




Margination of artificially stiffened red blood cellsRevaz D. Chachanidze,^{1,2} Othmane Aouane ,³ Jens Harting ,^{3,4}
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Margination, a fundamental process in which leukocytes migrate from the flowing blood to the vessel wall, is well-documented in physiology. However, it is still an open question on how the differences in cell size and stiffness of white and red cells contribute to this phenomenon. To investigate the specific influence of cell stiffness, we conduct experimental and numerical studies on the segregation of a binary mixture of artificially stiffened red blood cells within a suspension of healthy cells. The resulting distribution of stiffened cells within the channel is found to depend on the channel geometry, as demonstrated with slit, rectangular, and cylindrical cross sections. Notably, an unexpected central peak in the distribution of stiffened red blood cells, accompanied by fourfold peaks at the corners, emerges in agreement with simulations. Our results unveil a nonmonotonic variation in segregation/margination concerning hematocrit and flow rate, challenging the prevailing belief that higher flow rates lead to enhanced margination.

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Introduction. In physiological blood flow, leukocytes partake in a phenomenon known as “margination” [1–5]. This process plays a pivotal role in the immune response by enabling the interaction between white blood cells and the vessel wall, facilitating their subsequent migration to sites of inflammation [6,7]. Blood primarily comprises red blood cells (RBCs) constituting 45% of its volume, suspended in a predominantly Newtonian buffered protein solution called plasma. While other cell types, such as white blood cells, make up a mere fraction of the total volume, margination ensures that they tend to flow closer to the vessel wall. This phenomenon is believed to be driven by differences in size and stiffness among the various cell types. Moreover, margination is not confined to white blood cells; it also manifests in platelets and in pathological conditions like malaria and sickle cell disease, where rigidified RBCs exhibit margination [8–10]. From a physical perspective, it raises the question of how a binary mixture of flexible objects of different stiffnesses self-organizes in the nonequilibrium situation of a pressure-driven flow.

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Ordering of stiff particles in a highly dilute duct flow at low but finite Reynolds number ($Re \geq 1$) is described by the Segré-Silberberg effect [11]. The salient features are the shear-induced migration force and the inertial lift force experienced by rigid particles in bounded flow [12–16]. Moreover, efficient multiparticle separation, still at low concentrations, is achieved at the micrometric scale by coupling the inertial lift force to Dean drag forces in numerous devices [17]. In a square channel, a rigid sphere is localized at four-point attractors close to the centers of each facet/side [18]. Considering suspensions in an inertial regime, rigid particles can also accumulate in these fourfold symmetric points, leading to pattern formation [19].

In the limit of vanishing Reynolds number ($Re \ll 1$), symmetry arguments of the Stokes equation preclude any lateral migration across streamlines of solid particles. This mathematical statement breaks down for a soft particle, which, while deforming, breaks the fore-aft symmetry, leading to a viscous lift force that pushes the particle away from the wall. The softer it is, the greater the viscous lift force, a phenomenon highlighted by studying the dynamics of vesicles, microcapsules, and red blood cells [20–24]. In a monodisperse suspension of such soft core-shell particles, short- and long-range hydrodynamic interactions in a shear flow induce hydrodynamic diffusion, spreading any density heterogeneity in the bulk [25,26]. However, these physical effects lead to the creation of a particle-free layer at the periphery of the flow of a monodisperse suspension of soft core-shell particles: the so-called “cell-free layer” (CFL) in blood vessels. This phenomenon is well understood experimentally in blood flow [27–29] or suspensions of physiological and stiffened RBCs [30], theoretically by coarse-grained models [31,32], and numerically in biomimetic suspensions made of vesicles, microcapsules, and models of RBCs, thanks to the growing interest and intense activity of the high-performance computing (HPC) community in the past decade [30,33–37]. For the case of a binary dense suspension, i.e., white and red blood cells, the situation is less clear, and experiments are scarce and controversial. Aggregation of RBCs promotes margination [38], while recently, it was claimed that the viscoelastic properties of plasma (suspending fluid) are at the origin of margination [39]. From a theoretical perspective, it was found that shear-induced pair collisions should be the most dominant cause for margination [40,41]. Several numerical simulations of a mixture of two kinds of soft core-shell particles in bounded Poiseuille flow have demonstrated segregation of both species [42–47], but generally, they differ in shape, size, and stiffness simultaneously.

