Microrheologic dysfunctions in blood during malaria

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Among the Plasmodium variants that cause human malaria, vivax malaria is considered to be non-malignant. Recent research has indicated that severe vivax infection can turn out to be as pathological as falciparum. This review evidences microrheologic pathology in vivax malaria, similar to that as seen in malignant falciparum. The parasite invasion, internalization and growth in the RBC lead to membrane rigidification and progressive loss of deformability, rosetting and cytoadherence, enhanced aggregation, clumpy, non-deforming, sticky aggregates and chronic sedimentation profiles. A model that reflects the net effect of these changes is of clinical value to establish disease severity in specific malaria. In this respect an artificial neural network (ANN) model, implemented in malaria severity analysis, is discussed. Results of this model suggest that a good degree of severity classification (60 to 100%) can be achieved even with small sample size (malaria samples n=12, normal =10). With larger sample size, ANN may be very apt as microrheological model for severity analysis.

Keywords: Artificial neural networks (ANN), Microrheological dysfunctions, Red cell aggregation and deformability, Vivax malaria

Malaria in human host begins when an infected female anopheles mosquito injects the Plasmodium sporozoites during its blood meal. These first invade the liver cells and develop to produce parenchymal schizonts in the pre-erythrocytic phase1. The mature schizonts rupture release merozoites into the blood stream which readily attack the red blood cells (RBC) and develop in stages. The first identifiable stage of parasite by light microscopy is the ring stage, wherein signet like structures appears in the erythrocytes. The rings develop into trophozoites, which undergo multiple nuclear fissions leading to erythrocytic schizonts. These bursts release merozoites along with the merozoin pigment into the blood stream causing typical malaria symptoms. The parasite invasion, internalization and growth cause a myriad of erythrocytic changes that lead to different microrheological dysfunctions (MRD). These are, erythrocyte stickiness and rosetting, compromised, if not fully abolished, red cell deformability (RCD), hyper-aggregation and agglutination tendencies, non-deforming erythrocyte clumping, etc., resulting in low flow and clogging of blood vessels. These dysfunctions witnessed in Plasmodium falciparum (PF) malaria play a significant role in sequestering of parasitized red blood cells (PRBCs) in internal organs2-5, trapping of cells in reticuloendothelial system3, blocking of cerebral capillaries4, etc. Many of these severe pathologies attributed to the PF were seen in Plasmodium vivax (PV) infections as well5-8. Severities like pulmonary edema and respiratory complication7, anemia8, cerebral involvement and even coma6,7 were recently reported in cases of vivax malaria. Such observations call for indepth assessment of the microrheologic dysfunctions in blood due to vivax malaria; this forms the objective of this brief review, covering the changes in microrheological parameters and their relevance in predicting the severity of the disease.

Erythrocyte Alterations in Malaria

The invading malarial parasite is vulnerable to spleenic clearance3,5, which removes the infected erythrocytes. To dodge this human immune mechanism, the parasite modifies and restructures the host cell9-12. The various erythrocytic changes that occur during malaria can be broadly categorized into the membrane, morphological/cytoplasmic and the hemoglobin modifications. These pave ways to the intermediate changes namely, the erythrocyte stickiness, transport, surface-charge, shape and magnetic properties. Each of these changes has a direct bearing on the rheologic functioning of blood.

