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Original Article

Back to the "Gold Standard": How Precise is Hematocrit Detection Today?

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Abstract. ***Introduction*: The commonly used method for hematocrit detection, by visual examination of microcapillary tube, known as "micro-HCT", is subjective but remains one of the key sources for fast hematocrit evaluation. Analytical automation techniques have increased the standardization of RBC index detection; however, indirect hematocrit measurements by blood analyzer, the automated HCT, do not correlate well with "micro-HCT" results in patients with hematological pathologies. We aimed to overcome those disadvantages in "micro-HCT" analysis using "ImageJ" processing software.**
***Methods*: 223 blood samples from the "general population" and 19 from sickle cell disease patients were examined in parallel for hematocrit values using the automated HCT, standard "micro-HCT," and "ImageJ" micro-HCT methods.**
***Results*: For the "general population" samples, the "ImageJ" values were significantly higher than the corresponding values evaluated by standard "micro-HCT" and automated HCT, except for the 0 to 2 month old newborns, in which the automated HCT results were similar to the "ImageJ" evaluated HCT. Similar to the "general population" cohort, we found significantly higher values measured by "ImageJ" compared to either "micro-HCT" or the automated HCT in SCD patients. Correspondent differences for the MCV and MCHC were also found.**
***Discussion*: This study introduces the "micro-HCT" assessment technique using the image-analysis module of "ImageJ" software. This procedure allows overcoming most of the data errors associated with the standard "micro-HCT" evaluation and can replace the use of complicated and expensive automated equipment. The presented results may also be used to develop new standards for calculating hematocrit and associated parameters for routine clinical practice.**

Keywords: RBC indices, Microcapillary hematocrit, Image analysis.

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Introduction. The hematocrit (HCT) value represents the volume fraction of whole blood occupied by packed red blood cells (RBCs), while the residual fraction includes the plasma and white blood cells. Changes in

HCT reflect acute or chronic alterations in a patient's physical state. Therefore, when urgent therapeutic decisions have to be made, a quick HCT result is critical to establishing prompt and adequate treatment.[1,2] Due to its advantages over hemoglobin (Hb) analysis, HCT measurement is widely used in neonatology to decide whether to administer blood transfusions in cases of anemia or partial exchange transfusions in cases of polycythemia. The advantages of the HCT measurement are the small amount of blood required and the rapid results, which are often obtained at bedside analysis.

Today, two main approaches for HCT measurement are in clinical use: (i) direct manual HCT detection by centrifugation of a blood-filled microcapillary tube and manual examination by eye using a ruler (micro-HCT) and (ii) automated calculation of HCT performed by modern blood analyzers.[3,4] The automated method is used worldwide in routine practice, whereas micro-HCT detection is mostly applied in neonatology wards. The benefits of using the computerized approach over the traditional micro-HCT measurement are high throughput and high measurement standardization. On the other hand, blood analyzers give more precise results, with less than 1% coefficient of variation for the HCT index.[5]

On the other hand, automated HCT measurements have several significant limitations. First, they are indirect, using either a forward scatter-like approach in flow cytometry (ADVIA blood analyzers, Siemens) or impedance readouts (Sysmex/Beckman Coulter) for blood cell count detection. ADVIA blood analyzers calculate HCT values indirectly by multiplying RBC count by mean RBC corpuscular volume (MCV), which is also measured indirectly. In ADVIA analyzers, RBCs swell, lose their native morphology, and are chemically fixed before detection.[6,7] Therefore, this approach gives false MCV determinations of the HCT index for red cells with morphological abnormalities, such as sickle cells that are less spherical; other morphologically abnormal RBCs also affect HCT measurement.[8] Another possible source of erroneous, usually lower, HCT evaluations is the presence of RBC agglutinates, which are not counted as part of the RBC fraction by the automatic analyzers, mainly due to their strongly defined volumetric threshold.[8] Moreover, blood electrolyte and protein concentration abnormalities may affect the HCT evaluation.[9] Overall, the indirect measurement of HCT by blood analyzers may be poorly correlated with the results of the micro-HCT in patients with severe and diverse pathologies, including autoimmune hemolytic anemia such as cold agglutinin disease, sickle cell anemia, hereditary spherocytosis, and others.[10–12]

In addition, errors in the automated HCT calculation are more common in patients with polycythemia[13] or in cases of abnormal plasma osmotic pressures.[14] Previous studies in anemic adults and preterm infants found a lower correlation between circulating RBC volume and HCT than in healthy individuals, making automated analyzers inaccurate in these cases. Furthermore, many investigators[15–18] have detected a poor correlation between circulating RBC mass/volume and automatically determined HCT or hemoglobin in very low birth weight infants (with correlation coefficients varying between 0.3 and 0.7), making these measurements unreliable. For preterm infants, a correlation between RBC volume and HCT values ranged between 0.87 and 0.96;[19] therefore, using the automated method for these patients is also less appropriate. Similar correlation values (0.88–0.92) were reported for normal and anemic adults by Huber et al.[20] and by Bentley and Lewis.[21]

Moreover, extremely decreased RBC content, higher reticulocyte count, and elevations in hypochromic RBC or white blood cell counts may also result in a false HCT evaluation.[8,22,23] Thus, despite the common use of automated HCT measurements and their derived indices, results may be unreliable in numerous pathological conditions. Finally, the cost of automatic analyzers and consumables is high, making them less available in healthcare centers with limited resources or outside hospitals that lack equipped laboratories.

