Original articles

# The GEN.S: a fortuitous finding of a routine screening test for hereditary spherocytosis

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## Abstract

Abstract. As part of the evaluation of the GEN.S (Coulter®), we compared the Mean Corpuscular Volume (MCV) to the Mean Spherized Corpuscular Volume (MSCV) assessed during the reticulocyte count procedure under hypo-osmotic conditions. A sub-group of patients with hereditary spherocytosis (HS) was singled out in all of them, the MSCV became smaller than the MCV. As the cell volume normally increases in red cells derived from other patients in the same conditions, we decided to further study the reason for this particular behaviour of HS red cells. Whereas normal red cells are able to undergo an osmotic expansion, the spherocytes reach a critical osmotic volume leading to cell fragmentation consistent with the decrease of MSCV. This fortuitous finding is likely to be a reliable improvement for the routine screening of HS.

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While performing a routine evaluation of the new automated hematology analyzer Coulter® GEN.S (Beckmann Coulter Inc. Fullerton, CA), a sub-group of samples with unexpected red cell indices emerged. Most of them were actually found to have been drawn from patients affected with hereditary spherocytosis (HS). HS is a common red cell constitutional disorder related to various red cell protein defects [2]. Further investigations on this observation led us to define a new index for the routine detection of HS patients.

## Blood samples

286 samples obtained from adults and children in our general hospital were randomly tested after exclusion of patients transfused over the 3 preceding months. Our recruited population covered a wide variety of pathologies including patients with RBC disorders (hereditary elliptocytosis , auto-immune hemolytic anemia , sickle cell disease , alpha thalassemia, HS...). HS affected patients (31), had been previously diagnosed in our laboratory, as already described [1].

Blood samples were collected by venepuncture in Becton Dickinson tubes containing K3 EDTA. Tests were performed between 30 min and 36 h after collection. When assessed after 6 h, samples were stored at 4˚ C. All samples were simultaneously assessed with another blood cell counter (Bayer® Technicon) and results were compared [3].

## Material and method

The Coulter GEN.S (Beckmann Coulter Inc. Fullerton, CA) is a new hematology analyzer which provides a complete blood count (CBC), 5 part differential and reticulocyte analysis by means of Coulter impedance technology. Red blood cells (RBC) are counted in an iso-osmotic solution. Each cell passage through a

calibrated aperture modifies the electrical resistance thus allowing one to count the cells and determine their size. The pulses from 36 femtoLiters (fL) to 360 fL are sorted by size into 256 channels to build the RBC histogram. The system multiplies the number of RBCs in each channel by the size of the channel. The products of each channel are added. The sum is divided by the total number of RBCs between 36 to 360 femtoLiters (fL). The analyzer then multiplies by a calibration constant and expresses the Mean Corpuscular Volume (MCV) in fL.

Reticulocyte analysis on the GEN.S is performed through a flow cytometer [4]. While the reticulocyte assay is performed in automatic mode, the RNA of the reticulocytes is primarily stained by a vital dye derived from the new methylene blue. On a second phase, an acidic hypo-osmotic solution (reagent B = 95 mosmoles, pH = 1.4) is poured into the medium at 41˚ C leading to an hypo-osmotic spherization of RBC. When reticulocytes are counted, reticulocyte indices (Mean Reticulocyte Volume) are determined as well as the Mean Spherized Corpuscular Volume (MSCV) of all the gated red cell population.

The Coulter Multisizer® is a particular analyzer based on the principle of high resolution volumetric analysis. This instrument was used for the purpose of measuring RBC volume in various conditions. We reproduced the same conditions as reticulocyte procedure occurring on Gen.S in 2 steps. We performed the first step by adding the New Methylene Blue to the blood sample at 41˚ C. The second step consisted of addition of the reagent B at 41˚ C. The computer analysis provides the RBC histogram scattered in 256 channels.

We carried out a direct comparison between the 2 volumes before and after spherization by linear regression to obtain the regression coefficient R and the equation of the regression line according to the least squares method. In addition, studies were also undertaken to determine the sensitivity and specificity according to a classification of samples into four categories true positive (TP) or true negative (TN), respectively for HS and non-HS blood samples detected with our test false positive (FP) and false negative (FN) when the GEN.S data and the diagnosis were discordant. From the number of cases in each category, we used the following formulas- sensitivity (%) (TP/TP+FN) x 100

- specificity (%) (TN/TN+FP) x 100.

