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Venous stasis and routine hematologic testing

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Summary Prolonged venous stasis, as generated by a long tourniquet placement, produces spurious variations in several measurable analytes. To verify to what extent venous stasis influences routine hematologic testing, we assessed routine hematologic parameters, including hemoglobin, hematocrit, red blood cell count (RBC), main cell hemoglobin (MHC), main cell volume (MCV), platelet count (PLT), main platelet volume (MPV), white blood cell count (WBC) and WBC differential on the Advia 120 automated hematology analyzer in 30 healthy volunteers, either without venous stasis (no stasis) or after application of a 60 mmHg standardized external pressure by a sphygmomanometer, for 1 (1-min stasis) and 3 min (3-min stasis). Although the overall correlation between measures was globally acceptable, the mean values for paired samples were significantly different in all parameters tested, except MCV, MHC, PLT, MPV, eosinophils, basophils and large unstained cells after 1-min stasis and all parameters except MCV, MHC, MPV and basophils after 3-min venous stasis. As expected RBC, hemoglobin and hematocrit displayed a significant trend towards increase, whereas WBC and the WBC subpopulations were decreased. Difference between measurements by Bland and Altman plots exceeded the current analytical quality specifications for desirable bias for WBC, RBC, hemoglobin, hematocrit, lymphocytes and monocytes in samples collected after either 1- and 3-min stasis. These results provide clear evidence that venous stasis during venipuncture might produce spurious and clinically meaningful biases in the measurement of several hematologic parameters, prompting further considerations on the usefulness of adopting appropriate preventive measures for minimizing such influences.

Keywords Hemoglobin, hematologic analyzer, hematologic testing, preanalytic variability, tourniquet, venous stasis

Introduction

The utilization of laboratory medicine services has substantially increased over the past decades, enhancing usefulness to assist the medical decision-making process (Panteghini & Forest, 2005; Froom & Shimoni, 2006). Remarkable advances in technology and automation have allowed the development of innovative hematology analyzers, which have finally reduced the manual work, increased the throughput, improved precision and accuracy of measurements, providing reliable, fast, and cost-effective complete blood counts, white blood cell differentials (WBC diff) and reticulocyte measurements (Lippi *et al.*, 2005a,b). The main objective of any laboratory analysis was to produce results that are accurate and precise enough for clinical use (Plebani, 2002). Although automated hematology counters provide several test results to the clinicians rapidly and precisely enough, poor standardization and control of several pre-analytical variables might generate unreliable data, causing adverse events in the complex clinical decision-making process (McMullin *et al.*, 2005). It has been highlighted that the number of errors occurring within the analytic phase of

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the testing process is considerably less than those occurring during the preanalytical and postanalytical phases (Plebani & Carraro, 1997). There are consolidated evidences that a prolonged venous stasis, as generated by a long tourniquet placement, might produce spurious variations in several measurable hematologic parameters. Ideally, the tourniquet should be applied only if necessary, and quickly removed when the needle is safely in the vein. However, the tourniquet is rarely released before blood drawing is completed, to avoid vein collapsing. Although blood collection is supposed to be completed as quickly as possible, several concurrent causes might contribute to lengthen the tourniquet time up to 3 min, such as location of an appropriate venous access, selection of the most suited blood collection system, needle insertion into the vein and collection of many tubes. Thus, although the influence of the tourniquet time during blood collection is theoretically recognized as one of the main causes of variability in laboratory data, its precise contribution is uncertain, due to poor standardization and interindividual variability in procedures employed to reproduce the venous stasis (Junge *et al.*, 1978; Lippi *et al.*, 2005a,b). Therefore, the aim of the present study was to investigate the effect of a prolonged tourniquet time on the measurement of several hematologic parameters.