The manual micro-HCT measurement has been considered a cornerstone of hematology for many years. Almost all automated hematological analyzers are calibrated primarily based on these micro-HCT measurements. Therefore, the commonly accepted reference ranges for HCT and other RBC indices depend on the accuracy of this examination.[22,24] However, although the manual micro-HCT approach is simple and inexpensive, it has numerous disadvantages and may be affected by several variables. This manual procedure is relatively slow and requires skilled personnel to avoid artifacts when filling the capillaries and obtaining the HCT readouts.[25] Moreover, the technical aspects, such as duration of centrifugation and differences in angle rotor speed,[26] the plasma trapped between the cells, which can reach up to 4% of the total RBC volume.[27–30] leucocyte and platelet contamination of the RBC layer,[25] RBC dehydration[31] and oxygenation state[32] may significantly affect the results of the manual micro-HCT technique. Fortunately, most of these errors tend to counterbalance, so the real mistake is typically small.[22]

The subjective nature of the visual interpretation of the sample (due to personal visual specificities, non-controlled measuring tilt and distance, and more) remains one of the key sources for false HCT evaluations with the microcapillary method. We aimed to overcome these complications in HCT data analysis by using image-processing software to analyze microcapillary samples more precisely. ImageJ, an open-source software for imaging analysis provided by the US National Institutes of Health [33], has been recently used to quantify blood parameters in dried blood spots.[34,35] In the

present study, we suggest using this tool to precisely calculate HCT and HCT-derived blood parameters obtained from the routine microcapillary approach.

Several previous reports have discussed the inaccuracy of the automated measurements of HCT and HCT-derived parameters for hereditary hemoglobinopathies.[12,36] Here, we also compared the three approaches (micro-HCT with eye and image analyses and the automated HCT) for HCT calculation in blood samples obtained from sickle cell patients.

Methods.

Patients and Blood Samples. In total, 262 samples were included in the study; 243 fresh blood samples, termed the "general population group," in K3EDTA-supplemented tubes were evaluated by ADVIA® 2120i Hematology System (Siemens Healthineers AG). Samples arriving at the Emek Medical Center (EMC) central laboratory for measurement of complete blood count (CBC) were chosen randomly during the period 2018–2020. Manual HCT measurement was performed within 4 h of blood sampling (see **Table 1** for the demographic data). Adult subjects were considered anemic when Hb levels were <13.5 g/dL for males and <12 g/dL for females,[37] and polycythemic when HCT > 51% for males and > 48% for females.[38] The other 19 blood samples were from patients with sickle cell disease (SCD group), collected in the EMC Pediatric Hematology Unit (**Table 2**). The study was performed in accordance with the Declaration of Helsinki and approved by the EMC ethics committee (EMC-0123-18). In view that exclusively blood remnants after CBC evaluation at the EMC hematology laboratory were collected for the study and no specific blood sampling was performed, no informed consent was required to fill for the study participants.

Manual HCT Measurement (Micro-HCT). Sodium heparin-containing HCT capillaries (Heinz Herenz Medizinalbedarf GmbH) were filled with the blood samples, sealed, and centrifuged for 5 min at 12,000 rpm using a Sigma 1-14 laboratory centrifuge with micro-HCT rotor 11026 (Sigma Laborzentrifugen GmbH), following the commonly used protocol. For examination by eye using a ruler or microscale (hereafter referred to as examination by eye), the total height of the sample and the height of the packed RBC layer were visually examined using a micro-HCT reader or a ruler. The RBC layer height was divided by the total sample height and expressed in percent to obtain the HCT value. At the same time, images of these capillaries were captured by a 16MP camera (installed in a Samsung Galaxy S6 Model SM-G920F mobile phone). We performed a series of preliminary experiments to determine whether distance from the capillary and camera tilt will alter the HCT calculation (**Figure 1A**). So, we found that camera tilt (up to 30º incline and 45º decline with respect to the horizontally positioned capillaries) has no significant effect on the HCT calculations (**Figure 1B**). Distances of less than 10 cm and over 15 cm caused a strong blurring of the image and interfered with the accuracy of the imaging and subsequent image analysis (**Figure 1C**). Based on these preliminary findings, the camera was set up horizontally (with 0º tilt relative to the capillaries) at a 10 cm distance from the capillaries. A non-significant ($p > 0.05$) effect of image zooming [1X (100%) to 4X (400%) magnification] on HCT estimation was determined (**Figure 1D**); we chose 300% magnification as optimal in terms of image clarity and blur. We also found that using different cameras (16MP, 25MP, 13MP cameras) installed in various mobile phones (Samsung Galaxy S6 Model SM-G920F, Samsung Galaxy A50 SM-A505F, Huawei P9 lite 2017 mobile phones, respectively) causes a minimal difference in the HCT calculations (**Figure 1E**).

The images of the capillaries were then analyzed by the free-to-the-public Windows version of ImageJ software (ImageJ 1.52a; Wayne Rasband, National Institutes of Health, USA; downloaded from https://imagej.nih.gov/ij/download.html). The image analyses were performed as follows (**Figure 1F**):

- The height of the RBC fraction was estimated from the RBC–sealant border to the RBC–leucocyte border as length [in arbitrary units (AU) and keeping a 90º angle] using the ImageJ analyzing 'straight line' tool. First, the line is manually drawn after maximal enlargement, and then the line parameters (i.e., the length) are analyzed using the software. The examples of the analyzing procedure are shown in **Figures 1F** and **S1A**.
- The height of the total fraction was estimated from the RBC–sealant border to the plasma–air border as length in AU.
- Each tube's HCT value was calculated as the ratio of the corresponding RBC height to the total height.
- At least three independent measurements of the total sample height and the corresponding height of the packed RBC layer were performed for each capillary. The average value was compared to the measurement done by eye and to the HCT value from the automated analyzer.

Although the used Windows version of ImageJ software has a tool option to calculate the length measurement in cm unit, we performed our preliminary experiment to test the correlation between the length scales measured by the ruler and ImageJ software. First, the water-filled capillary was placed near a 5 cm ruler with marked 0.5 cm steps and then captured as described above. Next, the known lengths (with an increasing 0.5 cm step) were analyzed by ImageJ software

Table 1. Demographics and RBC Properties of the "General Population" Subjects and HCT Analysis Performed by the Three Methods (automated HCT, Micro-Eye and Micro-ImageJ) Variation in HCT values measured by ImageJ vs. eye methods and vs. automated HCT in the "general population" group subdivided into subject age, gender, MCV and anemic states. M, male, F, female; RBC, Red Blood Cell count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin. Subjects were considered anemic when Hb < 13.5 g/dL for males and <12 g/dL for females, and polycythemic when HCT > 51% for males and > 48% for females. Data are presented as average ± SE. *,^p < 0.05; ^^p < 0.01; ^^^p < 0.001. * for the automated HCT vs. corresponding measurement by eye, and ^, ^^, ^^^ for the automated HCT or measurement by eye vs. corresponding ImageJ micro-HCT measurements for the examined group.