Membrane modifications—The erythrocyte
membrane is composed of phospholipids bilayer, interposed with integral proteins. The cytoplasmic surface of the membrane is densely covered with the spectrin and actin and these connect to transmembrane glycoprotein and band 3 protein. The membrane modifications are important to the very survival of the parasite as the process of invasion, internalization, intercellular development, and evasion of host immunity are made possible by such modifications. The parasite entry into the host cell is a multistep sequence involving attachment, junction formation, movement and invagination of the merozoite released into blood stream by the rupture of hepatocytic schizont. The PF mediates the entry both by binding to sialic acid as well as non sialic-acid receptor proteins, while the PV is completely dependent on duffy binding protein. The enzymatic interactions, facilitating the invasion and internalization, are thought to be mediated by a family of parasites proteins and antigens, apical membrane antigen (AMA-1), duffy binding-like erythrocyte protein (DBL–EP), erythrocyte binding antigen (EBA-175), merozoite surface protein-1(MSP-1), PF subtilisin-like protease-1 (PfSUB-1), and PV reticulocyte-binding protein (PvRBP). A chymotrypsin like protease enzyme is found to be particularly involved in both membrane alteration and the host cell rupture. As a result of the interactions the erythrocyte surface invaginates allowing the parasite into an enlarging parasitophorous vacuole. The membrane modifications continue further in the form of degradation of membrane proteins, insertion of the malarial protein, alteration of transport property, and the membrane’s ability to undergo fusion. Spectrin, a cytoskeletal protein, is reduced to lower molecular weight protein through proteolytic activity using the parasite-specific protease or the activation of the host enzyme. Similarly actin and other membrane proteins are phosphorylated. In addition, malarial proteins: knob associated histidine rich protein (KAHRP), ring-infected erythrocyte antigen (RESA) and PF erythrocyte membrane protein-1, 3 (PFEMP1,3) are inserted in the membrane. These cause changes in composition and asymmetry of the phospholipid-bilayer and hence loss of structural integrity of membrane cytoskeleton. In this process the transport property of membrane is also altered. There is an increase in sodium influx and potassium efflux. The calcium uptake is also increased with the maturation of the parasite, indicating possibility of a greater requirement of calcium for its metabolism. The membrane modifications influence several microrheological factors. These make contribution in aggregation and deformability of erythrocyte and hence the bulk changes in the rheologic functioning of blood.

Morphological modifications—The parasite initiated restructuring of erythrocytes results in dramatic morphologic changes that play a role in the pathogenesis of the disease. After invasion, the parasite becomes enclosed in a thin parasitophorous vacule membrane, which serves as semi-permeable barrier for nutrient uptake and secretion of parasite factors. This enclosure appears as a signet ring structure associated with early ring stage of parasite. In contrast to the PF, in PV caveola-vesicle complex are found lining the membrane which is caveolae fused with vesicles and these correspond to the fine stippling pattern called Schiiffner's dots. Membrane enclosed clefts derived from the parasite are seen distributed throughout the cytoplasm. Functionally clefts and vesicles are engaged in micropinocytosis, transport and release of specific malarial protein and the membrane-lined vesicles of PV, fuse with the surface to promote expansion and enlargement of the infected RBC. In addition to clefts and vesicle, electrondense elevations and knobs are found evenly distributed on host membrane. The density of the red cell cytoplasm tends to decrease at an early stage of parasite development. These changes may be responsible for an increased fragility of the red cell.

Hemoglobin modification—The parasite for its growth feasts and degrades up to 75% of the host cell hemoglobin. Enzymology of hemoglobin degradation shows that aspartic proteases plasmepsins and cysteine proteases falcipains are found involved in the process. In malaria, the heme (met-heme) has a spin state higher than the deoxy-hemoglobin, thus making the parasitized erythrocytes paramagnetic by a greater degree. In a recent study the magnetic susceptibility of parasitized cell was found to be increasing with development of blood-stage parasite. Because of the paramagnetic property of RBC and PRBC, these show enhanced mobility under inhomogeneous magnetic field. Hemoglobin modifications influence shape of the erythrocyte which in turn may play a role in aggregation and deformability.

Microrheological alterations
Microreological changes are direct repercussions of erythrocytic alterations (membrane-morpho-hemoglobin) discussed above, the membrane alterations lead to loss of structural integrity and fragility, causing shape changes and RBC rigidification. The morphological and hemoglobin changes influence size, appearance and aid cytoadherence. The parasite derived proteins are probably involved in initiating these effects of cellular changes. Details of the microreological dysfunction and their cascading influence on the pathophysiology of malaria are discussed here.