Materials and methods

Study design

Venous blood specimens were drawn in the morning from 30 fasting voluntary physicians (17 women and 13 men; mean age: 33 years), who gave their informed consent for testing. Sequential venipunctures were performed on three different veins of the upper arms, alternating one arm with the other, to minimize either interference of previous stasis or contamination from endovascular material released from earlier venipunctures. The first venipuncture was carried on without venous stasis (no stasis), whereas the second and the third venipunctures were performed following the application of a standardized external pressure of 60 mmHg by a sphygmomanometer for 1 (1-min stasis) and 3 min (3-min stasis), as currently recommended (Thompson, 1992). The external pressure was maintained until venipuncture and blood collection was completed and the last sample (3-min stasis) was always collected within 10 min from the first specimen (no stasis). Blood was always collected by a single and expert phlebotomist, by venipuncture with 20 G straight needles (Terumo Europe NV, Leuven, Belgium), directly into 3.0 ml of siliconized vacuum tubes containing 5.4 mg K_2 EDTA (Becton-Dickinson, Oxford, UK). Each phase of sample collection was accurately standardized, including an identical time of resting of the subjects (>10 min) and the use of needles and vacuum tubes of the same lot. No specimen needed to be discarded due to unsatisfactory attempts, difficulty to locate a suitable venous access, vein missing, visible hemolysis or lipemia.

Laboratory testing

We assayed hemoglobin, hematocrit, red blood cell count (RBC), main cell hemoglobin (MHC), main cell volume (MCV), platelet count (PLT), main platelet volume (MPV), white blood cell count (WBC) and differential WBC (WBC diff) on the Advia 120 automated hematology analyzer (Bayer Diagnostics, Newbury, Berkshire, UK). Calculated indexes were hematocrit and MHC. The principles used on Advia 120 are photometric measurement for hemoglobin, optical laser light scattering for cell enumeration, myeloperoxidase staining coupled with flow cytometry-nuclear lobularity analysis for WBC diff, flow cytometry and laser diffraction for RBC and PLT. The WBC diff is accomplished by staining the white cells with peroxidase and separating them on the basis of the amount of peroxidase staining and cell size. The leukocytes are separated into five main cell clusters, corresponding to neutrophils, eosinophils, lymphocytes, monocytes, and large unstained cells (LUCs). Basophils are counted separately in a different (basophil) channel, as they do not stain with peroxidase. The instrument was calibrated against appropriate proprietary reference standard material and controlled daily with the use of proprietary controls. Specimens were maintained at room temperature and all measurements were performed within 1 h from venipuncture. A recent multicenter evaluation of the within-run precision of the Advia 2120 system, which employs technology identical to that of the Advia 120 analyzer, yielded coefficients of variation ranging from 1.6% to 2.3% for WBC, from 2.1% to 2.8% for PLT, from 0.6% to 0.9% for RBC and always lower than 0.7% for hemoglobin, MCV and MCH (Harris *et al.*, 2005).

Statistical analysis

Significance of differences between samples was assessed by paired Student's *t*-test; correlation of measures was assessed by Passing and Bablok regression analysis and Pearson's correlation. The level of statistical significance was set at $P < 0.05$. Bland and Altman, and limits of agreement analysis were used to compare the results of the independent measurements and differences were

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finally reported as percentage of averages. This option was selected as it appears more suited to verify the potential increase in variability of the differences as the magnitude of the measurement increases (Bland & Altman, 1999). The biases following 1 and 3 min venous stasis were finally compared with the current desirable quality specifications for bias, derived from biologic variation (Ricos *et al.*, 1999).

Results

Results of the present evaluation are given in Table 1. When compared with samples collected without stasis, the mean values for paired samples were significantly different in all parameters tested, except MCV, MHC, PLT, MPV, eosinophils, basophils and LUC after 1-min stasis and all parameters except MCV, MHC, MPV and basophils after 3-min venous stasis. Although RBC, hemoglobin and hematocrit displayed a significant trend towards increase, WBC and the WBC subpopulations displayed a consistent decrease following either a 1- or 3-min stasis. Passing and Bablok regression analysis and Pearson's correlation was globally acceptable for most of the analyses, although a lower correlation coefficient (r) was observed for basophils and LUC. Difference between measurements, as estimated by Bland and Altman plots and the 95% agreement interval in the set of differences between samples, exceeded the current analytical quality specifications for desirable bias for WBC, RBC, hemoglobin, hematocrit, lymphocytes and monocytes in samples collected after either 1- and 3-min stasis (Ricos *et al.*, 1999).