Both particle size and shape play important roles in determining margination behavior. Non-spherical particles, particularly those with a high aspect ratio, tend to marginate more efficiently than spherical particles [48,49]. This is due to the role of particle rotation in the margination process, which facilitates interactions with the vessel wall [50]. For example, oblate and rod-shaped particles show enhanced margination compared to spherical particles, especially in the presence of RBCs, which promote their migration toward the vessel wall [49]. Particle size also plays a crucial role, with several studies suggesting an optimal size for margination. Larger particles in the micrometer range tend to marginate more effectively [4], while the results for smaller particles have been mixed. Spherical particles larger than 500 nm exhibit pronounced margination, while those smaller than 200 nm are likely to remain trapped between RBCs in the core of the blood flow, away from vessel walls [51]. However, some studies have shown favorable margination behavior for nano-sized particles (< 100 nm) due to the influence of Brownian motion [48,52]. Additionally, particles around 1 μm in diameter have been found to exhibit the highest likelihood of forming ligand-receptor bonds with vessel walls [50]. The impact of particle stiffness on margination is still an area of active research with conflicting results reported in the literature. Some studies have shown that RBC elasticity does not significantly affect the margination of white blood cells (WBCs) [42]. In contrast, others have demonstrated that collisions between stiff and elastic particles enhance margination [40,53]. Experimental work using stiffened cells in animal models has also shown that increased particle stiffness leads to a higher degree of margination [39,54,55]. However, the isolated effect of particle stiffness remains an open question, as most studies involve interactions with other factors such as size and shape.

Here, we address the problem of margination combining *in vitro* and *in silico* investigations (Fig. 1). We quantify blood flow consisting of two populations of red blood cells—healthy and

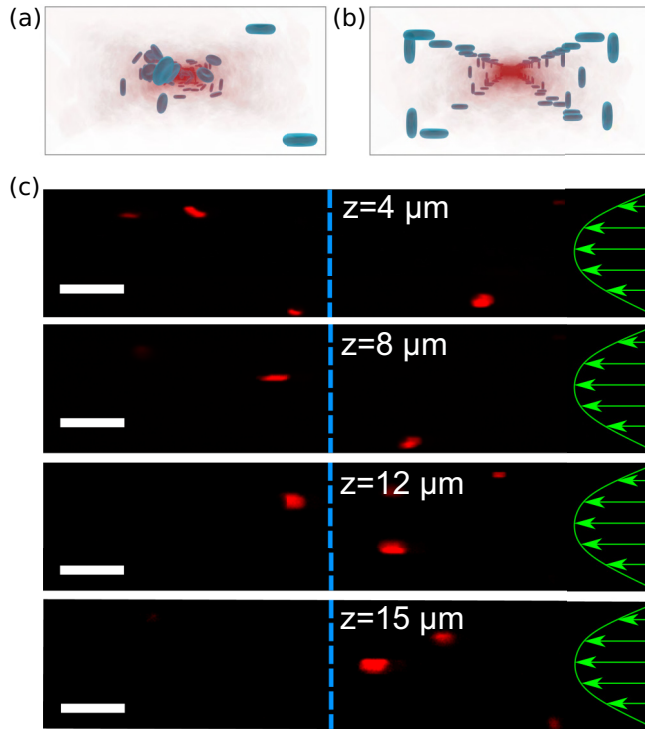


FIG. 1. (a) and (b) Numerical simulations. (c) Experiments. All conducted in a channel with a height of $h = 30 \mu\text{m}$ and $w = 60 \mu\text{m}$. (a) Initially, soft (light red) and stiff (blue) particles with the typical discoidal reference shape of RBCs are distributed randomly. (b) At steady state, the stiff cells are positioned in the four corners of the channel. (c) Confocal images show fluorescently labeled stiffened RBCs (red) at different z -focal planes. Scale bar = $5 \mu\text{m}$.