**Red cell deformability**—The erythrocyte deformability, dependent on size, shape, surface to volume ratio, membrane viscoelasticty and the state of cytoplasm, is probably the first of the microrheologic dysfunctions that occur during malaria. The changes in the red cell deformability (RCD) are detectable as early as the ring stage of the parasite growth. The contributions of geometric parameters to the RCD are well documented. The surface area, volume and their ratio (S/V) were altered in PF malaria. In their micropipette technique, Nash et al. aspired the infected cells into a micropipette and studied the surface area, volume and membrane viscoelaticity. While there was no significant change in the surface area of PRBC, their volume was found to be higher by 11.3 ± 5%, and hence their S/V ratio reduced significantly (8±2%). Associated with this reduction, the viscoelasticity of the PRBC was also altered. The membrane stiffening was seen during the growth and maturation of the parasite as indicated by the rise in the membrane shear elastic modulus. This value was 13% higher in early stage PRBC and 50% higher in mature forms. Among other factors, the parasite proteins, knob associated histidine rich protein, and PF erythrocyte membrane protein-3, significantly contribute to the rigidification of erythrocytes. The net effect of these factors was progressive loss in deformability of the PRBC’s with growth of the parasite from ring form to mature trophozoites and schizonts forms.

In a separate study by rheoscopy, it was found that in the PF malaria the ‘tank treading’ phenomenon of RBC membrane was abolished. This is probably a direct consequence of membrane cytoskeleton changes of PRBC that lead to stiffening of the cell membrane and the reduced shear induced elongation. Dondrop et al. have studied the change in deformability in the PF using ektacytometry, which reveals that in all severe cases the deformability is drastically reduced, particularly in cases where patients succumbed to the infection subsequently. Although these techniques gave valuable information on deformability of PRBC on isolated/cultured cells, one needs to know how changes in individual PRBC show up in a larger cell population with both infected and uninfected cells.

A simple method that allows deformability assay of cellular suspensions is the initial flow rate based filtration technique. Employing this technique, the relation between deformability and the severity of malaria in PV malaria was reported. The malarial samples were analyzed based on the PRBC count in the blood smears. The low, medium and high parasitaemia (LP, MP, and HP) were classified as non-severe malaria (NSM), mildly severe malaria (MSM), and highly severe malaria (HSM) infections, respectively. Such a classification holds good as higher parasitaemia corresponds to greater severity of malaria. The mean filtration times (MFT) of the normal and malaria samples, flowing through the microporefilter were measured by an optical hemorheometer. The filtration rate of all malarial samples (n=16) were slower by 38%, when compared to the normal samples. Further comparison of MFT among different severity levels revealed that the filtration time progressively increased with the increase in severity or parasitaemia of malaria. The MFT was increased by 32.26 and 61.84% in MP and HP, respectively, as compared to normal samples (Table 1). Similarly the NSM (LP) showed 14% increase in the MFT. Larger filtration time indicates decreased deformability, as the PRBC of given sample take longer time negotiating the torturous path of the filter. These results thus show that the bulk deformability of infected cellular suspensions reduces drastically with increasing parasitaemia. A recent report suggests that the deformability of vivax infected/cultured RBCs, is increased in mature amoeboid forms as determined by shear elastic modulus measurement. Such an increase could not be determined in other parasite stages and uninfected RBCs. Considering the relatively greater number of other parasite stages and uninfected cells in the blood, an increased RCD of amoeboid stage alone may not show significant effect on the net deformability of infected suspensions. This may be the reason as to why the parasitized blood samples showed slower filtration rate indicating reduced RCD in micropore
filtration assay. Thus the issue of individual, stage specific and bulk RCD in vivax malaria needs to be further explored.

Red cell adhesion and rosetting—The red cell adhesion to vascular endothelium and rosetting are not normal physiological phenomena. They are induced by the parasite to possibly evade the host immune system and peripheral clearance. While vascular adhesion is reported only in the PF, rosetting is found in the PF and PV species. Rosetting could also occur between an infected erythrocyte and another infected or uninfected erythrocytes. These two phenomena are now commonly known as cytoadherence and are considered to be the main reasons for microvascular blockage. A number of studies/reviews have elaborated on the molecular basis and implicated malarial induced protein for inducing cytoadherence. The microhemorheological measurement of cytoadherence and the force necessary to detach adhered cells was carried out by micropipette aspiration or by increasing the flow rate. It was observed that parasitized cell required three times more detaching force than required by sickle cells attached under similar conditions. The strength by which the non-parasitized cell attached with the parasitized ones was 5 times greater than the attachment among parasitized cells and parasitized cell with endothelium. Also the rosettes did not detach even at high shear rates that correspond to arterial flow. Even if the rosettes were disrupted during in vitro flow, these were later on attaching to the parasitized cells that are already stuck to the endothelium, worsening the vascular occlusion. These indicated that rosetting along with vascular adherence and reduced RCD had the potential to inhibit perfusion in microcirculation, causing vascular occlusion in malaria.