	n	Age (years)	M/F	RBC (10^6/μL)	Hb (g/dL)	MCV (fL)	MCH (pg)	Automated HCT (%)	Micro-Eye (%)	Micro-ImageJ (%)
Total	243	47.8 ± 1.6	113/130	4.44 ± 0.06	12.6 ± 0.2	86.9 ± 0.5	28.39 ± 0.23	38.43 ± 0.48^^^	38.63 ± 0.53^^^	41.31 ± 0.52
Age										
0-2 mo	11	18 ± 5.2 days	6/5	4.1 ± 0.4	13.9 ± 1.5	100.4 ± 2.9	33.8 ± 0.9	41.57 ± 4.7*	39.08 ± 4.3^^^	40.33 ± 4.14
2 mo-2 y	10	1.11 ± 0.2	7/3	4.5 ± 0.15	11.5 ± 0.3	77.4 ± 2	25.6 ± 0.8	34. 75 ± 1.12^^^	34.01 ± 1.54^^^	36.43 ± 1.43
2-20 y	18	13.3 ± 1.4	5/13	4.42 ± 0.16	11.8 ± 0.5	82.7 ± 2	26.8 ± 0.7	36.36 ± 1.37^	36.53 ± 1.37^^^	40.1 ± 1.3
21-40 y	54	30.7 ± 0.8	19/35	4.33 ± 0.12	12.2 ± 0.3	85.7 ± 0.9	28.2 ± 0.4	36.93 ± 0.95^^^	37.53± 1.11^^^	40.21 ± 1.05
41-60 y	57	51.4 ± 0.7	26/31	4.69 ± 0.12	13.4 ± 0.3	87 ± 0.9	28.4 ± 0.6	40.9 ± 0.9^^^	41.22 ± 1.04^^^	43.75 ± 0.99
61-80 y	72	68.8 ± 0.7	35/37	4.53 ± 0.09	12.8 ± 0.3	87.3 ± 0.9	28.3 ± 0.4	39.42 ± 0.81^^^	39.49 ± 0.87^^^	42.37 ± 0.97
>80 y	21	86.1 ± 0.9	15/6	3.93 ± 0.16	11.3 ± 0.5	89.1 ± 1.5	28.8 ± 0.6	34.83 ± 1.3^^^	35.49 ± 1.42^^	38.1 ± 1.54
Gender										
Male	113	50.7 ± 2.5		4.72 ± 0.09	13.52 ± 0.27	87 ± 0.7	28.4 ± 0.4	40.95 ± 0.74^^^	41.19 ± 0.82^^^	43.7 ± 0.81
Female	130	45.2 ± 2.1		4.2 ± 0.06	11.87 ± 0.2	86.8 ± 0.8	28.4 ± 0.3	36.24 ± 0.55^^^	36.41 ± 0.62^^^	39.24 ± 0.61
MCV (for adults ≥20 years old only)										
<80	24	53.5 ± 4	12/12	4.69 ± 0.25	11.2 ± 0.7	75.2 ± 1.1	23.7 ± 0.5	35.2 ± 1.93^^^	36.34 ± 2.19^^^	38.44 ± 2.16
80-95	159	54.1 ± 1.5	72/87	4.54 ± 0.06	13.1 ± 0.2	87.2 ± 0.3	28.6 ± 0.2	39.53 ± 0.51^^^	39.89 ± 0.59^^^	42.54 ± 0.58
>95	22	65.2 ± 4.1	11/11	3.55 ± 0.13	11.3 ± 0.5	99.2 ± 0.9	31.9 ± 0.4	35.1 ± 1.3^^^	34.57 ± 1.51^^^	38.33 ± 1.47
Non-Polycythemic vs. Polycythemic (for adults ≥20 years old only)										
Non-Polycythemic	194	55.5 ± 1.4	105/127	4.36 ± 0.06	12.4 ± 0.2	87.1 ± 0.5	28.4 ± 0.2	37.78 ± 0.47^^^	38.05 ± 0.52^^^	40.84 ± 0.53
Polycythemic	11	51.5 ± 3.8	8/3	6.08 ± 0.19	17.1 ± 0.3	86 ± 2	28.3 ± 0.7	52 ± 0.57^	53.88 ± 1.31^^	55.13 ± 1.4
Non-Anemic vs. Anemic (for adults ≥20 years old only)										
Total										
Anemic	91	54.9 ± 2.3	41/50	3.79 ± 0.07	10.4 ± 0.2	87 ± 1	27.5 ± 0.5	32.72 ± 0.5^^^	32.63 ± 0.57^^^	35.49 ± 0.6
Non- anemic	114	55.6 ± 1.5	54/60	4.98 ± 0.06	14.5 ± 0.1	86.9 ± 0.5	29.1 ± 0.2	43.19 ± 0.5 ^^^	44 ± 0.6^^^	46.55 ± 0.55
Male										
Anemic	42	62.4 ± 3.3		3.93 ± 0.09	10.9 ± 0.3	87.3 ± 1.3	27.3 ± 0.9	34.18 ± 0.79^^^	34.08 ± 0.87^^^	36.81 ± 0.94
Non- anemic	54	56.6 ± 2.3		5.4 ± 0.08	15.7 ± 0.2	86.9 ± 0.8	29.2 ± 0.3	46.68 ± 0.48 ^^^	47.62 ± 0.64^^^	50.04 ± 0.6
Female										
Anemic	49	48.4 ± 3		3.69 ± 0.09	10.1 ± 0.2	87.1 ± 1.4	27.7 ± 0.5	31.69 ± 0.61^^^	31.48 ± 0.73^^^	34.46 ± 0.74
Non- anemic	60	54.6 ± 2		4.59 ± 0.05	13.3 ± 0.1	86.9 ± 0.5	29 ± 0.2	39.88 ± 0.45^^^	40.48 ± 0.68^^^	43.22 ± 0.65

