host cell membrane rupture using high-speed video microscopy, as represented in **Figure 2**. At the instant of schizont rupture, a pore opening in the red cell membrane allows only 1 or 2 merozoites to emerge. Once the pore reaches a critical radius, the host cell membrane rapidly curls outwards to form a circular toroid around the initial opening, and then buckles, undergoing eversion to push out and disperse the rest of the merozoites. Finally, the everted membrane spontaneously forms vesicles (Lew, 2011). This curling-buckling-eversion-vesiculation sequence is completed in about 400-800 ms (Callan-Jones et al., 2012; Braun-Breton and Abkarian, 2016) and resembles, on a shorter time scale, the spontaneous vesiculation of uninfected RBCs found previously by Lew et al. (Lew et al., 1988; Tiffert and Lew, 2014). This similarity may be evidence of the parasite exploiting an intrinsic host cell property and remodelling the cortical cytoskeleton for a rapid response during egress (Kabaso et al., 2010).

The three main phases in which invasion is usually divided i) pre-invasion, ii) recoil phase, and iii) internalisation (Gilson and Crabb, 2009; Groomes et al., 2022) are described in **Figure 2**, highlighting the biophysical techniques developed to measure both the parasite and the RBC physical quantities at each phase. During pre-invasion, which lasts between 2-50 s, merozoites make reversible contacts with RBCs triggering transient deformations of the cell membrane from the contact site. The merozoite re-positions itself on the surface of the RBC until it is apically aligned, and binds to the RBC using adhesins on the merozoite coat (Tham et al., 2015; Weiss et al., 2016). After a recoil phase where RBC deformations cease and the biconcave shape is restored, the secretory organelles located in the merozoite apex release ligands into the RBC and the merozoite is irreversibly attached (Treeck et al., 2009; Crosnier et al., 2011). The tight junction is then formed, and penetration begins driven by the merozoite actomyosin motor. Merozoite internalisation is frequently, but not always, accompanied by echinocytosis (taking approximately 5-15 minutes (Gilson and Crabb, 2009) where the RBC outer surface curls and forms spikes, before restoring their original biconcave shape with the parasite-ring inside. Transition of RBCs from discocytes to echinocytes results from the expansion of the outer leaflet of the plasma membrane with respect to the inner one (Lim et al., 2002), producing convex structures on the cell surface (echinocytic spicules) to accommodate the extra area, and resulting in increased bending modulus, shear modulus, and surface area (Park et al., 2010) (**Table 1**). Computational modelling of this invasion processes is a huge and still open challenge since invasion ligand expression is controlled in time and space, and ligand-receptor interaction binding rates are modulated by membrane properties (Mognetti et al., 2019; Hillringhaus et al., 2020).

The latest imaging upgrade to investigate *Plasmodium* invasion is given by the 4D lattice light-sheet microscopy (LLSM), described by the pioneering work of Chen and colleagues (Chen et al., 2014). LLSM provides volumetric information at hundreds of images per second, and a distinct advantage of this method over the conventional 2D microscopy consists of looking at parasites and RBCs from multiple orientations given by the near isotropic resolution (Geoghegan et al., 2021). Compared to confocal laser scanning microscopy, the thinness of the sheet produces a high axial resolution (the ability to discern between two points at different depth along the optical axis of the imaging objective) and larger field of view, a better signal-to-noise ratio when using multiple fluorescent probes, and less photobleaching. LLSM has succeeded in characterising all stages of invasion quantitatively, including parasite internalisation and parasitophorous vacuole membrane (PVM) formation (Geoghegan et al., 2021) (**Figure 2**). Despite many shape changes throughout the invasion process, the membrane area and volume of host RBCs were previously thought to remain essentially unchanged (Mauritz et al., 2009; Waldecker et al., 2017). Here the authors showed a stepwise decrease in RBC surface area which coincides with the total sum of surface areas lost for the PVM of each parasite invading the same RBC.