Red cell aggregation and aggregate sedimentation dynamics—The red cell aggregation (RCA) is a physiological phenomenon wherein erythrocytes form 3D chain-like structure under low flow conditions, similar to venous circulation, in the presence of plasma proteins. The RCA is a well understood phenomena and a number of excellent articles explain its measurement, specific details of its alterations, etc. In malaria the alteration in RCA was not focused, except for the aggregate sedimentation dynamics (ADS) studies and a report on erythrocyte sedimentation rate (ESR). One reason could be that in most research, the RCA is indirectly determined either by the ESR method or rheoscopic method, while in other advanced techniques, RCA is evaluated through the dynamics of the sedimentation of aggregates. The need though was for an imaging technique wherein cellular participation in aggregation, aggregate morphology and their sedimentation dynamics, could be visualized in conditions that mimic venous flow. Various imaging modalities were developed for aggregation imaging, however those needed external shearing or flow control mechanisms to achieve low flow conditions. To circumvent the requirements of additional mechanisms to generate flow, gravitational sedimentation approach was implemented and entire aggregation dynamics was analyzed by a computerized video-microscopic imaging and image analysis system. Figure 1 (a) shows the block diagram of the aggregation imaging, hereafter referred to as microhemofludic visualization system (µHVS) considering its usefulness as platform for studying various cellular interactions.

The aggregate images of red cells obtained from the µHVS for normal and various categories of malaria patients are shown in Fig. 2. These images are

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Table 1—Comparison of microrheological parameters in healthy and vivax malaria infected subjects. The parameters are: Aggregate sedimentation velocity (ASV), Effective number of cells (ENC), Process completion time (PCT) Mean filtration time (MFT) (from ref 37, 57, 49)

[Values are mean ±SD, Figures in parentheses are number of samples]

<table>
<thead>
<tr>
<th>Sample category</th>
<th>ASV (pixel/sec)</th>
<th>ENC × 10^5 (number)</th>
<th>PCT (min)</th>
<th>MFT (sec)</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>18.8 ± 3.9 (5)</td>
<td>5.23±0.7 (10)</td>
<td>33.8±4.2 (10)</td>
<td>1.73±0.29 (10)</td>
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<tr>
<td>Low parasitaemia (LP)</td>
<td>24.5 ± 1.4* (5)</td>
<td>5.01±0.7 (6)</td>
<td>30.0±4.1* (6)</td>
<td>1.98±0.29 (6)</td>
</tr>
<tr>
<td>Medium parasitaemia (MP)</td>
<td>27.6±2.2* (4)</td>
<td>3.90±0.7** (6)</td>
<td>21.5±1.5** (6)</td>
<td>2.29±0.25** (6)</td>
</tr>
<tr>
<td>High parasitaemia (HP)</td>
<td>30.4±0.4** (4)</td>
<td>3.02±0.4** (4)</td>
<td>15.8±0.1** (4)</td>
<td>2.8±0.52** (4)</td>
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P values: * < 0.01, ** < 0.005
from video-clipping recorded during sedimentational process in a micro-flow chamber (Fig. 1b) designed for this purpose. As the membrane properties are subjected to changes in parasitized samples, the formed aggregates show irregular shapes and compactness in MP and HP categories when compared to the normal aggregates. The video recording of HP showed that the erythrocytes formed clumps/mass of cell and did not undergo usual disc shaped deformation that is seen in normal aggregates. The clumps rolled while sedimenting with no deformation or change of shape on account of local shear, whereas, the normal aggregates showed deformation, changes in shape and orientation while sedimenting. At times normal aggregates fragment or break up and then fragmented parts re-aggregate among themselves or with other aggregates in the vicinity. Such deformation of rouleaux network was not seen among the clumped cells of parasitized samples. In fact erythrocytes were found adhering to each other leading to agglutination, which is clearly seen from the sub-images of Fig. 2 (c1, c2, d1). A close-up of aggregate clumps showed speculed and wavy membraned cells, not deforming into the aggregate favouring disc shape, yet sticking with each other (Fig. 2d1). This clumping may be attributed to the enhanced cellular adhesion in malaria on account of cytoadhesion and platelet glycoprotein mediated auto-agglutination and other cellular aspects. In a study on adhesive phenotypes involved in auto-agglutination and rosetting in malaria, Pain et al. through electron micrographs confirmed that the auto-agglutinates or clumps were also composed of infected erythrocytes and platelets. These platelets acted as bridges between the parasitized erythrocytes.