## Results

An unexpected result emerged from the overall comparison between MCV and MSCV of 286 samples [R = 0.594] as only a weak correlation was found. We thus set up a line defined by MCV = MSCV (Fig. 1). We obtained a sub-group of 48 patients with MSCV < MCV (values ranging from 0.1 to 22 fL). In contrast, the majority of samples (238) displayed, as expected, a higher MSCV when compared to the MCV. The sub group of 48 patients comprised all the 31 HS included patients (Fig. 1). Such data led us to further study this particular pattern of HS red cells. We reproduced on a Coulter Multisizer® an assay using the same chemical conditions as in the reticulocyte procedure on the GEN.S. The Multisizer® provided histograms representing the number of red cells as a function of their volume between 10 and 300 fL. In the acidic and hypo-osmotic conditions induced by the addition of reagent B (step II), the normal red cells were able to accept an osmotic expansion leading to a right shift of the RBC volume distribution curve and subsequently an increased MSCV (Fig. 2). The same experiment applied to HS red cells shown a non-gaussian widened histogram with particles reaching 10 fL, illustrated by the modification of the RBC volume distribution curve, leading to decreased MSCV (Fig. 3).

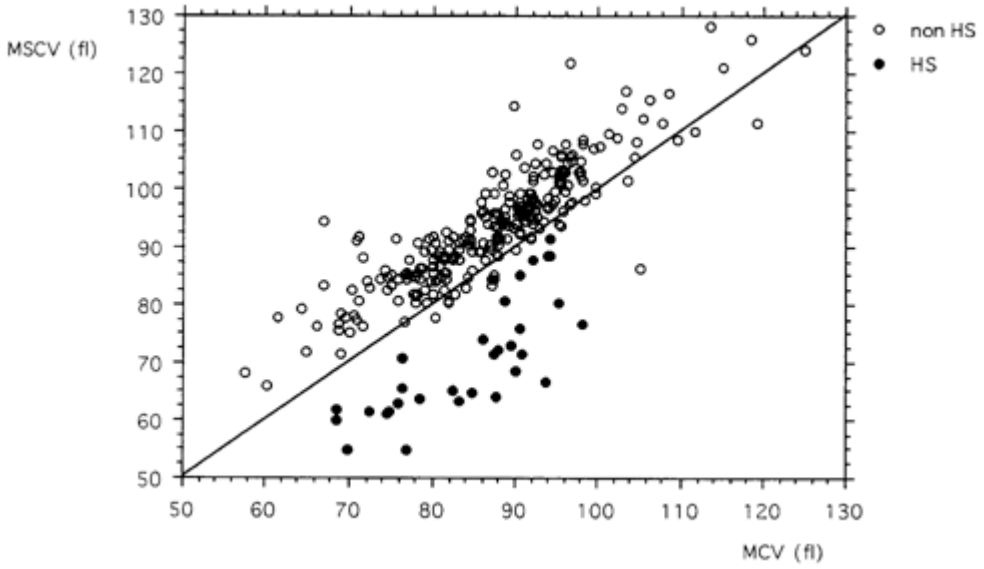


Fig. 1.
Direct correlation between MCV and MSCV in 286 samples. Plots reveal a significant sub-population under the line defined by MSCV = MCV

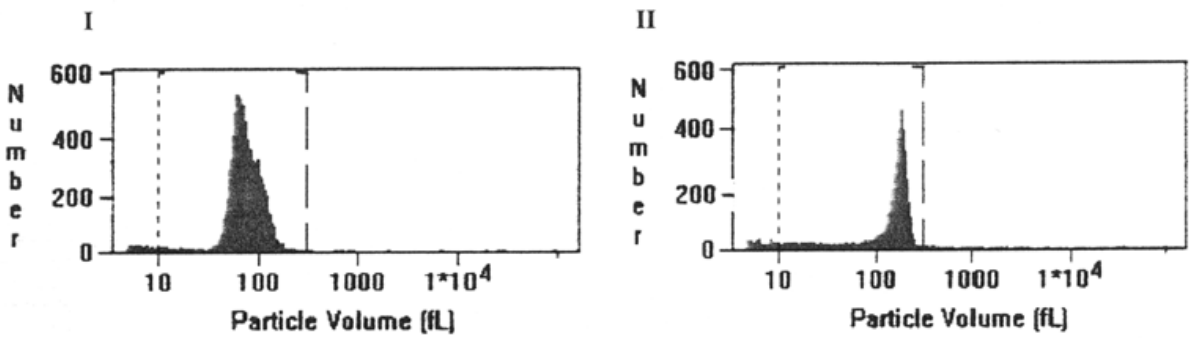


Fig. 2.
Multisizer® assay control sample. I. First step blood sample with new methylene blue at 41˚C. II. Second step blood sample with new methylene blue and reagent B at 41˚ C a red cell spherization is induced