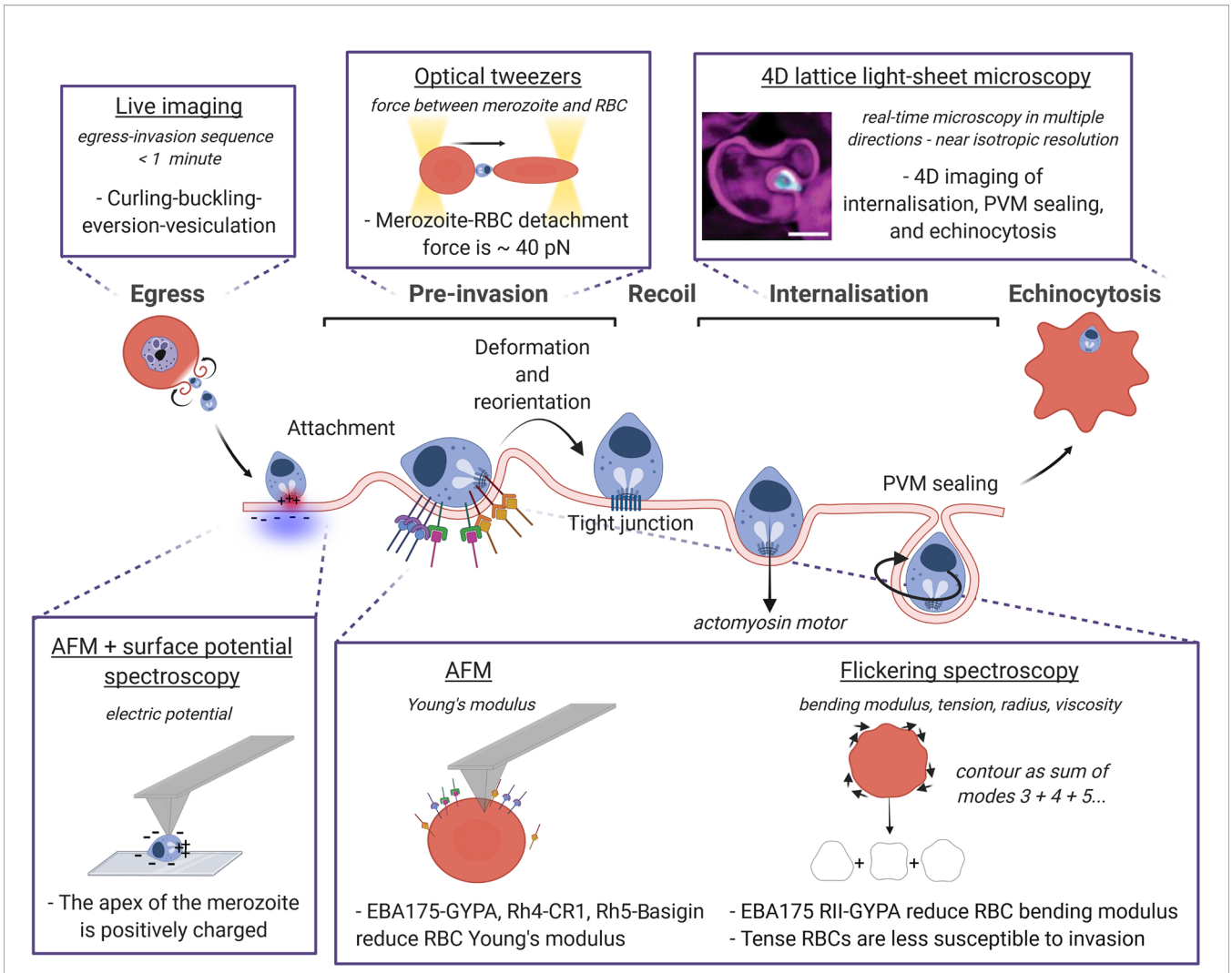

FIGURE 2 | Biophysical techniques and methods to probe the mechanics of *P. falciparum* invasion. The sequence egress, pre-invasion, recoil phase, internalisation and finally echinocytosis is represented with a focus on the biophysical approaches used to measure the mechanical properties of the RBC and the parasite, and their interactions. The technique is underlined, the quantity measured with the relative technique is in italics, and the dashed sentences report the main findings. Image made using ©BioRender (https://biorender.com). LLSM image was taken from Geoghegan et al. (Geoghegan et al., 2021).

Recent imaging of CRISPR Cas9 engineered parasite lines with fluorescent tags (Lee et al., 2019), has boosted the study of specific ligand-receptor interactions at the molecular level. This has permitted the localisation of apical ligands that take part in the invasion (Treeck et al., 2009), and showed that the invasion proceeds with the wider end of the merozoite leading, rather than the narrower pointed end, highlighting a remarkable motion of the merozoite that until now was mostly overlooked (Yahata et al., 2021). Moreover, machine learning algorithms for image recognition have been employed to automatically detect infected cells and their stage (Davidson et al., 2021).

One challenge for live-cell imaging is the long, repeated exposure to light which affects both the fragile parasites and the RBCs. Phototoxicity induces lysis of the acidic food vacuole of the parasites (Wissing et al., 2002) and damages RBC haemoglobin inducing autofluorescence (Shrirao et al., 2021), making it very difficult to follow the parasites during the entire intraerythrocytic cycle. Using DIC and confocal microscopy, Gruring et al. (Grüring et al., 2011) were able to visualise the 48-hour development of *P. falciparum*, elucidating the trafficking mechanism of exported proteins to the host membrane and revealing a fascinating morphology of the parasite at the ring stage. Contrary to the familiar features visible with Giemsa staining, rings are not always circular but assume transient amoeboid forms during the first 5-10 hours post invasion. This phenomenon would require further investigations in real time to explain this initial amoeboid cytoplasm.

Subsection 1.2. Biophysical Analysis of Receptor-Ligand Interactions

The initial merozoite-red cell adherence can occur at any point on the merozoite surface due to low-affinity and reversible

interactions between Merozoite Surface Proteins (MSPs) and receptors on the red cell membrane - with coat filaments of 20-150 nm length joining the two surfaces (Bannister et al., 1986). Subsequent reorientation and final attachment require Erythrocyte-Binding-Like (EBL), Reticulocyte binding homologous (Rh), and AMA-1 ligands that span the red cell membrane and link to the merozoite cytoskeleton. These adhesive ligands are secreted from the organelles located at the apex of the 1 μm ovoidal merozoite, which was for a long time presented and modelled as being the narrow end, but recently this depiction has been questioned (Yahata et al., 2021). In hindsight, if the parasite is pear-shaped it will rotate to have the less curved side attached to the membrane. This will reduce bending energy, and allow a larger adhesive area per merozoite. The merozoite shape and orientation therefore play a key role in the mechanics of invasion, which needs further investigation.

Beyond mediating attachment, such ligand families can also transduce signals and modify the physical properties of the host cell membrane (described in **Table 1**). For example, recombinant proteins Rh5 binding to Basigin mediate Ca^{2+} signal in the RBC triggering phosphorylation of specific proteins and the subsequent rearrangements of the RBC cytoskeleton that facilitate the insertion and anchoring of rhoptry proteins and junction formation (Aniweh et al., 2017), and increases deformability, i.e. decreases the Young's modulus of RBCs, albeit to a lesser extent than binding of MSPs, Rh4, or EBA175, hence favouring invasion (Sisquella et al., 2017) (**Figure 2**). EBA175 RII binding to the RBC receptor Glycophorin A (GYPA) induces a reduction in the host cell bending modulus that increases invasion efficiency, by lowering the energy necessary to bend the RBC surface and facilitate merozoite attachment. The possible caveat is that the local concentration of EBA175 (or any other ligand) on the merozoite surface is unknown, so it is hard to predict how well the experimental set up mimics the actual host RBC-merozoite interaction during invasion. These measurements were performed with AFM, and confirmed using flickering spectroscopy (Koch et al., 2017), as described in **Figure 2**. Flickering spectroscopy is an optical technique that extracts the cell bending modulus (the energy required to bend the membrane) and tension (the resistance of the membrane to stretch) by analysing the plasma membrane thermal fluctuations. By extending this approach into the dynamical domain and measuring the relaxation rates, it is possible to extract the cytosolic viscosity (Helfrich and Servuss, 1984; Monzel and Sengupta, 2016) (example in **Figure 2**) Flickering experiments served to investigate the biophysical properties of RBCs depending on oxygenation level, cell morphology, hydration state (Yoon et al., 2009), glucose concentration (Tapia et al., 2021) and parasite infection (Davies et al., 2020); as well as exploring the biophysical impact of malaria-protective polymorphisms affecting RBCs (such as hemoglobinopathies S and C (Froühlich et al., 2019), Dantu blood group (Kariuki et al., 2020) and beta-thalassaemia Introini et al., 2022). The advantages of this method to measure deformability over the more traditional tools, as AFM and optical tweezers, are i) the easy set-up where only a good quality camera is required, ii) the non-invasiveness since samples are not directly manoeuvred, and finally iii) the ability to decouple bending modulus and tension, while other techniques yield the effective shear modulus of the composite system (Kariuki et al., 2020) (see **Table 1**). It is notable that the theoretical assumptions behind this method are valid only for circular-like closed objects like vesicles and RBCs.