The ESR is a clinical test prescribed to determine the chronic condition of a given disease. As an advanced approach to sedimentational study, an optical aggregometer was indigenously developed. The laser transmitted intensity after passing through erythrocyte suspension placed in a glass chamber, is analyzed to obtain a multi-parametric description of the entire aggregation sedimentation dynamics, than just ESR. This study showed that the formed aggregates in malaria were of larger size than the normal ones. Out of the parameters that described the ASD, the parameter process completion time (PCT) directly correlated to the ESR. This was 68% less in HSM when compared to normal (Table 1), indicating chronically faster sedimentation rates.

For MSM and NSM, the PCT compared to normal was lower by 36 and 11%, respectively. Another parameter that represents the effective number of cells (ENC) present in observed volume indicated that with the progression of malaria severity, the aggregates sediment at a faster rate right from the beginning of the sedimentation process. Conventionally, loss of deformability is considered to be inhibitory factor to aggregation, but in this study despite RCD decrease, the aggregation was enhanced in malaria, similar to as reported in other studies. Such a phenomenon may be contributed by cytoadhesion and platelet induced agglutination tendencies and hypercoagulatory tilt in the hemostasis during severe malaria.

Microrheological ANN model—The observation of enhanced aggregation, cellular clumping and agglutination tendencies and the altered deformability describe the rheological pathology in malaria. Considering the extent of these microrheological alterations and their relation to severe pathology, it is imperative to employ the rheological data for disease severity modeling. To emphasize the clinical importance of MRD in indicating the disease severity, an artificial neural network (ANN) model, using aggregation and deformability data, was implemented.

The ANN is a distributed network of computing elements that is modeled after biologic neural system and implemented as computer software. The neural network algorithm can predict relations, found in the newer data based on the knowledge acquired during learning. The methodology has extensively been used for recognition of a particular pathology in radiology, urology, laboratory medicine and cardiology for improvement in health care systems. Generally most medical data are characterized by missing elements, noise, randomness and uncertainty errors. Test results in any two cases for the same disease may

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**Fig. 1**—Schematic of the aggregation imaging system (a) and the sample chamber (b) (from ref. 48).
not be exactly identical. Diagnosis in medicine generally involves interpretation of large, intricate and complex data, often based on the expertise of the clinician. Therefore, medical diagnostic knowledge, known a priori, can be automated by applying expert systems to aid the clinician in achieving reliable results. The ANN serves as an excellent tool in this respect. Therefore an ANN based microrheological model, was proposed for severity analysis of malaria.

The microrheological model uses a multi-layer feed-forward ANN with an error back-propagation learning law. The aggregation data obtained from laser aggregometry (process completion time and effective number of cells), aggregate imaging system (aggregate sedimentation velocity), and deformability data (mean filtration time) formed the input vectors to the ANN. A priori knowledge of what severity/class (normal, NSM, MSM, HSM) did the input vector [ASV, MFT ENC, PCT] belonged was used for training the ANN model in supervisory mode. The number of training epochs (iterations), based on the least mean square error between the desired output and the actual output, was chosen. For testing phase, also referred to as the recall or cross-validation phase, a new set of aggregation and deformability data patterns, different from those used for training, was presented to check the identification of the severity class. The number of training epochs (iterations), based on the least mean square error between the desired output and the actual output, was chosen. For testing phase, also referred to as the recall or cross-validation phase, a new set of aggregation and deformability data patterns, different from those used for training, was presented to check the identification of the severity class. The ANN produced the maximum output at that node to which it assigned the given test pattern. This network output was compared with the test pattern class and the accuracy of classification was determined. Results of severity classification showed 100% correctness for the normal class, meaning that no normal sample was misdiagnosed as one infected with malaria. Similarly in HSM, no pattern was wrongly classified. For the NSM and MSM class the correctness of classification were 60 and 80% respectively. However when misclassified samples in these categories were examined, they were found to be classified into other malaria category than the category they belonged to, showing that none of them were classified as non-malarial one. The lower classification correctness may be attributed to the inter-class overlap of rheological data of these classes. The performance of network could be improved if number of samples in each of the classes were large. In this respect one unique advantage the ANN offers is that once a network is developed based on a given set of samples, it could always be trained to update its knowledge as and when newer data are available and the subsequent performance of the network could be improved. This study demonstrates usefulness of microrheological data in disease severity classification in malaria via an ANN model.