rigidified with the cross-linking agent (glutaraldehyde) [56]—in microfluidic channels for different flow rates, hematocrits, and vessel geometries. RBCs are soft core-shell particles with a discoidal shape. They are approximately $8 \mu\text{m}$ in diameter and $3 \mu\text{m}$ thick. Such an experimental model allows us to examine margination caused exclusively by the rigidity contrast between two subpopulations of cells. We use confocal microscopy to reconstruct three-dimensional (3D) distributions of labeled cells across the section [5]. We identify similar observations *in vitro* and *in silico*: similar patterns of segregation and entrance lengths, as well as the presence of an unexpected maximum of segregation according to the flow rate and the volume fraction (H_t) of physiological RBCs.

Materials and methods. Whole blood is taken from healthy volunteers via venipuncture and collected into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). First, blood is centrifuged (Hermle Labortechnik GmbH Z36HK) at 1400 g for 10 min to separate RBCs from plasma and other blood components. After that, the RBC pellet is thoroughly washed three times in physiological buffer solution [(PBS) Gibco Life Technologies], acquired as a stock solution and not pills, containing 10 mg/ml of bovine serum albumin (BSA) and 5.5 mM of glucose (Sigma-Aldrich). Washed cells are finally resuspended and adjusted to the required hematocrit in an isodense iodixanol-based solution (OptiPrepTM, Sigma-Aldrich). This suspending medium prevents RBCs from sedimentation and contains $35\% \text{ v/v}$ concentration of OptiPrepTM in PBS. A rheological characterization with a rotational rheometer (MCR702, Anton Paar, Austria) showed no relevant differences between the samples in PBS and OptiPrepTM/PBS mixtures [57]. The necessary RBC concentration is achieved by carefully pipetting a required volume of the washed RBC pellet. The hematocrit level of a blood sample is verified with a micro-hematocrit

centrifuge. Then, a small portion of RBCs ($<1\%$) is rigidified with glutaraldehyde (Sigma-Aldrich, Saint Louis, USA) for 1 h, thoroughly washed, and then fluorescently labeled with CellMask™ Deep Red Plasma membrane stain (Molecular Probes Life Technologies). Labeled stiff cells are added to the sample to create a binary suspension with two populations of cells with different rigidities. Atomic force microscopy (AFM) measurements showed that the fixed cells have a Young modulus that was at least three orders of magnitude larger than that of the healthy cells, i.e., they can be considered as completely rigid [58]. Fixed cells are kept as a stock solution at 4°C for an indefinite period in PBS containing 20 mg/ml of BSA to passivate any residuals of glutaraldehyde. Blood collection and realization of the experiment take place on the same day.

Polydimethylsiloxane (PDMS) channels are constructed using a standard soft lithography technique. Confocal measurements are performed with a spinning disk-based confocal head (CSU-W1, Yokogawa Electric Corporation) coupled with an inverted microscope (Nikon Eclipse Ti). A solid-state laser (with a wavelength of 647 nm, Nikon LU-NV Laser Unit) is used as a light source for irradiating fluorescently labeled cells. Image sequences are acquired with a digital camera (Orca-Flash 4.0, Hamamatsu Photonics, Hamamatsu City, Japan).

Numerical simulations are performed using our in-house hybrid lattice Boltzmann/immersed boundary 3D code. A finite element method is incorporated to calculate the elastic force the red blood cells exert on the fluid. Our code has consistently predicted the complex behavior of soft and rigid particles in simple and complex geometries in the past [30,41,59–61]. A detailed description of the algorithm is provided in our previous works [60,62], and the numerical setup is described in the Supplemental Material [58] (including Refs. [56,57,60,62–78]). For this paper, we simulated up to 18000 red blood cells to achieve the largest volume fraction (40%). Each cell is discretized with a triangular mesh with 2000 faces and 1002 nodes. Although our code is parallelized, some of the simulations corresponding to low velocities and large volume fractions require run times of several weeks on a little more than 4000 CPU cores.