But how strong is the attachment between the RBC and the invading merozoite? Crick *et al.* measured for the first time the detachment force between a merozoite and an RBC using optical tweezers (40 ± 8 pN) (Crick et al., 2014). Within few seconds from egress, a merozoite is brought in contact with nearby RBCs forming a chain of RBC-merozoite-RBC, and the two trapped RBCs are pulled away simultaneously using optical tweezers (sketch in **Figure 2**). For a given rate of extension, RBC have been shown to behave like linear springs, i.e. they have a well - stiffness. Thus, the detachment force can be calculated knowing this RBC stiffness and observing the maximum elongation of the cells just before detachment. Generally used for mechanical measurements, optical tweezers are a powerful method for positioning μm-size colloids and cells, and therefore optimal for manipulating individual merozoites and RBCs during invasion (Mauritz et al., 2010). The main difficulty of this technique comes from the low yield due to the short viability of free *P. falciparu*m merozoites after egress (< 2 minutes). Only treatments like heparin that block invasion at the early stage show a reduction in the detachment force attesting that the force measured comes from ligands and receptors participating in the pre-invasion phase (Crick et al., 2014). The electrostatic attraction of the positively charged apex of the merozoite towards the negative surface of the RBC measured using AFM with surface potential microscopy (Akaki et al., 2002) adds to the force generated by EBL and Rh ligands, although the exact ligand responsible for the measured force has not been identified yet, as most EBL and Rh ligands mediating attachment have a redundant function (Menard et al., 2013; Weiss et al., 2015) (**Figure 2**).

A hallmark of invasion videos is the dynamic deformations of the RBC membrane elicited by merozoite adhesion during pre-invasion. These RBC deformations wrap the merozoites with different strength which correlate with successful invasion (Weiss et al., 2015; Kariuki et al., 2020; Geoghegan et al., 2021) and have been categorised visually from weak to strong by using a scale from 0 to 3 (Weiss et al., 2015). Calcium influx from the merozoite to the RBC was hypothesised to trigger deformations to assist parasite reorientation (Lew and Tiffert, 2007), but this has been disproved by using simultaneously bright-field and fluorescence live microscopy, suggesting instead that local membrane deformations might simply be a passive byproduct of parasite adhesion to the RBC (Introini et al., 2018a; Hillringhaus et al., 2020). This view was supported by Dasgupta et al.'s numerical simulations (Dasgupta et al., 2014) showing that merozoite shape and apical adhesion gradient contributed extensively to wrapping, with an indentation force during the early stage of the invagination estimated of 13 pN (Abdalrahman and Franz, 2017). This is in fact what happens

when placing spent merozoites close to RBCs using optical tweezers, they in fact had lost the ability to invade 2-3 minutes post-egress but nonetheless adhere to RBCs and still induce transient local membrane deformations (Crick et al., 2013; Crick et al., 2014).

Altogether, these approaches show that biophysical measurements are necessary to understand how the strength of interaction between specific ligands and RBC receptors affects the host cell mechanics.

Section 1.3. Contribution of Host Cell Mechanics to Invasion

Not only do merozoite interactions modify the biomechanical properties of the RBC to facilitate parasite entry, but naturally occurring mutations in proteins of RBC membrane can impact the host cell biophysics and confer protection against malaria. Malaria has provided a strong selective force on the human genome, leading to the selection of many polymorphisms that provide some level of protection against severe disease. Given that all the symptoms and pathology of malaria occur during the intraerythrocytic stage, unsurprisingly most of these polymorphisms relate to the structure and function of red blood cells (Kariuki and Williams, 2020). *Plasmodium* merozoites are less able to invade RBCs with higher membrane rigidity (Mohandas et al., 1984; Chasis et al., 1985), as documented in a plethora of mutations in the RBC membrane associated with protection against malaria (Mohandas et al., 1984; Froühlich et al., 2019). A recent example is the rare Dantu variant, present at appreciable frequency almost only in East Kenya, which is caused by a rearrangement of Glycophorin A and B genes that results in the expression of a hybrid Dantu protein with a GYPA extracellular domain/GYPB tail fusion that does not interact with the RBC cytoskeleton (Malaria Genomic Epidemiology Network., 2015; Leffler et al., 2017; Ndila et al., 2018). By combining real-time imaging with flickering spectroscopy, Kariuki *et al. (*Kariuki et al., 2020) were able to quantitate for the first time both the biophysical properties of the host cell and the invasion outcome by *P. falciparum* merozoites, and compare these properties across Dantu genotypes. These data suggested that there is a threshold for RBC membrane tension, above which the chance of successful invasion is significantly reduced. Knowing the membrane tension and bending modulus of RBCs it is possible to calculate the relative wrapping energy, which is higher for both Dantu and heterozygous beta thalassaemia individuals (Introini et al., 2022). Using a mechanically realistic model of a deformable RBC and an attractive potential to mimic the adhesion gradients at the surface of the merozoite, Hillringhaus *et al. (*Hillringhaus et al., 2019) found that an increased membrane rigidity results in poor merozoite alignment which could explain why several other RBC polymorphisms associated with membrane tension such as Southeast Asian hereditary ovalocytosis (Mohandas et al., 1984) protect people from malaria infection.

Merozoites were often observed laterally attached to Dantu RBCs but seldom poised for invasion, a recurrent feature observed also in invasion attempts of denser RBCs (Tiffert et al., 2005). Invasion is indeed reduced in dehydrated RBCs (hence higher haemoglobin concentration and cytoplasmatic viscosity), and mutations related to RBC hydration also have effects on RBC deformability (e.g., PIEZO1 (Ma et al., 2018) and the ATP2B4 (Timmann et al., 2012; Lessard et al., 2017; Villegas-Mendez et al., 2021) gene that encodes the plasma membrane calcium ATPase 4 responsible for the intracellular calcium level). PIEZO 1 is a mechanosensitive channel that can be activated when RBCs are stretched in the circulation, causing small-dehydration events *via* brief surges in RBC calcium (Lew and Tiffert, 2017; Rogers and Lew, 2021). An analogous phenomenon may happen during the deformation phase if merozoite contact is sufficient to mechanically activate the PIEZO 1 ion channel. However, studies on the mechanics and susceptibility to invasion of the human gain-of-function PIEZO1 allele variant, E756del, show discordant results (Thye et al., 2022): some did not indicate RBC dehydration or hinder *P. falciparum* invasion and growth *in vitro (*Nguetse et al., 2020), others found that cell density and shear stress contribute to lower parasite replication in E765del PIEZO1 blood *in vitro (*Glushakova et al., 2022). Since the mechanical activation of PIEZO 1 happens when RBCs squeeze within capillaries, future studies under flow might shed light on the role of PIEZO 1 in malaria invasion (see **Section 2**).

Section 1.4. Merozoite Motility

In general, invasion can be explained as a combined effect of passive processes such as merozoite adhesion-driven wrapping (**Section 1.2**), and active parasite-induced processes such as RBC cytoskeleton remodelling by merozoite ligand injection (Dasgupta et al., 2014) (**Section 1.2**) and merozoite motor contribution (deformations are inhibited when impairing the parasite motor with cytochalasin D) (Weiss et al., 2015; Geoghegan et al., 2021). Merozoites do not have flagella or cilia but instead use a substrate-dependent locomotion called gliding motility to invade target host cells. The motor complex (glideosome) that powers invasion in *Plasmodium* merozoites (Baum et al., 2006) is the same that allows *Plasmodium* sporozoites to traverse tissues, and is also present in tachyzoites of *Toxoplasma gondii* and conserved across other Apicomplexan parasites including *Cryptosporidium (*Wetzel et al., 2005) and *Babesia (*Asada et al., 2012). As a result, this motility mechanism plays a key role in determining the variety of hosts and tissues targeted by these pathogens. Merozoite penetration is driven by the actomyosin motor that drags the tight junction adhesins rearward, thereby creating a traction force that propels the parasite forward into the RBC. The last act is the PVM and host membrane sealing which seems also common among Apicomplexa: *Toxoplasma gondii* shows a twisting (corkscrew-like) motion (Pavlou et al., 2018) and this is probably alike to the helical motility of *Plasmodium* merozoites (Blake et al., 2020; Geoghegan et al., 2021).