Table 2. HCT Calculations and RBC Properties in Sickle Cell Disease Patients and HCT Analysis Performed by the Three Methods (Automated HCT, Micro-Eye and Micro-ImageJ) Variation in HCT values measured by ImageJ vs. eye methods and vs. the automated HCT in the SCD patients subdivided into subject age, gender, genotype and HbF content (%). M, male, F, female; RBC, red blood cell count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; SCD, sickle cell disease; SS, sickle cell homozygous; S/β-thalassemia, sickle cell β thalassemia. ^p < 0.05; ^^p < 0.01; ^^^p < 0.001 for the automated HCT or measurement by eye vs. corresponding ImageJ micro-HCT measurements for the examined group.

	n	Age (years)	M/F	RBC (10^6/µL)	Hb (g/dL)	MCV (fL)	MCHC (pg)	Automated HCT (%)	Micro-Eye (%)	Micro-ImageJ (%)
Total	19	26 ± 2.5	9/10	2.98 ± 0.15	9.11 ± 0.35	96.6 ± 3.3	31.2 ± 1.2	28.29 ± 1.09^^^	28.73 ± 1.07^^^	30.41 ± 1.12
Age (years)										
<20	8	27 ± 3.3	5/7	2.82 ± 0.14	8.59 ± 0.38	92.9 ± 4.5	30.7 ± 1.6	25.98 ± 1.15^^	26.69 ± 1.29^	28.37 ± 1.43
>20	11	34.2 ± 1.7	5/6	3.1 ± 0.24	9.5 ± 0.52	99.3 ± 4.7	31.5 ± 1.8	29.98 ± 1.54^^	30.22 ± 1.48^^^	31.9 ± 1.52
Gender										
Male	9	25.1 ± 4.1		3.26 ± 0.24	9.38 ± 0.49	91.9 ± 5.4	29.6 ± 2	29.32 ± 1.68^^	29.4 ± 1.55^^	31.11 ± 1.65
Female	10	26.7 ± 3.2		2.74 ± 0.16	8.87 ± 0.5	100.9 ± 3.7	32.6 ± 1.3	27.37 ± 1.44^^	28.13 ± 1.54^^	29.79 ± 1.57
Genotype										
SS	10	25.7 ± 3.9	4/6	2.74 ± 0.13	9.46 ± 0.43	105.1 ± 4	34.8 ± 1.2	28.57 ± 1.44^^	29.07 ± 1.41^^	30.81 ± 1.42
S/β-thalassemia	9	26.2 ± 3.4	5/4	3.26 ± 0.26	8.73 ± 0.55	87.2 ± 3.2	27.2 ± 1	27.99 ± 1.75^^	28.35 ± 1.71^^	29.97 ± 1.83
HbF content (%)										
<15	7	24.2 ± 4.1	4/3	2.86 ± 0.23	8.66 ± 0.42	95.3 ± 5.3	31.1 ± 2	26.73 ± 1.57^^	27.11 ± 1.25^	29.07 ± 1.36
>15	12	27 ± 3.3	5/7	3.06 ± 0.2	9.38 ± 0.49	97.4 ± 4.4	31.3 ± 1.5	29.21 ± 1.45^^^	29.67 ± 1.5^^^	31.2 ± 1.58