Image processing of the experimental photographs and the evaluation of the numerical data are performed using the MATLAB™ (MathWorks, MA, USA) computing environment. Apart from the velocity calculation of labeled cells, this custom-developed MATLAB software is used to evaluate the coordinates of the moving cells and their velocity-corrected probability density as a function of the distance from the wall. To characterize the margination strength (wall concentration in percent), the ratio of the integrated probability density from the wall to $8\ \mu\text{m}$ into the bulk is compared to the integrated probability density in the inner part of the channel.

The hydrodynamic behavior of a red blood cell with a radius of R and a shear elastic modulus of G_s is influenced by two dimensionless parameters. The first parameter is the Reynolds number at the scale of the particle, which measures the ratio of inertial forces to viscous forces and is defined as $\text{Re}_p \equiv \text{Re}(\frac{2R}{D_h})^2$, with D_h being the hydraulic diameter. In our experiments, Re_p does not exceed 0.007, while it is fixed to 0.5 in numerical simulations. The second important dimensionless parameter is the capillary number. It describes the ratio of external shear stress to the deformability of the red blood cell. It is defined as $Ca \equiv \rho\nu R\dot{\gamma}_w/G_s$ with $\dot{\gamma}_w$ representing the wall shear rate. While Ca is fixed to approximately 0.2 for physiological RBCs in the numerical simulations, its value varies in the experiments between 0.05 and 1.2.

Results. First, we consider a hematocrit of $H_t = 20\%$ and rectangular microchannels in two limiting cases, i.e., with aspect ratios such as $h/w \ll 1$ and $h/w \approx 1$, where h and w are the height and the width, respectively. In rectangular channels of $h = 30\ \mu\text{m}$ and $w = 60\ \mu\text{m}$, the distribution of stiff RBCs is evaluated employing confocal microscopy [see Fig. 1(c)]. Cells passing through the section of a channel are tracked in each focal plane, and the final probability density function is shown in Fig. 2(a). It is noteworthy that segregation appears at the four corners where the shear rate within the near to the wall regions is minimal. This statement is assessed by considering other geometries. In rectangular microchannels with a slit-like geometry (aspect ratio $h/w = 8\ \mu\text{m}/60\ \mu\text{m} \approx 0.13$), a quasi-mono-layer of particles flows along the slit. Again, we observe a significant level of cell segregation. Stiff particles accumulate near the side walls of the channel

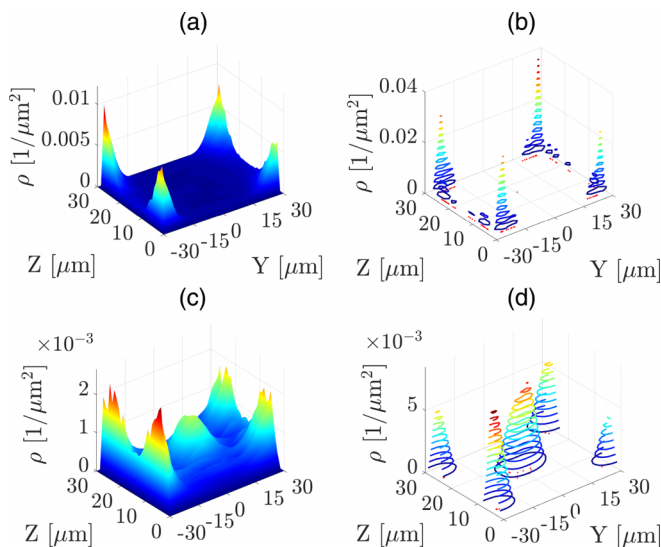


FIG. 2. The distribution of stiff cells at steady state. (a) Experimental results at $H_t = 20\%$ hematocrit, the mean velocity of the flow is $v_m \approx 900 \mu\text{m/s}$, and $Ca = 0.24$. (b) Numerical results at $H_t = 20\%$, $v_m \approx 1300 \mu\text{m/s}$, and $Ca = 0.2$ for the soft RBCs and $Ca = 0.002$ for the stiffened RBCs. (c) Experimental results at $H_t = 40\%$ and $v_m \approx 2850 \mu\text{m/s}$ and $Ca = 0.76$. (d) Numerical results at $H_t = 40\%$, $v_m \approx 500 \mu\text{m/s}$, and $Ca = 0.2$ for the soft RBCs and $Ca = 0.002$ for the stiffened RBCs.