P. falciparum merozoites were for a long time thought to not display the gliding motility of other stages, but Yahata et al. (Yahata et al., 2021) recently demonstrated that they glide on specific polymer-coated substrates *in vitro*. Gliding behaviour

depends on the substrate coating: the percentage of motile parasites on glass coverslip is very low and merozoites follow a Brownian motion when released, this is possibly the reason why this phenomenon has not been observed before with the same microscopy techniques. Tracking merozoite directions after egress with time-lapse imaging shows that *P. falciparum* merozoite average gliding speed is 0.6 μm/s and the longest gliding time is 43 s, significantly lower than *Plasmodium* sporozoites, and *Babesia bovis* merozoites (Yahata et al., 2021). The short duration of *P. falciparum* merozoite motility corresponds to the 2-minute viability of merozoites post egress already shown using optical tweezers (Crick et al., 2014), and indicates declining of motor function over time. It is not clear if this motility is actively used during invasion to discriminate between RBCs, for example based on their mechanical properties, and thus direct invasion in selected cells.

Section 1.5. Future Perspectives of Biophysical Approaches in Merozoite Invasion

It remains experimentally challenging to investigate how physical traits impact *P. falciparum* invasion due to the short duration of merozoite invasion and the heterogeneity of RBC mechanical properties. Nonetheless, ground-breaking technological improvements are already helping to answer some of the urgent outstanding questions in malaria formulated by Groomes and co-authors (Groomes et al., 2022). Invasion is a balance between parasite and host cell contributions, evolved toward maximal efficient use of parasite force and modulation of the host cell biophysical properties. Merozoite attachment alters the biomechanical properties of the RBC membrane and triggers dramatic surface deformations that are thought to accommodate the parasite for invasion. The mechanism of these large deformation responses remains an open question for future research. A first step forward would be the quantification of the deformation strength based on more standardised physical parameters such as curvature variation and duration, and not only by subjective observation. This can be achieved by continuous advances in imaging technologies (e.g., LLSM) that help reveal novel features of RBC remodelling and clarify concealed mechanisms through previously unachieved resolutions. High-speed video microscopy combined with flickering spectroscopy has played an important role to establish a membrane tension threshold for *Plasmodium* invasion, and in principle it could be optimised to measure the changes in the biophysical properties of the invaded RBC at every step of merozoite reorientation and internalisation. However, flickering spectroscopy relies on the detection of the entire cell contour, and the drastic changes in the RBC membrane during the pre-invasion phase and echinocytosis limit the use of this technique. Finally, if flickering experiments, from video recording to complete analysis, became fully automated and higher throughput, it could be employed to screen compounds that inhibit *P. falciparum* invasion by modifying the host cell membrane biomechanics. If feasible, it should be more difficult for Plasmodium parasites to develop resistance to such a host-targeted approach.

An emerging area of research is the development of fluorophores that allow the measurement of forces (Valanciunaite et al., 2020), and they could be in future applied to the study of RBC membrane biophysics or even food vacuole and endoplasmatic reticulum in living parasites. Probes that are sensitive to lipid order (Niko et al., 2016) and "Flippers" (Goujon et al., 2019) that respond to the change of plasma membrane tension by changing their fluorescence lifetime could be applied to measure lipid organisation and tension of RBCs before and during invasion. However, these techniques rely on Fluorescence Lifetime Imaging Microscopy (FLIM) (Deo, 2020) to measure the probe fluorescence decay time that might be slower than invasion, and therefore their use might be limited by the short duration of malaria invasion.

In malaria, optical tweezers were used directly on RBCs attached to a merozoite to obtain the system detachment force, but optical tweezers have been typically used to quantify the stretchability of cells by trapping and pulling beads conjugated to a target membrane. Future lines of research at the interface between soft matter and cell biology could involve biomimetic approaches to investigate host-pathogen interactions, such as the use of DNA-functionalised colloids of micrometre dimension, vesicles or beads coated with proteins (Mognetti et al., 2019). These synthetic systems provide a better control of ligand adhesion rate and diffusion, overcoming challenges of real biological systems like the wide variability of biophysical properties within a single population of RBCs, even from a single donor. The use of engineered parasites in optical tweezer experiments could help dissecting the orientation of each EBL and Rh families and their contribution to the strength measured.

Finally, future studies should explore how host-pathogen interactions change when the invasion occurs in flow. All studies presented so far have been performed in static cultures, but looking at invasion under flow is important because shear forces can alter ligand-receptor interactions and activate mechanosensitive channels in RBCs. It is known that shaking malaria cultures increases the efficiency of invasion, reduces asynchronous development and multiple invasions (multiple merozoites invading the same RBC) (Allen and Kirk, 2010). This is due to a more uniform distribution of metabolites and nutrients among parasites, as well as a more efficient dispersal of the merozoites after egress (Collins et al., 2017). Shaking can even have a profound effect on the invasion phenotype: *P. falciparum* strains cultured in moving suspension as opposed to static conditions switch from sialic acid-dependent to independent invasion pathways accompanied by upregulation of the corresponding key invasion genes (Awandare et al., 2018; Nyarko et al., 2020). Moving suspension culture may possibly select for parasites with stronger interactions to fasten onto the RBC surface prior to entry, and therefore a thorough study of the hydrodynamic forces at play and how they regulate this switching mechanism will provide insight into the nature of invasion, as occurs *in vivo*. In this view, microfluidic devices and deformation cytometry (Xavier et al., 2016), a technique that

relates cell shape to its stiffness based on the chamber flow rate, would be ideal to systematically control the rheology and physical environment while assessing if the familiar features of static invasion, such as RBC deformations, merozoite reorientation and invasion rate, remain the same in flow settings.