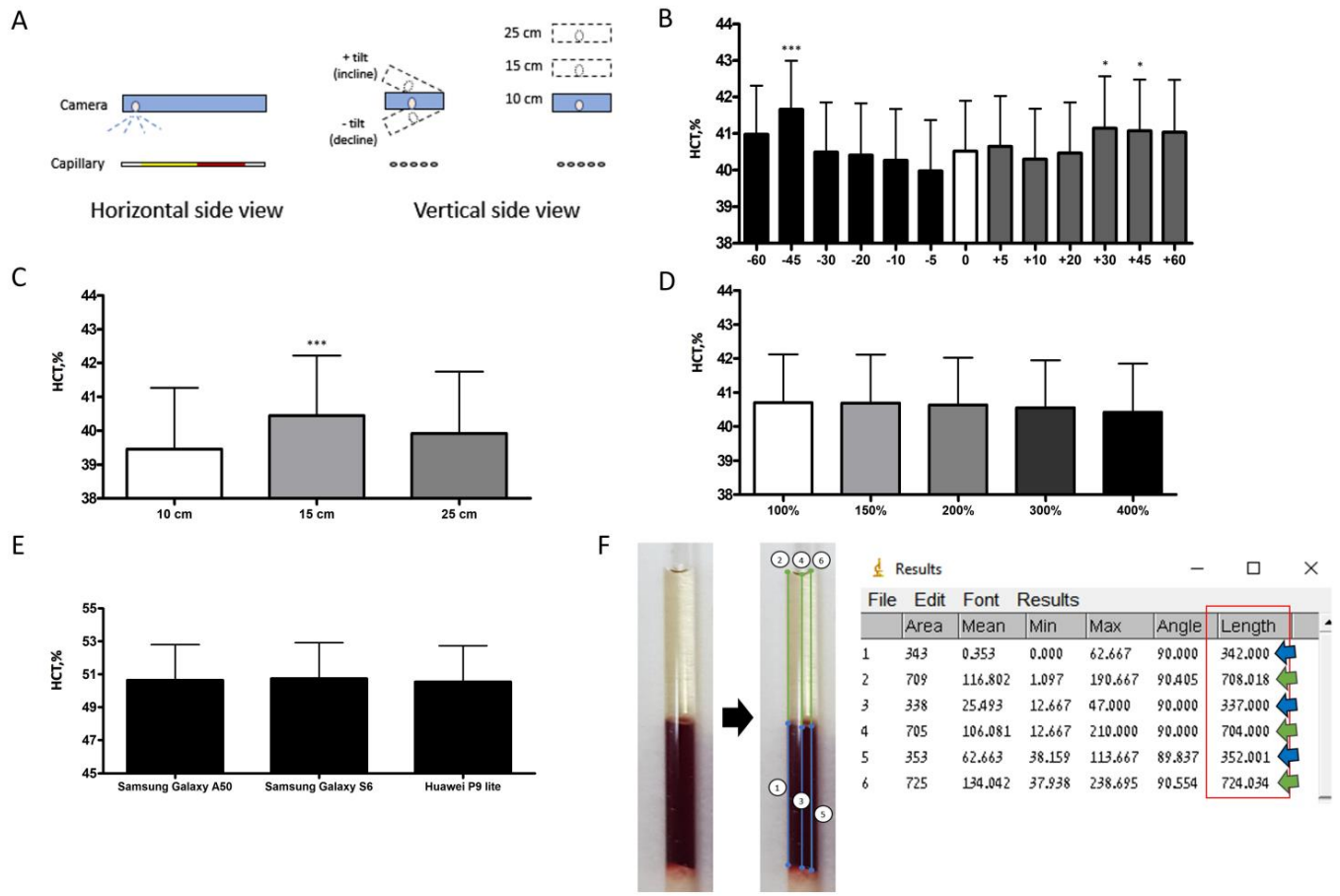

Figure 1. Determination of technical conditions for optimal capillary imaging and analysis. (**A**) Schematic presentation of the preliminary experiments to determine optimal camera tilt and distance. (**B**) Effect of camera tilt (-60º to +60º) relative to the capillary axis. Significance was estimated relative to calculated image values obtained when the camera was not tilted (0º). (**C**) Camera held at distances of 10, 15 and 25 cm from the imaged capillary. Significance is shown relative to images taken at 10 cm distance. (**D**) Effect of 1X to 4X (100–400%) image magnification pre-analysis by ImageJ software. No significant changes in calculated HCT value were found relative to the 3X (300%) magnification. (**E**) The comparative HCT analysis in the capillary images taken by different cameras installed in various mobile phones. (**F**) HCT analysis of the capillary image. At least three independent measurements of total sample height measured from the RBC–sealant to plasma–air borders (sum of green and blue segments), and of the corresponding height of the packed RBCs, from the RBC–sealant to RBC–leucocyte borders (blue segments), were performed for each capillary using the free public version of the ImageJ application. The average value was compared to the measurement by eye and to HCT value evaluated with the automated analyzer. *p = 0.01–0.05; ***p < 0.001.

(Supplementary Figure 1A). As a result, we found complete correlations (R=1) between the ImageJ length scale (in pixels) and the ruler's cm length as well as between the ImageJ length scales calculated in pixels and cm **(Supplementary Figure 1B)**, thus confirming a complete numerical comparison between HCT estimated by ruler and ImageJ approach.

The indices derived from the HCT were calculated using the formulas: mean corpuscular volume (MCV) = HCT x 100/RBC number; and mean corpuscular Hb concentration (MCHC) = Hb x 100/hematocrit, where RBC number and Hb values were from the CBCs.

Statistics. All data are presented as mean values ± SEM. One-way ANOVA followed by Friedman post-test (GraphPad Prism 4) was performed to compare the same indices measured by different approaches. The level of statistical significance was indicated as $p < 0.05$ (* or $^{\wedge}$), $p < 0.01$ (** or $^{\wedge\wedge}$) or $p < 0.001$ (*** or $^{\wedge\wedge\wedge}$), and $p > 0.05$ as nonsignificant (NS).

Results. We first examined what was the contribution of the subjectivity (i.e., the precision by the test with the ruler/eye) on the HCT measurement by the routine

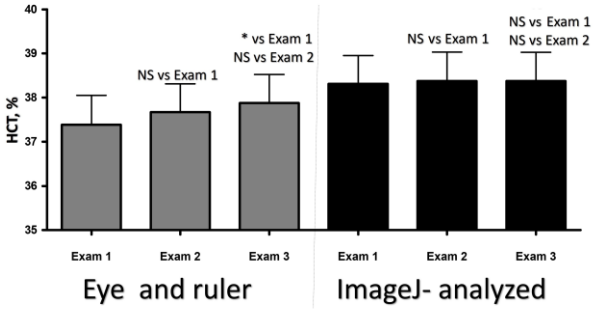

Figure 2. Subjectivity effect of measurement by eye (eye and ruler) and ImageJ evaluation of the HCT values. Significant differences were found for HCT values estimated by Examiner 1 vs. Examiner 2 vs. Examiner 3 when the evaluations were performed by eye; *p = 0.017. Differences were also significant for all three examiners between ImageJ estimation and estimation by eye (p < 0.001). NS, not significant.