where the shear rate is highest [Fig. 3(a)]. Finally, we consider a circular cross section. A glass microcapillary of $50 \mu\text{m}$ is set vertically along the optical path of the objective and is plunged into a transparent cuvette filled with a fluid of higher density to prevent any growing deposit of RBCs. This approach allows us to visualize flowing cells at a capillary's exit and directly estimate their spanwise distribution [58]. Stiff particles are mainly allocated along the capillary wall [Fig. 3(b)], where the shear rate is at its maximum in contradiction with the experimental results of [39]. However, we also observe a small accumulation at the center, where the shear rate is minimal. These results can seem puzzling if the shear rate, a scalar quantity, is considered. Indeed, such a peak can also be found if we vary the flow rate and the hematocrit in a rectangular microchannel with $h/w = 0.5$. When the hematocrit is increased from 20% to 40%, again, the four previously evoked peaks of stiff

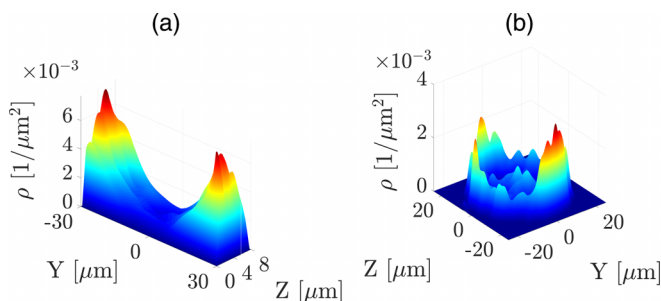


FIG. 3. The distribution of stiff cells at steady state. (a) Margination in a slit geometry obtained in an $8 \mu\text{m}$ high and $60 \mu\text{m}$ wide channel at $H_t = 20\%$ hematocrit, a mean velocity of the flow $v_m \approx 900 \mu\text{m/s}$, and a capillary number $Ca \approx 0.24$. (b) Segregation in a glass microcapillary of the inner diameter of $50 \mu\text{m}$ at $H_t = 20\%$ hematocrit, a mean velocity of the flow $v_m \approx 1000 \mu\text{m/s}$ and a capillary number $Ca \approx 0.32$.

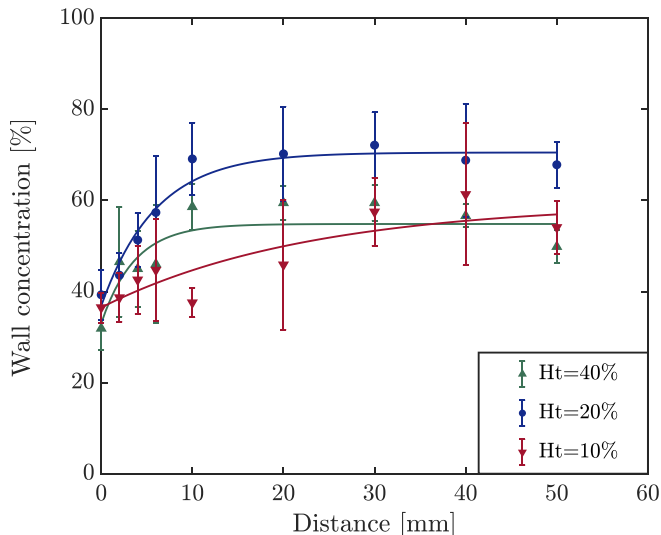


FIG. 4. Experimental results of the evolution of segregation (near wall concentration of stiffened cells) along the flow in the microchannel at different hematocrits. The mean flow velocity of flowing cells $v_m \approx 900 \mu\text{m/s}$, corresponding to a capillary number $Ca \approx 0.24$. Error bars are 95% confidence interval of the mean calculated using the sample rate of distinct experiments as a weighting coefficient.

particles appear at the four corners, and at the same time, the central peak appears where the shear rate vanishes [Fig. 2(c)]. This leads to a more complex view of margination.