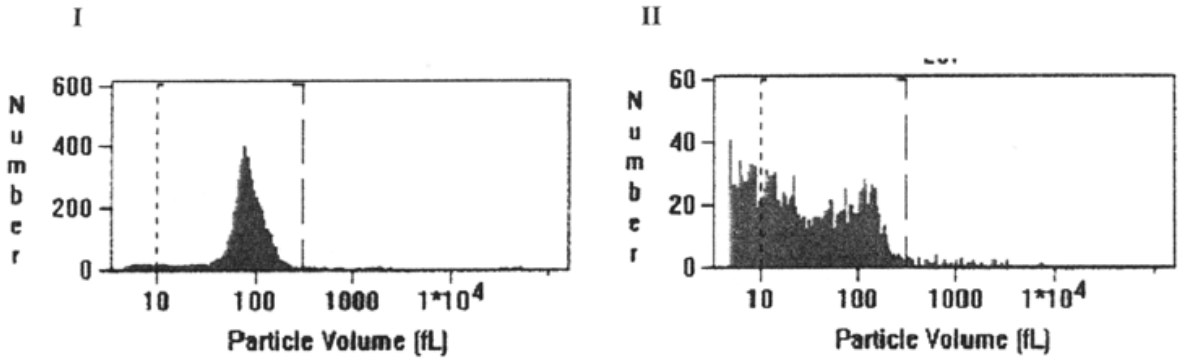


Fig. 3.
Multisizer® assay HS sample. I. First step blood sample with new methylene blue at 41˚C. II. Second step blood sample with new methylene blue and reagent B at 41˚ C a red cell fragmentation is induced

In order to settle the optimal efficiency of this new screening test for HS, we have calculated the sensitivity and the specificity according to different levels of MSCV. Table 1 shows that the level defined by MSCV < MCV leads to the best sensitivity (100%) and 93.3 % of specificity. When considering MCV - MSCV > 10, we obtained better specificity (99.6 %) but lower sensitivity (71 %). Conversely, when recalculating the MSCV/MCV correlation after exclusion of the 31 HS samples, we found a close correlation between these two

indices [R = 0.802].

| | MSCV < MCV | MSCV < MCV | | |
|---|---|---|---|---|
| | | MCV – MSCV >0 | MCV – MSCV > 5 | MCV – MSCV > 10 |
| Total samples N = 286 | N = 238 | N = 48 | n = 31 | N = 23 |
| HS samples n = 31 | n = 0 | N = 31 | n = 28 | n = 22 |
| Sensitivity (%) | | 100 % | 90.3 % | 71 % |
| Specificity (%) | | 93.3 % | 98.9 % | 99.6 % |

*MCV*: mean corpuscular volume, *MSCV*: mean spherized corpuscular volume

**Table 1.** Sensitivity and specificity of HS detection as assessed from MSCV and MCV values, respectively

## Discussion

We performed an evaluation of different parameters on 286 samples available on a routine basis. The automateprovides 2 measurements of red cell volumes the MCV obtained in the CBC procedure and the MSCV measured during the reticulocyte count analysis. The MSCV is an artificial volume due to the hypo-osmotic solution used in the processing of the reticulocyte count. The hypo-osmotic solution creates a flow of water into the red cells transforming discocytes into osmotic spherocytes. Fig 1 clearly shows that in most samples (238/286) the procedure results in MSCV higher values than the MCV. In contrast, in 48 samples the ratio was different in such samples the osmotic volume was smaller than the MCV. 31 of these were from HS patients. When considering other diagnoses with MSCV<MCV, red cell disorders were prominent such as AHAI, SCD, HE the other patients presenting with miscellaneous non hematologic diagnoses. HS is a heterogeneous common inherited hemolytic anemia [2]. The primary erythrocyte membrane defect may concern alpha or ß spectrin, ankyrin, band 3 or protein 4.2. They all involve one of the two " vertical interactions " between the skeleton and the bilayer. Destabilisation of the bilayer results in vesiculation, and progressive loss of surface area. The red cells become more spherocytic and less deformable. As a result of decreased surface/volume ratio, the cell osmotic fragility is increased. Based on our observation, we tried to better understand the underlying mechanism leading to the special behaviour of HS red cells during the reticulocyte count procedure. When analyzing the histogram of the RBC volume provided by the Multisizer® in the same conditions, we assumed that in hypo-osmolar medium the critical volume for spherocytes occurs for an osmolality which is higher than for normal RBC. This phenomenon probably results in cell fragmentation, which is illustrated by the presence of small particles on the Multisizer® histogram and accounts for the significant difference between MSCV and MCV in these patients.

Therefore, our finding, based on the comparison of MSCV and MCV, allowed us to observe and analyze the particular behaviour of HS red cells. Beyond the technical advantage of GEN.S, this fortuitously discovered screening ability is of interest it is likely that MSCV < MCV is highly indicative of HS and probably of acquired immune spherocytosis, which must both be confirmed by other methods.

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