SECTION 2. INFECTED RED BLOOD CELL MECHANICS AND CYTOADHESION

Subsection 2.1. Understanding Mechanical Properties of Infected Red Blood Cells With Microfluidic Devices

A remarkable feature of human RBCs is their high deformability when traversing small diameter capillaries, thus exposing maximal surface area and minimal distance to tissue cells for optimal gas exchange across capillary walls. The principal techniques employed to investigate the biophysical properties of iRBCs described in **Sections 2.1**, **2.2** are summarised in **Figure 3A**. The use of microfluidics has vastly improved our understanding on the mechanical properties of uninfected and iRBC, as well as their interactions with the microvasculature. Microfluidic devices are fabricated by lithography patterning of optically clear flexible polymers, such as poly-dimethylsiloxane (PDMS), and provide control of the size and geometry of microfluidic networks at micrometre resolution (Antia et al., 2008). For example, early studies with microfluidic devices consisting of 2-8 μm microchannel constrictions revealed that *P. falciparum*-infection prevented iRBC passage through small diameter constrictions. Later, wedged PDMS capillary constrictions were used to determine the smallest diameter a cell can pass through without increasing its surface area or lysing. Overall, this study found that schizonts lose the property of flowing through the narrowest constrictions (Herricks et al., 2009) (**Figure 3A**). Moreover, Namvar and colleagues demonstrated using similar chips that surface area-to-volume ratio, not cellular viscoelasticity, determines RBC traversal through small capillaries, the opposite is true for obstruction caused by *P. falciparum*-iRBC (*Pf*-iRBC) trophozoites, which are a result of changes in viscosity and not surface area-to-volume ratio (Namvar et al., 2021).

Late-stage Pf-iRBCs-iRBCs become less deformable and get trapped in the splenic endothelial slits, a narrow 2 μm passage between cell junctions in splenic sinusoids. Microsphiltration assesses the ability of iRBCs to squeeze through narrow fissures

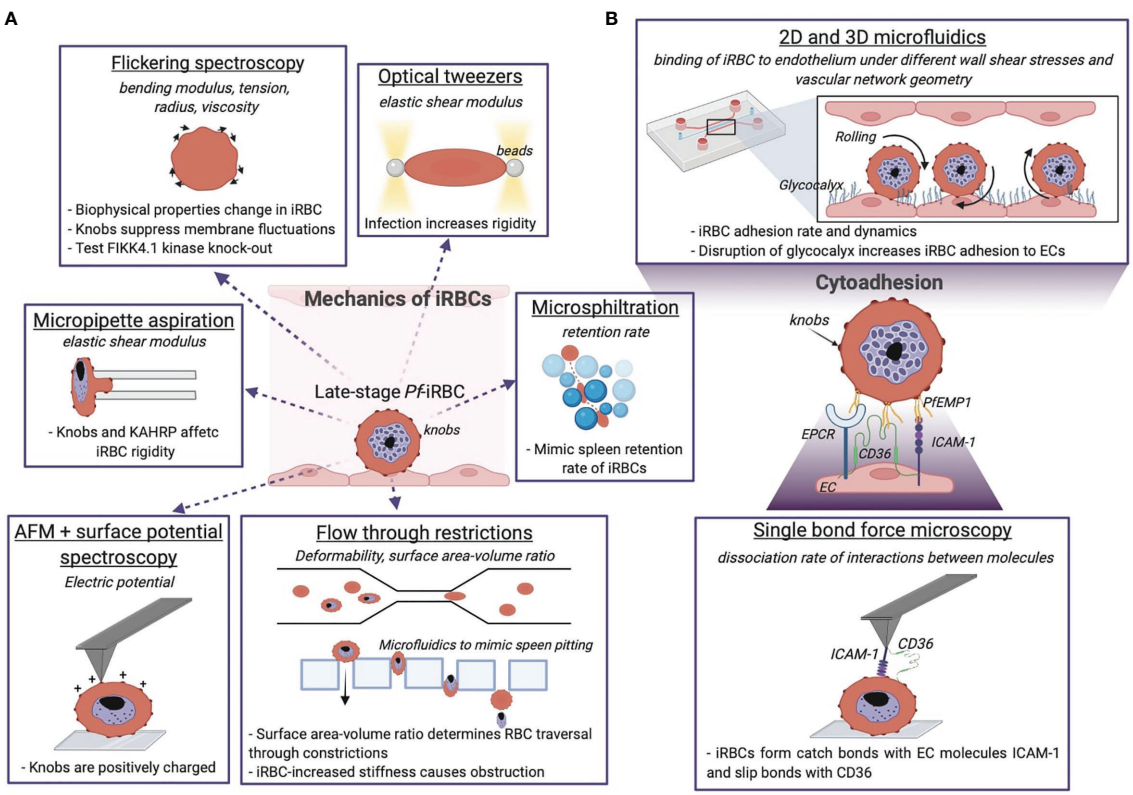

FIGURE 3 | Biophysical methods give insights into the mechanics of *P. falciparum* iRBCs and their cytoadhering features. **(A)** Optical and mechanical techniques have been employed to investigate the effect of the parasite infection onto the host cell membrane biophysics. **(B)** Adhesion between the iRBC and the endothelium is studied at the molecular level and using microfluidic devices, the latter give information about the iRBC dynamics and pathogenesis of malaria by reproducing the physiological *in vivo* situation. The technique is underlined, the quantity measured with the relative technique is in italics, and the dashed sentences report the main results achieved. Image made using ©BioRender (https://biorender.com).

between metallic microspheres of 5-25 μm in diameter arranged in a matrix, by computing the retention rate from the upstream and downstream proportions of iRBCs (Deplaine et al., 2011; Lavazec, 2017; Ndour, 2017). It has been applied as high-throughput screening of drug-induced parasite stiffening (Duez et al., 2018). Additionally, microfluidic devices have been used to mimic spleen filtration and the process of pitting with an array of rectangular pillars of PDMS with tuneable slit sizes down to few micrometres (**Figure 3A**) (Rigat-Brugarolas, 2014). Elizalde-Torrent and colleagues showed that by regulating the slit size and the blood flow it is possible to remove the solid parasite from the cytoplasm of an iRBC without destructing the cell (Elizalde-Torrent et al., 2021). Taken together, *Pf*-iRBCs become more rigid as the parasite progresses through the blood developmental cycle, increasing their vulnerability to spleen clearance as shown by the use of microfluidic devices.

Subsection 2.2. Biophysical Analysis of Infected Red Blood Cell Mechanics

The increase in parasitised RBC rigidity is in part due to the presence of a growing nondeformable intracellular parasite (Fedosov et al., 2011a), and to a number of parasite-encoded proteins, that are exported into the infected cell where they associate with the RBC membrane and cytoskeleton. Plasma membrane deformability of iRBCs has been measured by optical tweezers, as reported in **Figure 3A**, following the thermal motion of single RBCs held in a laser trap (Paul et al., 2019) or by stretching cells attached to beads (Suresh et al., 2005; Mills et al., 2007). The shear modulus of RBCs is found to increase by up to 10-fold during parasite development (Suresh et al., 2005), in the range of 20-60 μN/m at the trophozoite stage and 25-90 μN/m at the schizont stage (Suresh, 2006) (**Table 1**). Another non-invasive optical technique, tomographic phase microscopy, has been used to obtain information about the morphology of parasite vacuoles and decreased haemoglobin content by mapping the refractive index of iRBCs (Park et al., 2008).