In order to verify that only the differences in stiffness of the cells cause the margination, we perform numerical simulations of a binary suspension of soft and stiff microcapsules that flow in the rectangular channel with the same dimensions as in experiments, i.e., the one of aspect ratio $30/60 = 0.5$. As shown in Figs. 2(b) and 2(d), our numerical simulations confirm the qualitative picture with stiff particles gathering at the four corners. And very comparable to the experiments, the simulations predict as well a peak in the center of the flow when the hematocrit is increased to 40%.

To gain insight into the dynamics of segregation, we perform experiments to evaluate the entrance length, i.e., the distance required to establish a fully developed distribution of segregated stiff cells. For this purpose, we evaluate their distribution at different positions from the entrance of the large aspect ratio microchannel to a distance of 5 cm for three different hematocrits. The applied pressure is adjusted to obtain the same flow rate ($U \approx 900 \mu\text{m/s}$) in all three cases. The data are well fitted with a simple exponential function where the entrance length is the characteristic length, as shown in Fig. 4. The entrance length in the experiments, i.e., the distance to reach the saturation of the segregation pattern, is equal to 2.71, 0.67, and 0.55 cm for $H_t = 10, 20$, and 40 %, respectively, i.e., typically two orders of magnitude larger than the width of the microchannel. These lengths are comparable to the ones that are needed to form the cell-free layer [30]. The saturation level in the experiments depends non-monotonically on the hematocrit H_t . This can be observed even better if we focus on the dependency of the margination on the velocity, as shown in Fig. 5. We find that segregation is most expressed at a mean flow velocity of $v_m \approx 1000 \mu\text{m/s}$. With further increase of the velocity, the degree of segregation is attenuated for all studied hematocrits. Cell segregation is obviously more expressed at 20% concentration of deformable cells. This is also well reproduced by our numerical simulations. Unfortunately, reaching full convergence of the simulations corresponding to low velocities and large volume fractions was not possible due to limited available computational resources. As such, we carried out the simulations at a higher speed of 5 mm/s. We find a higher amount of cell segregation at a steady state in the simulations and assume that the main reason for this discrepancy is the fact that the cell-free layer (CFL) in

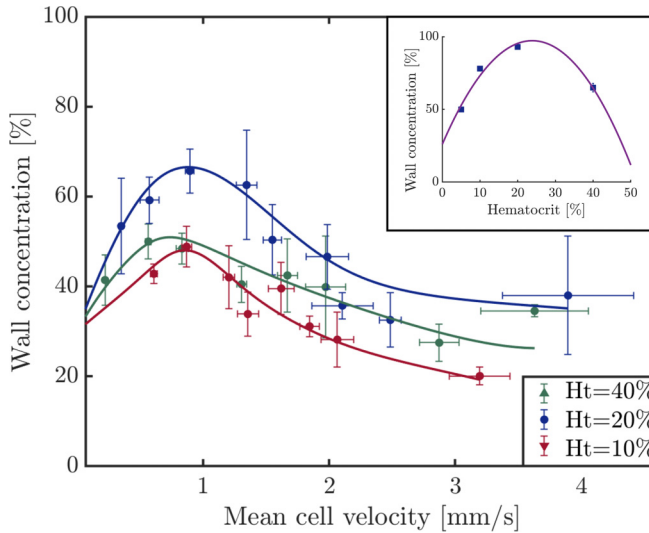


FIG. 5. Experimental results of the maximum segregation according to flow rate and hematocrit. Symbols are as in Fig. 4. Effect of varying velocity on cross-stream particle migration in experiments. Solid lines are only guides for the eye. Error bars are 95% confidence interval of the mean calculated using the sample rate of distinct experiments as a weighting coefficient. In the main figure, the capillary number Ca varies approximately between 0.05 to 1.2. Inlet: Numerical results for the maximum segregation for different hematocrit at a centerline bare fluid velocity of $\bar{v} \approx 5000 \mu\text{m/s}$ and a capillary number $Ca = 0.2$ for the soft RBCs and $Ca = 0.002$ for the stiffened RBCs.