Conclusions and future directions

The virulence and mortality in malaria can be associated to the extent of microrheologic dysfunction. Alteration in the erythrocytes plays a definite role in microvasculature of many internal organs. The MRD in blood are a consequence of the membrane, morphological, surface charge and hemoglobin modification in erythrocytes due to the parasite. It is apparent that a number of authors have investigated various aspects of the microrheological changes and have focused mainly on the mechanism of cytoadhesion and contribution of parasite proteins to deformability and rigidification of erythrocytes and elaborated on the molecular basis of cytoadherence. Nash et al., quantified the deformability of the PRBC rosettes and their adhesiveness to endothelium. While Sutera and Krogstad demonstrated that with the internalization and growth of the parasite, the deformability of PRBC reduces progressively. Dondrop et al. have studied the change in deformability in malaria (PF) using ektacytometry, which reveals that in all severe cases, the deformability was drastically reduced. The focus of all these studies has all along been the PF malaria. The PV research, compared to falciparum has been sparse and the microrheological aspects in PV have not been elucidated. This may be due to the conventional understanding that vivax is non-malignant and less fatal.

However, considering the reports, that severities attributed to falciparum (sequestering, anemia, respiratory, cerebral involvement etc) are seen in vivax malaria as well, makes the study of rheological mechanism in vivax infections imperative. In this respect, recent microrheological research, viz., the influence of PV on erythrocyte aggregation, deformability, and the ANN approach to analysis of malaria severity in PV, were probably the first of their kind in vivax research. These clearly demonstrate the extent of MRD during malaria in terms of enhanced aggregation, erythrocyte clumping and reduced deformability. The in vitro imaging technique (µHVS) provides the visual evidence to
Fig. 2—Aggregate images of normal (a) and malarial sample of low parasitaemia (b), medium parasitaemia (c) and high parasitaemia (d), and their corresponding sub images (b1, c1, c2 d1 d2) (from ref. 64).
the aggregational pathology and such direct evidential studies in vivax malaria are not yet reported. The µHVS can be used to study cellular interactions in falciparum and mixed infections under flow/sedimentation conditions. Rheologically, the intercellular parasite and its mediated membrane-morpho-hemoglobin changes contribute to altered deformability, cytoadherence, rosetting and enhanced stickiness of cells prompting hyper-aggregation and agglutination tendencies. These microrheological dysfunctions afflict the microcirculation in many ways. The significant reduction in lumen diameter in capillaries and post-capillary venules, low in vivo shear stress encountered on the venular side, increased vascular resistance may all contribute to sluggish flow and microvascular clogging. The MRD during malaria need to be further explored, for one, what would be the rheological properties of rosetting in vivax malaria? What is the role of cell adhesiveness, leukocyte aggregation, visa vie the aggregation clumps observed in the PV malaria? The issue of individual, stage specific and bulk RCD in vivax malaria also needs to be further studied. An in vitro culture and in vivo animal model study would be the best approach to understand these. If at all the rheological assessment has to be accepted as diagnostic aid in disease staging and recovery assessment during therapy, then there is a need to develop a model/index that reflects the net effect of MRD discussed above. Such a model would be of clinical value to establish disease severity in specific malaria. With attempts of using ANN methods as medical diagnostic aid based on rheological data, development of such a disease severity model may be very much on the horizon. This may be speeded up by developing a 'single platform solution' to characterize the MRD. Drug research aimed at improving deformability of less deformable cells will be a promising future development. The future research by means of novel microcirculatory and in vivo models may finally lead to newer treatment paradigms to ameliorate microrheological dysfunctions.

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