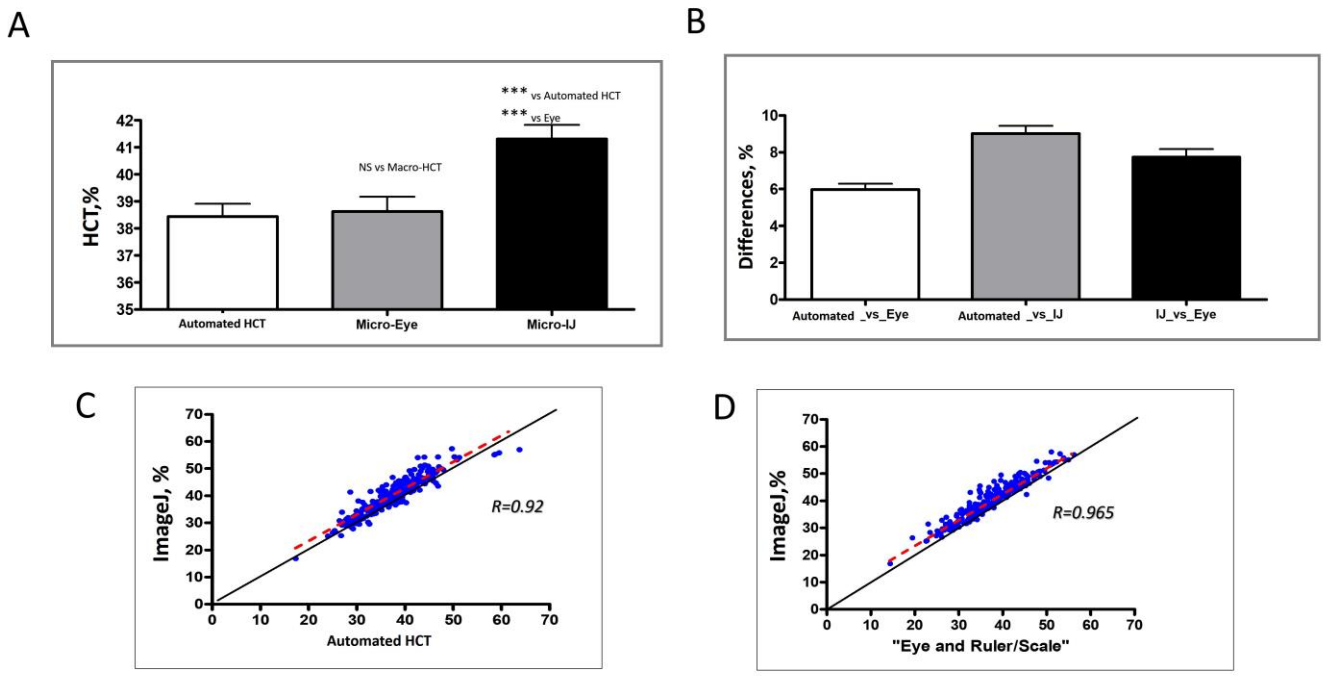

Figure 3. Comparison of HCT values measured by macro-HCT, and by micro-HCT measured by eye (micro-eye) and analyzed by ImageJ (micro-IJ) for the general population samples (n = 223). (**A**) Average absolute values and (**B**) differences in % between HCT measured in parallel by the three approaches. (**C**) and (**D**) High correlation, with a small upper shift, between values calculated by macro-HCT or by eye (eye and ruler/scale) and those obtained with the ImageJ approach. ***p< 0.001; NS, not significant.

micro-HCT method and, if this is found to be significant, minimize the differences by enlarging the picture and analysis using the routinely used ImageJ software. For that, the HCT results of 12 randomly received blood samples were compared by three independent examiners. The three examiners were experts in the field of hematology laboratory methods and research and performed two micro-HCT analyses: by eye and using the ImageJ approach (**Figure 2**). All three examiners performed the eye evaluation without being aware of the results of the other two examiners. Their evaluation by ImageJ software was performed independently, including using their computers. For the measurement by eye, each examiner gave slightly but significantly different values [Examiner 1, 37.38 ± 0.66; Examiner 2, 37.67 ± 0.64 (NS vs. Ex#1); Examiner 3, 37.88 ± 0.65 (p = 0.017 vs. Ex#1 and NS vs. Ex#2). All data are the mean values ± SE]. When the same examiners assessed the HCT by ImageJ, the variations were minimized: 38.31 ± 0.64, 38.38 ± 0.65, and 38.38 ± 0.65 for Examiners 1, 2 and 3, respectively; NS for all comparisons between examiners. However, the examination of HCT by ImageJ vs. the by-eye approach for the two first examiners revealed big and significant differences (all p < 0.001). In contrast, for Examiner 3, no significant differences were observed.

We then compared the HCT values measured by the three methods: (i) the automated HCT, (ii) examination by eye, and (iii) examination by ImageJ in a large and heterogeneous cohort. In total, 242 blood samples were tested—223 randomly collected "general population" samples from the hematology laboratory (**Figure 3** and **Table 1**) and 19 samples from SCD patients **(Table 2)**. For the "general population" samples, we did not find any differences between the automated HCT and measurements by eye. However, the ImageJ-measured HCT values were significantly higher than the corresponding values evaluated by eye (p < 0.001) and the automated HCT (p < 0.001) (**Figure 3A** and **Table 1**). In addition, the absolute (in percent) variance analysis revealed important differences between the corresponding values measured by these three approaches, 5.7 to 8.8% (**Figure 3B**). Despite these variations, the obtained automated HCT and eye-HCT values were strongly correlated (R = 0.918 – not shown), and each was strongly correlated to the ImageJ-evaluated index (R = 0.92, **Figure 3C** and R = 0.965, **Figure 3D**, for the automated HCT and by eye-HCT, respectively).

We compared the HCT values calculated by these three approaches for the examined cohort, subdivided into individual groups according to age, gender, MCV, and anemic conditions in general and divided by gender. We found significantly higher values of ImageJ- vs. either by eye- or the automated HCT-measured values in all examined subgroups, except for the 0- to 2-month-old newborns (**Table 1**). The latter was the only subgroup in which the automated HCT index was similar to the ImageJ-evaluated HCT (average difference 6.4 ± 1%, p = 0.26), and the results were higher when compared to the values measured by eye.

Since several blood indices are mathematically associated with or extrapolated from HCT values, as described in the Methods section, our next goal was to examine the ImageJ-evaluation effect on the values of MCV and MCHC compared to the other two methods **(Supplementary Figure 2A and C**, respectively**)**. We found differences in the by-eye vs. ImageJ estimations for both indices **(Supplementary Figure 2B and D).**

When we compared the three methods for HCT calculation in blood samples obtained from SCD patients, we observed important differences in the absolute variance between the corresponding values measured by these three approaches: for the automated HCT vs. by eye measurements, 4.6 ± 0.8%; for by eye vs. ImageJ measurements, 5.9 ± 0.5%; and for the automated HCT vs. ImageJ measurements: 7.8 ± 1.3%. In addition, similar to the non-SCD cohort, we found significantly higher levels for values measured by ImageJ compared to either eye-HCT or the automated HCT values ($p < 0.001$) in SCD patients **(Table 2** and **Figure 4)**. Moreover, we found significant differences when we analyzed the data in SCD patients in subgroups according to age, gender, genotype, and fetal Hb (HbF) content **(Table 2)**.

Discussion. The HCT measurement, regardless of the method, is crucial for the medical management of patients with anemia or polycythemia. However, despite its high throughput and complete blood count test, the indirect measurement (the automated HCT) has numerous limitations, such as a required volume of the tested sample and examination of blood cells with abnormal morphologies. In addition, automated measurement has the added limitation of being calculated and not directly measured and requiring expensive equipment and skilled laboratory personnel; the automatic equipment may not be suitable for use in rural areas or in situations where the medical staff moves from one site to another, for example, in the battlefield or disaster areas. This is why direct measurement (micro-HCT) is still commonly used.