A unique feature of *P. falciparum* is sequestration of iRBC in the microvasculature, a survival mechanism to prevent clearance by the spleen and prolong the time in the vasculature to allow the parasite to complete its intra-erythrocytic life cycle. Although sequestration has evolved as a protective mechanism for the parasite, it can lead to severe disease when large numbers of iRBC accumulate in specific microvascular beds. This phenomenon is linked to severe disease complications such as cerebral malaria or placental malaria. Parasite binding to endothelial cells (ECs) is mediated by the variant adhesion protein family *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), which is encoded by a repertoire of approximately 60 *var* genes, and expressed at the surface of the iRBC (Smith et al., 2013). *P. falciparum* has evolved a complex protein machinery and subcellular structures to support trafficking of *Pf*EMP1 to the membrane of iRBC. For example, *P. falciparum* has expanded a family of FIKK kinases implicated in this function. Flickering spectroscopy (**Figure 3A**) seems to suggest that a member of this family, FIKK 4.1, plays also a role in rigidification of the iRBC cytoskeleton (Davies et al., 2020). Other *P. falciparum* erythrocyte-binding proteins such as STEVOR and the ring-infected erythrocyte surface antigen (RESA) have been proved to concur to iRBC membrane rigidity as assessed by microsphere filtrations and ektacytometry (Sanyal et al., 2012). Other parasite features responsible for the rigidification of RBCs are the knobs: 50-150 nm structures formed by the self-assembly of the parasite knob-associated histidine-rich protein (KAHRP). Knobs act as cytoadhesive platforms responsible for the correct display of *Pf*EMP1, while interacting with the host cytoskeleton proteins (Aikawa et al., 1996). KAHRP interactions with the RBC cytoskeleton are highly dynamic; super resolution approaches have recently revealed that it interacts with both β-spectrin and actin in ring stages, while being exclusively associated with actin in trophozoites (Sanchez, 2022). The presence of positively charged knobs partially contributes to iRBC binding to the negative membrane of ECs (Aikawa et al., 1996) (**Figure 3A**), and their increased membrane density in the last 24 hours of parasite maturation has been identified as one of the primary stiffening factors of the host cell (Herricks et al., 2009; Zhang et al., 2015). The role of KAHRP in the increased rigidity of parasitised RBCs has been determined by micropipette aspiration (**Figure 3A**), by measuring the membrane elastic shear modulus of iRBCs. Transgenic *kahrp* knockout parasites presented significant lower membrane shear elastic modulus than wild type lines, and this effect was attributed to the high transcriptional expression of KAHRP and to its clustering at the membrane (Glenister et al., 2002). Recently, the use of flickering spectroscopy by Fröhlich *et al. (*Froühlich et al., 2019) has confirmed iRBC stiffening during the parasite intra-erythrocytic cycle. In particular, trophozoites present a fourfold increase in surface tension increased, and a sevenfold rise in cytosol viscosity compared to uninfected RBCs. Mathematical simulations have revealed that knob formation strongly affects the tension by suppressing membrane fluctuations and increasing membrane-cytoskeleton coupling. In this study, flickering spectroscopy has been also used to better understand how changes in RBCs mechanical properties in hemoglobinopathies might confer protection to severe malaria. Uninfected RBCs containing haemoglobin S (HbAS) and C (HbAC) display surface tensions significantly higher than wild type RBC (HbAA), in line with the idea that higher membrane rigidity hampers parasite infection, and HbAS and HbAC trophozoites show an increased bending modulus and fewer but enlarged knobs (Froühlich et al., 2019).

Subsection 2.3. Red Blood Cell Interactions With Endothelial Receptors Under Flow

Pf-iRBCs bind to a broad array of endothelial receptors. Among these, *Pf*EMP1 binding to ICAM-1 (Ockenhouse et al., 1992) and EPCR (Turner et al., 2013) has been associated to cerebral malaria (Lennartz et al., 2017), binding to CD36 (Barnwell et al., 1989) to uncomplicated malaria (Storm et al., 2019) and binding to the glycan CSA has been linked to placental malaria. **Figure 3B** zooms on iRBCs interactions to endothelial receptors. Early studies of iRBC sequestration under fluid shear stress were

performed in bulky flow chambers, usually made of rigid materials impermeable to gas exchange. In these devices, iRBC binding was perfused with a flow pump over a channel seeded with ECs or coated with endothelial receptors. Parasite binding was usually quantified under flow or after fixation of the experiment.

In order to avoid splenic clearance iRBC-EC bonds must withstand considerable forces (2-100 dyne/cm^2) due to fluid shear stress caused by blood flow (Koutsiaris et al., 2013). There are three types of bonds that can be formed between a receptor-ligand pair: slip bonds, catch bonds, and ideal bonds. Slip bonds break faster when an external force is applied; catch bonds, on the other hand, take longer to dissociate when subjected to an increasing force, and finally the lifetime of an ideal bond is independent on the force acting on it. Lim et al. studied the single molecule interaction between iRBCs and ICAM-1 and CD36 by functionalising an AFM cantilever with these human recombinant proteins (Lim et al., 2017) (**Figure 3B**). They found that indeed ICAM-1 forms catch bond interactions with iRBCs that persist even under flow (single and multiple bonds), while iRBCs formed slip bonds with CD36. Surprisingly the rate of association of iRBC-ICAM-1 bonds is ten times lower than iRBC-CD36 (Lim et al., 2017). This result suggests that ICAM-1 is not the sole mediator for malaria cytoadhesion in the brain, and highlights the importance of other brain receptors such as EPCR.

Over the years, experimenting with the impact of flow has become much more accessible due to the commercialisation of easy to use small single-channel flow chambers. These commercial devices are being used to identify endothelial receptors that mediate binding of *P. falciparum*-tissue culture adapted parasite lines (Lennartz et al., 2017) or field isolates (Storm et al., 2019). These experiments have been crucial to reveal static and rolling interactions with the aforementioned receptors (**Figure 3B**). Flow experiments revealed that the rolling of schizont-stage infected cells under physiological flow conditions resembles the behaviour of leukocytes during acute inflammation (Helms et al., 2016; Dasanna et al., 2017). Mathematical simulations are in agreement with the experimental results (Dasanna et al., 2019), and indicate that the peculiar flip of trophozoites is due to the inertia of the solid parasite mass localised on one side of the host cell (Fedosov et al., 2011b). The rolling behaviour of late stage iRBCs can be linked to knob distribution and the stiffer plasma membrane, which is less prone to elastic deformation in flow (Lansche et al., 2018). Conversely, deformable uninfected RBCs undergo different motions near the vessel wall like tumbling, tank-treading, and rolling depending on the flow rate.