Discussion

The introduction automation in laboratory hematology has disclosed new horizons in routine hematologic testing, improving throughput and global quality of the testing process and abating the burden of potential diagnostic errors in patient care. Nevertheless, quality improvements mainly concerned the analytic phase and mostly consisted in accomplishment to optimal analytic performances (accuracy, precision and diagnostic efficiency), aligned with international guidelines (Buttarello, 2004). Owing to innovative fully automated analyzers and improved testing techniques, the overall prevalence of analytic error has been consistently reduced, accounting for <10% of all mistakes in the testing process. Accordingly, preanalytical errors are now more prevalent within the laboratory testing process, highlighting the need for a more rigorous process of defects identification and prevention, calling for exhaustive programs for quality assessment that should encompass many additional aspects related to the pre- and postanalytical phases (Plebani & Carraro, 1997).

Laboratory errors can be introduced in any phase of the testing process, including sample collection, transport and storage. Accurate standardization of the preanalytical phase of hematologic testing is hence pivotal to ensure reliable results and prevent inappropriate decision making. Although there is general consensus that the tourniquet should be applied if necessary and quickly removed when the needle is safely in the vein, only few studies evaluated the influence of a prolonged tourniquet time on the variability of hematologic testing (Reeder, 2001; McMullin *et al.*, 2005). Results of our investigation provide clear evidence that a prolonged tourniquet time, which may frequently occur in the phlebotomy practice, can produce clinically meaningful variations in several hematologic parameters. In agreement with previous observations, we have noticed a statistically significant and clinically meaningful increase in RBC, hematocrit and hemoglobin concentration, even after 1 min venous stasis. This is not surprising as hemoconcentration is recognized as a major factor contributing to increased concentration of large molecules, such as proteins and protein-bound substances, cells and coagulation factors (Lippi *et al.*, 2005a,b). However, we have first demonstrated that even a short venous stasis can substantially decreased WBC and leukocytes subpopulations. This is consistent with the evidence that the number of marginating leukocytes and the density of blood cells sticking to venous endothelium are both inversely correlated with shear rate and blood flow velocity within the vein (Nazziola & House, 1992; Kuhnle *et al.*, 1995).

Several local hemorheological disturbances in the vessels are likely to alter the rheological properties of blood. Although leukocytes and platelets are not primarily involved in the normal blood flow structuring, they can be largely influenced by substantial modifications in flow velocity (Mchedlishvili, 1998). In particular, blood stasis and venous hypertension can trigger the endothelium to release inflammatory mediators and growth factors, especially interleukin 1-beta, interleukin 6, tumor necrosis factor-alpha (Signorelli *et al.*, 2000), intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and angiotensin-converting enzyme (Michiels, Bouaziz & Remacle, 2002). These mediators are essential in the processes of leukocyte recruitment and activation, promoting leukocyte migration, rolling and infiltration of the venous wall (Kuhnle *et al.*, 1995). Thus, endothelial and leukocyte activation following venous stasis and hypertension may explain the slight but analytically and clinically meaningful decrease

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Table 1. Statistical analysis of hematologic testing in plasma specimens collected without venous stasis (no stasis) and after application of a standardized external pressure by a sphygmomanometer for 1 (1-min stasis) and 3 min (3-min stasis)