simulations is literally free from any healthy and thus flexible cells. In contrast, in the experiments, we observe (in classical bright field mode) under the microscope, both more “abnormal” healthy cells, such as spherocytes, echinocytes, or trilobes in the CFLregion [79]. In other words, in the experiments, there is a certain contamination of rigid cells in the suspension of flexible cells, which might lead to a certain reduction of the absolute amount of margination. Despite this discrepancy, the numerical model captures the dependency of the degree of segregation on the concentration of the stiffened cells qualitatively well (inset of Fig. 5).

Summary. The segregation of rigid cells is studied experimentally and numerically in a wide range of flow velocities. Notably, our numerical simulations are in full qualitative agreement with our experimental findings. However, simulations of more than 10 000 cells are extremely costly in terms of computing resources, and a steady state can only be reached for a few runs at large velocities.

Our findings reveal an unexpected nonmonotonic behavior in particle segregation, a phenomenon previously unobserved due to the constrained range of flow rates in earlier studies [4,42,43,45,51,80–82]. Rigid cells concentrate at the four corners of a rectangular channel and in the center of the flow, at least at higher hematocrit. Interestingly, these initially centrally located rigid cells seem to become “trapped” and resist lateral migration at elevated hematocrit values. A similar observation was done for the case of small spherical particles in a suspension of RBCs [5]. The lateral migration of rigid particles is found to depend nonmonotonously on the volumetric fraction of the dominant population of deformable particles. The lateral migration of rigid particles is intricately linked to the volumetric fraction of deformable particles and exhibits non-monotonic behavior, a trend reminiscent of previous numerical [43] and experimental [38] studies. A mechanistic model for margination proposed in Ref. [83] underscores the role of two competing factors: wall-induced hydrodynamic migration for deformable particles and particle pair collisions, both homogeneous and heterogeneous. Collisions in this context do not necessarily mean that the cells get into direct contact, but they interact by their surrounding hydrodynamic stress field which is different for soft

and for solid objects While this model sheds light on the migration of stiff particles toward the walls, their accumulation in the center and the nonmonotonic behavior as a function of velocity or hematocrit remains a puzzle [41]. Furthermore, our results show that a two-dimensional shear gradient has a stronger effect on the margination than a one-dimensional one, even if the absolute value of the latter might be bigger.

In some experimental investigations, a link has been drawn between margination and rouleaux formation, the reversible aggregation of red blood cells induced by plasma proteins at low shear rates [38,84]. However, in our case, rouleaux formation is conspicuously absent, and consequently, aggregation does not play a pivotal role in particle segregation within our experiments. This underscores the complexity of the margination phenomenon and the various factors at play.

Lastly, it is worth noting that both our simulations and experiments have highlighted that margination necessitates travel distances on the order of centimeters to achieve a steady state, a finding consistent with previous work [85]. These distances are considerably longer than the average spacing between bifurcations in *in vivo* capillaries, arterioles, and venules. This underscores the need for future studies to incorporate more intricate flow geometries for a realistic depiction of margination. Of course, the behavior of white cells and platelets might also differ severely from the behavior of stiffened red blood cells, and a detailed study on their specific margination behavior should be performed in future investigations.

Ethical statement. The project was approved by the ethical commission of the Ärztekammer des Saarlandes, Kenn-Nr. 51/18 and the agreement between partners IRPHE and EFS (French Establishment for Blood Research).

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R. Chachanidze, O. Aouane, M. Leonetti, and C. Wagner contributed equally to this work.

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