In this study, we show that a simple technology allows overcoming most of the errors and variations in the data associated with the subjective (by eye with a microscale or ruler) micro-HCT evaluation and can replace the use of complicated and expensive automated equipment where it is unavailable. Furthermore, the ability to analyze any capillary at large magnification with an almost unlimited number of corresponding RBC vs. total blood heights allows taking into consideration capillary defects, centrifugation-affected blood distribution, and the roughness of the seal material in the capillary. Thus, these disadvantages of the microcapillary method for HCT estimation are resolved. Moreover, the invariably higher values of ImageJ-measured capillary HCT vs. the corresponding values obtained by eye ($p < 0.0001$) can also be explained. On the other hand, the lack of magnification with inspection by the eye does not allow examining capillary defects or the roughness of the seal material in the capillary. **Figure 5** schematizes some of the areas of just non-estimated RBCs and overestimated plasma fractions in measurements by eye and their precise detection by ImageJ analysis (arrows in **Figure 5**). The false approximation of the fractions by eye results in an incorrect and underestimated evaluation of the HCT parameter.

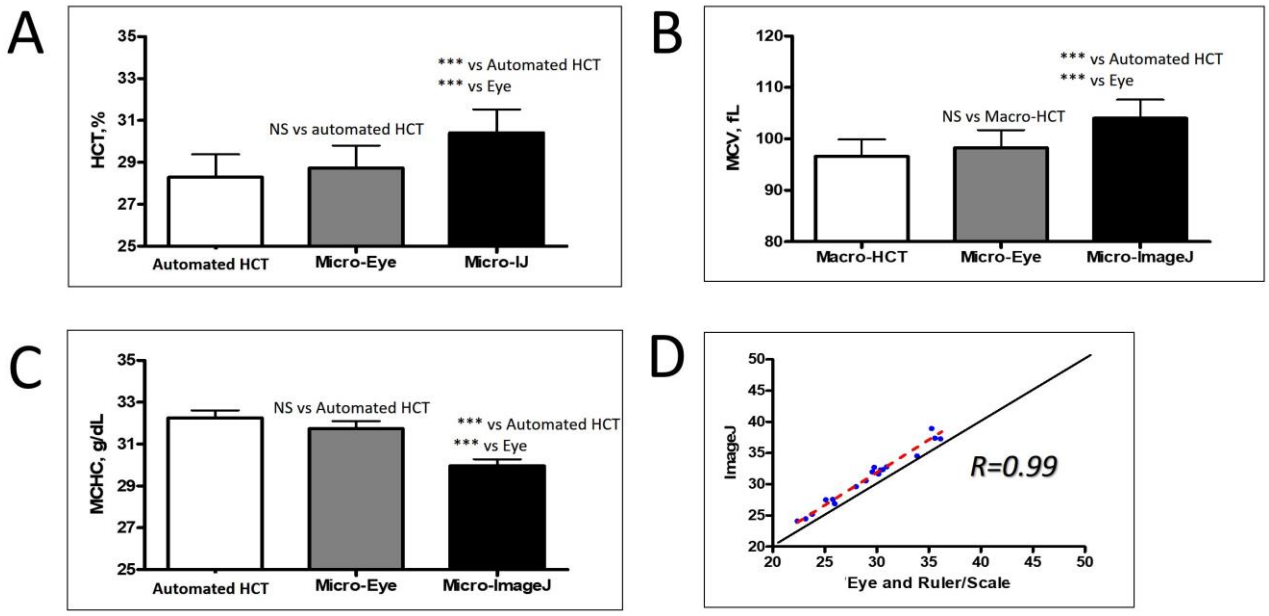

Figure 4. Comparison of HCT and HCT-derived values measured by manual (micro-eye, micro-IJ) and automated (macro-HCT) approaches in sickle cell disease (SCD) patients (n = 19). Average values for HCT (**A**), MCV (**B**) and MCHC (**C**) measured by automatic and manual methods. (**D**) Correlation between eye (eye and ruler/scale)- and ImageJ-evaluated HCT (R = 0.99). ***p < 0.001; NS, not significant.

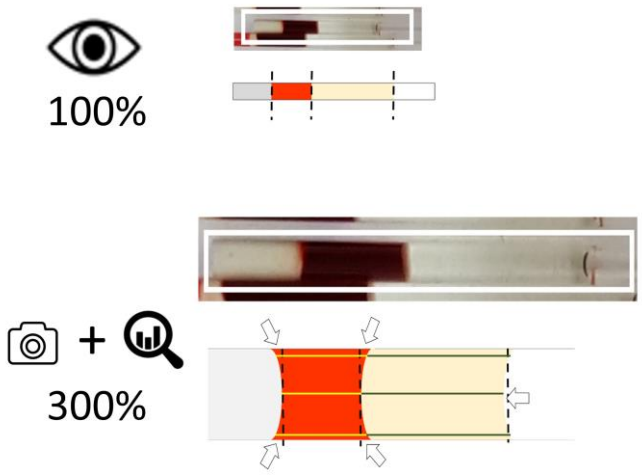

Figure 5. Schematic presentation of the differences in the visual evaluations of HCT by eye and ruler/microscale vs. image analysis approaches. Dashed black lines mark the borders of the RBC (red) and plasma (yellow) fractions evaluated by eye. The yellow and green lines show the borders of the RBC and plasma fractions, respectively, when evaluated by ImageJ analysis.

The only exception to the higher ImageJ vs. by eye and automated HCT values was observed in newborns. The newborns were the only subgroup for which the automated HCT index was similar to the ImageJ-evaluated manual HCT and significantly higher than the value measured by eye. Since, in current clinical practice, HCT in these patients is almost exclusively evaluated by the microcapillary method, mainly due to very limited amounts of blood for the sample, this finding is highly important. Compared to the significantly lower values observed by eye, the lack of variation in the automated HCT vs. ImageJ results may be explained by the unique characteristics of neonatal RBCs. In healthy infants, mild anisocytosis and poikilocytosis are frequently observed; the neonatal RBCs differ from adult RBCs in their deformability and fragility.[39,40] In addition, high numbers of pitted cells, echinocytes, spherocytes, and other abnormally shaped erythrocytes are seen in neonates, especially in premature infants.[41,42] Specifically, the fraction of stomatocytes is more than twice as high in neonates compared to adult blood, 40% vs. 18%, respectively.[43,44] Thus, such native "swelling" may result in a considerable decrease in the difference between the morphological properties of RBCs that are de-facto examined by the automatic and manual approaches, and, correspondently, similar HCT values will be obtained. Because neonatal blood is less available for automated examination, the only possibility to test HCT in these patients is the micro-HCT; the ImageJ analysis can provide the necessary accuracy for HCT evaluation in neonates. Since the results of the ImageJ approach are slightly higher than those obtained by eye, this approach may require some adjustment in policy by the neonatologists regarding the threshold for giving blood transfusions in neonates.