Additionally, microfluidic platforms have proven to be a reliable and physiologically relevant *in vitro* alternative to study the role of endothelial glycocalyx (**Figure 3B**) (Haymet et al., 2021). Glycocalyx breakdown has been recently associated with severe malaria (Hempel et al., 2016). For instance, brain swelling associated to cerebral malaria in children (Seydel et al., 2015), and fatal outcomes in Indonesian adults with *falciparum* malaria (Yeo et al., 2019; Georgiadou and Cunnington, 2019) can be traced to glycocalyx degradation. Nevertheless, the study of glycocalyx on malaria cytoadherence has been hindered by the experimental difficulty to obtain a mature glycocalyx *in vitro (*Barker et al., 2004; Tsvirkun et al., 2017). The glycocalyx participates in sensing mechanotransduction and shear stress influences its structure and thickness (Moore et al., 2021). Glycocalyx thickness varies from hundreds of nm to 1 μm depending on the techniques used to measure it - including electron and confocal microscopy (Kolářová et al., 2014), atomic force microscopy (Marsh and Waugh, 2013), and lately super resolution stochastic optical reconstruction microscopy (STORM) (Fan et al., 2019); and *in vivo* intravital microscopy (Liu et al., 2014; Kataoka et al., 2015). Initial studies on glass microcapillaries coated with a polymer brush to experimentally mimic the glycocalyx layer, found that RBCs travel with a velocity significantly lower than in bare capillaries (Lanotte et al., 2014), leading to the conclusion that the excessive flow resistance found *in vivo* can be attributed to the glycocalyx, as hemodynamic simulations previously suggested (Pries and Secomb, 2005). Perfusion systems lined with a human umbilical vein endothelial cell (HUVEC) monolayer have been instrumental to produce cell alignment (Sinha et al., 2016) and a glycocalyx layer similar to the one seen *in vivo (*Gouverneur et al., 2006). The loss of the sialic acids of the EC glycocalyx by enzymatic treatment increases adhesion of iRBCs without affecting their motion in proximity to the endothelium but decreasing their velocity by 10-14% (Introini et al., 2018b).

Subsection 2.4. Bioengineered 3D Microvessel Models to Study Malaria Pathogenesis

A main limitation of flow chambers is that they generally consist of a 1 mm-wide single channel in a linear display, and are therefore far away from recapitulating the complex hierarchical microvascular geometry or small vascular constrictions such as 5-10 μm capillaries or 2 μm endothelial slits. Recently, brain organoids have emerged as important 3D multicellular models (Velasco et al., 2019; Adams, 2021), but they have very heterogeneous non-controllable architecture and until today these systems lack perfusable vasculature.

Bioengineered 3D microvessel models are physiologically relevant alternatives to understand vascular dysfunctions and infections, as they reproduce the microvasculature architecture, geometry, and fluidic properties (Tan et al., 2020). These devices are built using microfabrication techniques, in hydrogels such as collagen or fibrin enclosed in PDMS or acrylic housing jigs. The dimensions of the endothelial microvessel networks can be controlled with micrometre precision by soft lithography patterning (Long et al., 2021). Bernabeu et al. examined the binding affinity of *Pf*-iRBC to 3D brain microvessels along a shear gradient (Bernabeu et al., 2019). This revealed that infected cells expressing *Pf*EMP1 that bind to ICAM-1 were more adherent at shear forces greater than 1 dyne/cm^2, further demonstrating iRBC-ICAM-1 catch bond behaviour. Binding of iRBC to EPCR was enhanced under low shear stress, suggesting that this bond might display catch-bond behaviour

at lower shear forces (Bernabeu et al., 2019). However, future AFM approaches are needed to confirm this finding.

Further advances in microfabrication have generated endothelialised capillary-size vessels down to 5 μm in diameter by using multiphoton ablation (Rayner et al., 2021). This cutting-edge approach was used to study malaria microvascular obstruction and revealed that the rigidity of *Pf*-iRBC alone is not responsible for capillary blockade (Arakawa et al., 2020). Knobless parasites expressing *Pf*EMP1 accumulated in the boundary between capillaries and venules, and the presence of knobs increased the affinity to capillary regions exposed to high shear flow. This system revealed highly distinctive spatial and temporal kinetics of iRBC accumulation and reproduced sequestration patterns observed in fatal cerebral malaria patients (Milner et al., 2015).

The study of malaria pathogenesis in more physiological systems is important, as endothelial transcription is modulated by both flow and stiffness of the extracellular matrix substrate. ECs possess mechanosensitive molecules such as caveolins, PIEZO1 or the glycocalyx that activate transcriptional pathways that modulate receptor expression as well as junctional proteins in response to culture under flow (Raasch et al., 2015; Reinitz et al., 2015). Furthermore, ECs can sense the mechanics of the surrounding tissue *via* the formation of focal adhesions (Wang et al., 2019), and when grown on stiff substrates become more permeable and sensitive to barrier disruptive molecules (Gordon et al., 2020). For example, ECs cultured on stiffer substrates have reduced glycocalyx (Mahmoud et al., 2021) and ICAM-1 (Scott et al., 2016) expression. Collectively this implies that the mechanics of the extracellular matrix may impact endothelial function and malaria pathology. While no studies to date have directly compared the effect of substrate stiffness to iRBC binding, future research needs to take these variables into account, as *Pf*-iRBC sequestration in the microvasculature of humans occurs at different stiffnesses (Zhang and Reinhart-King, 2020) and under a wide range of flow properties. As highlighted in **Figure 3B**, the study of malaria pathogenesis in bioengineered 3D microvessels with tunable flow and substrate biomechanical properties offers the opportunity to answer these questions.

Subsection 2.5. Future Perspective on Biophysics of Red Blood Cell Interactions With Host Cells

Microfluidics is essential for the study of cytoadhesion because it allows systematic tuning of biological (haematocrit, parasitaemia) and physical (flow rate, geometry, substrate stiffness, temperature) parameters (Krishnan et al., 2011), however many aspects need to be explored further. For example, malaria cytoadherence increases at an elevated temperature (Udomsangpetch et al., 2002; Zhang et al., 2018), while deformability of trophozoites and schizonts decreases irreversibly (Mills et al., 2007; Park et al., 2008), and this plastic behaviour of iRBCs in response to fever could have implications when treatments are used to suppress fever episodes. The possibility of adopting customised designs and materials that reflect a range of vascular organisations, architectures, and cellular compositions across different organs (Budday et al., 2015) could shed light on the heterogeneity of malaria pathology. With the support of computational modelling (Lykov et al., 2015; Ishida et al., 2017), it could deliver a comprehensive picture of malaria disease to direct drug intervention. Another important bottleneck of current imaging experiments, both static and in flow, is image analysis. This is currently done almost exclusively manually, selecting individual cells, and following their behaviour over time. There is an urgent need for automated image analysis methods that can handle large image datasets in different conditions and extract key cell properties. This advancement would improve experiment reproducibility and provide more quantitative information.

An important host response to malaria infection is the activation of multiple immune pathways to facilitate parasite clearance. However, the subsequent release of cytokines can activate the endothelium, promote iRBC sequestration and severity (Pais and Penha-Gonçalves, 2018). While these immunological interactions play crucial roles in influencing malaria pathogenesis, the biomechanical processes that underpin them remain unknown. Instead, the biomechanics of phagocytosis and immune synapse formation have been well studied in the context of other diseases, as reviewed in (Chua et al., 2021). Monocytes, macrophages and neutrophils have all demonstrated various abilities to phagocytose *Pf*-iRBC and merozoites. Phagocytes recognise target cells, particles, or debris which stimulates the cell to form a cup-like protrusion that spreads over the target and resulting in its eventual engulfment. The spatiotemporal forces that are exerted on a particle during phagocytosis have recently been determined by microparticle traction force microscopy and LLSM, revealing that macrophages generate F-actin and myosin-I rich "teeth"-like protrusions (Vorselen et al., 2021). Interestingly, macrophages were three times more efficient at phagocytosing stiffened microparticles (Jaumouillé et al., 2019), and need longer period of time to internalise ellipsoid particles over spherical ones (Paul et al., 2013). It is therefore likely that significantly stiffer late stage iRBCs are more easily phagocytosed during infection. Likewise, CD8+ T cells and natural killer cells show greater activation (Liu et al., 2021), form larger synapses (Friedman et al., 2021), and demonstrate greater cytotoxicity against stiffer targets (Tello-Lafoz et al., 2021). Critically, cytotoxic T cells and macrophages accumulate in the cerebral vasculature during cerebral malaria, and are thought to be at least partially responsible for disease severity (Riggle et al., 2020). Understanding the biomechanics and the functional consequences of iRBC interactions with the human immune system in the microvascular context will shed light on malaria pathogenesis.

SECTION 3. MALARIA SPECIES BEYOND *PLASMODIUM FALCIPARUM*

Only five out of over 200 known *Plasmodium* species are clinically relevant to humans: *P. falciparum, P. vivax, P. malariae, P. ovale,*

and P. knowlesi. Of these, *P. falciparum* and *P. vivax* contribute the majority of cases. *P. falciparum* remains the only species for which all stages have been cultured *in vitro*, and most research efforts and antimalarial therapies/vaccine candidates are focused on it. The lack of research in other species, particularly *P. vivax* and the zoonotic species *P. knowlesi*, will be the limiting factor that will hold malaria elimination back. These species are quite distantly related to each other, and rely on both unique and conserved molecular interactions to invade human cells and cause a broad spectrum of clinical presentations, posing quite different challenges for disease control and antimalarial development (Keeling and Rayner, 2015). For example, *P. vivax* can remain in dormant forms in the liver causing a relapse of the disease at a later time (Prudencio et al., 2006).

Plasmodium species have different durations of the intraerythrocytic cycle, ranging from 27 (*P. knowlesi*), 48 (*P. falciparum, vivax, and ovale*), and 72 hours (*P. malariae*), and number of daughter merozoites in a schizont (up to 16 for *P. knowlesi* and to 24 for *P. vivax*). *P. knowlesi* merozoites are 2-3 μm, twice the size of their *falciparum* counterparts, and therefore easier to follow by video microscopy. The first images of malaria merozoites were recorded in 1975 by Dvorak et al. (Dvorak et al., 1975) were in fact of *P. knowlesi* infecting monkey RBCs. Thanks to their larger dimensions and by using *P. knowlesi* merozoites expressing fluorescent-tagged apical membrane antigen-1 (AMA-1), it was demonstrated for the first time that the apical complex is located at the wider end of the zoite (Yahata et al., 2021), contrary to what previously thought from early images of invading parasites by transmission electron microscopy (Miller et al., 1979). *P. knowlesi* have longer-lived (316 s) merozoites that glide onto RBC surfaces at 1.1 μm/s, faster than *P. falciparum*. Moreover, they can glide across multiple human RBC, up to 7, before selecting a cell to invade, and this behaviour resembles the travel of sporozoites along several hepatocytes before invading one (Mota et al., 2001; Tavares et al., 2013). Gliding could be advantageous for the parasites to sense suitable RBCs for invasion, facilitating the identification of preferred cells like young RBCs called reticulocytes which make up for less than 2% of circulating RBCs.

Mechanical and rheological differences among *Plasmodium* species are of pathological relevance. *P. vivax* has a distinct preference for immature reticulocytes, identified by the transferrin receptor (CD71) on their surface that is progressively lost during reticulocyte maturation into erythrocyte. Invasion accelerates the ejection of host material, including CD71, completing the maturation by 3 hours post invasion (Malleret, 2015). Similarly to *P. falciparum (*Narla and Mohandas, 2017*), P. knowlesi*-infected human RBCs (Barber et al., 2018) become more rigid progressing throughout the blood stage. Instead, both bulk and single-cell techniques (micropipette aspiration, laminar shear flow system, microfluidics) show that *P. vivax*-rings are more deformable than uninfected cells (Suwanarusk et al., 2004; Handayani et al., 2009; Malleret, 2015). This is possibly related to a two-fold increase in surface area of *Pv*-iRBCs, and hence a marked increase in their surface area to volume ratio (Suwanarusk et al., 2004). Methods to measure the RBC mechanical properties can assist in establishing whether higher RBC tension confers protection across red cell ages and genetic backgrounds, and explore whether *Plasmodium* species and strains are differently affected by this mechanism. This will be critical to understanding whether tension is a potential cross-species intervention target.

Cytoadherence phenomenon was long believed to be restricted to *P. falciparum* infection, however some evidence now supports that also other *Plasmodium* species undergo cytoadhesion events (Totino and Lopes, 2017). The occurrence of severe forms of *P. vivax* infections, such as cerebral and placental malaria, which were previously reported to be exclusively associated with *P. falciparum*, suggests that *P. vivax* can, to some extent, present adhesive phenotypes, even if it does not present any protein homologous to *Pf*EMP1 *(*Totino and Lopes, 2017). Rosette formation, when uninfected RBCs adhere to iRBCs, appears in *P. falciparum*, *P. vivax (*Lee et al., 2014*), and P. knowlesi*, even though in the latter with less frequency and in the presence of human serum (Lee et al., 2021). Knob formation and sequestration are distinct characteristics of Pf-iRBCs. Pk-iRBCs *(*Russell and Cooke, 2017) do not develop knobs, but still can bind to lung and kidney endothelial cells primed with *P. knowlesi* culture supernatant *via* a functional ortholog of *Pf*EMP1, although not to the cerebral endothelium (Lee et al., 2021). Such novel findings on the capability of other *Plasmodium* species to undergo cytoadhesion beyond *P. falciparum* will require further investigations that can be addressed by the use of biophysical approaches and concepts.

CONCLUDING REMARKS

In this review we have emphasised the state-of-the-art technologies developed to probe the mechanics of malaria infection, specifically during *Plasmodium* invasion, maturation, and cytoadhesion, and how these would shape the future directions of malaria research. The contribution of RBC mechanics to invasion and vascular sequestration has recently become more evident thanks to the numerous biophysical techniques developed and applied to malaria. Some methods from soft matter, surface physics, and biomimetic nanotechnology (Wei et al., 2022) have been implemented to develop alternative therapeutic strategies, and together with novel ideas from the physics area that can be optimised for malaria research (Mognetti et al., 2019; Saint-Sardos et al., 2020) they should become part of our future toolbox to tackle new research questions.

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