The presented method has several limitations, which should be solved by the future users. One is related to the technical settings of the set-up and measuring conditions. We indicated that the camera's tilt, the distance between the camera and the capillary, and zooming would impact the image. Clearly, improper positing of the camera may introduce another source of variability, and more significant validation and fixing of the optimal measuring conditions are necessary prior to its certification as a standard application. The same is related to possible variations in the software versions. In addition, we compare the ImageJ-measured capillary HCT and the automated HCT results evaluated by only ADVIA® 2120i Hematology System. Although our preliminary experiment did not reveal any difference between HCT measurements performed using different cameras, it is obligatory in the future to compare the ImageJ-measured HCT with the automated HCT measured by other devices and approaches.

As a general comment and as a possible target for further studies, we note that the accuracy of the HCT determination by any (macro or micro) approach is still under debate. Thus, despite the objective benefits of the presented technology, we need to confirm that the key source of the micro-HCT error, i.e., the evaluation of the trapped plasma, cannot be corrected by the presented approach. To the best of our knowledge, no current routinely used camera may provide a necessary zoom to detect the separate RBC and the plasma surrounding them. Of course, it may be possible to obtain precise HCT values by more advanced methods, such as biotin- and radioactive-labeling, optical, impedance, or ultrasonic approaches;[45–50] but to apply any of these methods as a routine procedure in clinical practice is unrealistic, mainly due to technical and economic considerations. However, in contrast to the presented here approach, the routinely used eye/ruler method falsely considered, on the one hand, the trapped plasma (that lead to falsely elevated HCT evaluation), and, on the other hand, capillary defects, the roughness of the seal material in the capillary and indistinct margin between red and white cell layers, all mainly result in falsely lower HCT values. Although it is impossible to overlap the trapped plasma's challenge, we strongly suggest the method to minimize other sources of error.

Therefore, the manual micro-HCT approach with the improved measuring protocol can be a more reliable and inexpensive solution for the routine clinical practice of HCT measurements. Furthermore, because the parameter is clinically important and used as a prognostic factor,[23,36,51] its accurate evaluation is highly important. Moreover, the suggested approach will be beneficial for determining novel clinical standards for HCT and its associated parameters.

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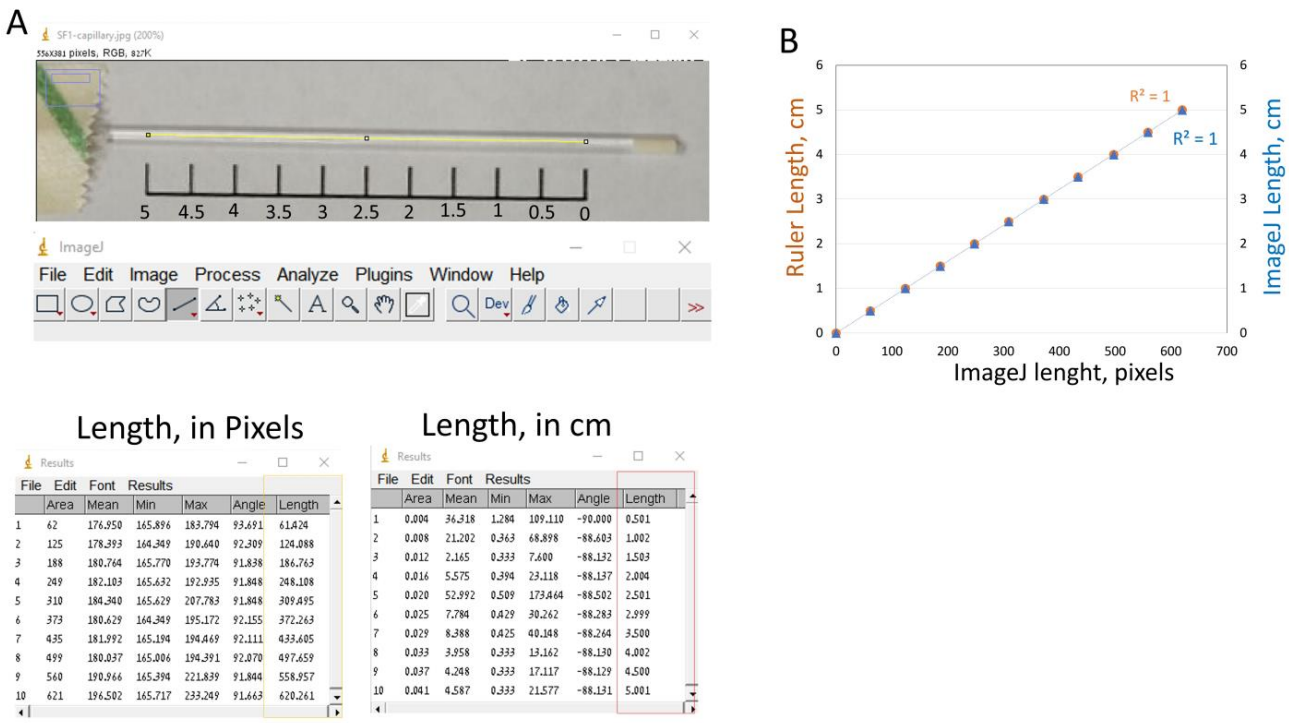
Supplementary files:

Supplement Figure 1. Correlation between the length measured by the ImageJ software scale (in AU) and by the ruler (in cm). The 5 cm distances with the period of 0.5 cm were gradually measured by means of ImageJ software (**A**) and correlated vs the correspondent ruler values (**B**).



Supplement Figure 2. Comparison of estimated blood indices derived from the HCT values. Average values and correlations between MCV (**A** and **B**) and MCHC (**C** and **D**) when HCT was measured in parallel by the three approaches. ***p < 0.001; NS, not significant.