			1-min stasis			3-min stasis		
	Desirable bias (%)	No stasis		Passing and Bablock regression (r)	Percentage bias and 95% CI limits of agreement		Passing and Bablock regression (r)	Percentage bias and 95% CI limits of agreement
White blood cell count (10^6/l)	±5.6	6.52 ± 1.53	6.13 ± 1.52*	$y = 0.986x - 2.4$ 0.978	−6.7 (−17.9 to 4.6)	5.98 ± 1.58*	$y = 1.03x - 0.74$ 0.954	−10.1 (−30 to 9.8)
Red blood cell count (10^{12}/l)	±1.7	4.58 ± 0.38	4.73 ± 0.34*	$y = 0.916x - 0.50$ 0.969	3.6 (−1.3 to 8.4)	4.92 ± 0.39*	$y = 1.051x + 0.07$ 0.942	7.4 (1.4 to 13.4)
Hemoglobin (g/dl)	±1.8	136 ± 12	140 ± 12*	$y = 0.937x + 12$ 0.983	3.0 (−1.0 to 6.9)	145 ± 12*	$y = 1.000x + 8$ 0.969	6.2 (1.5 to 11.0)
Hematocrit	±1.7	0.41 ± 0.03	0.42 ± 0.03*	$y = 0.944x + 0.03$ 0.967	3.7 (0.8 to 8.1)	0.44 ± 0.04*	$y = 1.048x + 0.08$ 0.940	7.3 (1.2 to 13.4)
Main cell volume (fl)	±1.2	89 ± 4	89 ± 4	$y = 1.00x + 0.1$ 0.996	0.0 (−0.8 to 0.8)	89 ± 4	$y = 0.980 + 1.67$ 0.995	−0.1 (−1.1 to 0.9)
Main hemoglobin content (pg)	±1.4	29.8 ± 1.8	29.6 ± 1.6	$y = 0.944x + 1.54$ 0.905	−0.9 (−6.1 to 4.4)	29.4 ± 1.5	$y = 0.89x + 3.11$ 0.870	−1.3 (−7.7 to 5.1)
Platelet count (10^9/l)	±5.9	285 ± 59	289 ± 58	$y = 1.049x - 11.6$ 0.955	1.9 (−12.2 to 16.0)	298 ± 58*	$y = 0.989x + 12.8$ 0.960	4.8 (−8.7 to 18.3)
Mean platelet volume (fl)	±2.3	7.9 ± 0.8	7.9 ± 0.7	$y = 0.909x + 0.67$ 0.967	−0.4 (−4.9 to 4.1)	7.9 ± 0.8	$y = 1.00x$ 0.939	0.4 (−6.6 to 7.4)
White blood cell differential								
Neutrophils (10^6/l)	±9.1	3.95 ± 1.15	3.75 ± 1.11*	$y = 0.962x - 0.02$ 0.989	−5.5 (−15.2 to 4.3)	3.68 ± 1.15*	$y = 0.972x - 0.11$ 0.975	−8.2 (−27.7 to 11.3)
Lymphocytes (10^6/l)	±7.4	1.98 ± 0.60	1.84 ± 0.56*	$y = 0.984x - 0.06$ 0.969	−7.9 (−22 to 6.0)	1.80 ± 0.56*	$y = 0.990x - 0.11$ 0.937	−10.7 (−33 to 12)
Monocytes (10^6/l)	±13.2	0.29 ± 0.08	0.26 ± 0.09*	$y = 1.00x - 0.03$ 0.902	−14.4 (−46 to 17)	0.24 ± 0.08*	$y = 1.00x - 0.05$ 0.923	−22 (−55 to +11)
Eosinophilis (10^6/l)	±19.8	0.12 ± 0.09	0.11 ± 0.09	$y = 1.00x - 0.01$ 0.958	−4.3 (−63 to 55)	0.10 ± 0.08*	$y = 0.890x - 0.01$ 0.976	−17.7 (−74 to +38)
Basophilis (10^6/l)	±15.4	0.04 ± 0.02	0.04 ± 0.01	$y = 0.967x + 0.01$ 0.736	−7.0 (−97 to +83)	0.04 ± 0.02	$y = 1.00x$ 0.804	−7.5 (−85 to 70)
Large unstained cells (10^6/l)	Non-available	0.14 ± 0.04	0.13 ± 0.04	$y = 0.875x$ 0.771	−8.8 (−48 to 31)	0.11 ± 0.03*	$y = 0.809x$ 0.753	−25.8 (−65 to 13)

Differences are evaluated by Student's paired t-test, Passing & Bablok regression analysis and Pearson coefficient of correlation (r). Differences of 1 and 3 min collected samples are also evaluated by Bland and Altman plot analysis and results are expressed as percentage mean bias and relative 95% CI limits of agreement. Values are finally compared with the current analytical quality specifications for desirable bias derived from biological variation (Ricos *et al.*, 1999).
*$P < 0.001$.

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observed in WBC and WBC subpopulations observed in our investigations.

Hematologic and clinical chemistry variations following the application of a standard tourniquet are barely reproducible; poor standardization and interindividual variability in procedures employed to reproduce the venous stasis occurring during venipuncture are major limiting factors, which might produce contradictory or controversial results. The innovative value of the present investigation is hence represented by the exclusive approach, which involved reproduction of a constant and highly standardized venous stasis by application of an external pressure of 60 mmHg by a sphygmomanometer.

Identification of spurious changes in hematologic parameters arising from preanalytical variability is advantageous for designing intelligent system approaches to prevent, control and eliminate many of these errors (Young, 2003). A suitable blood drawing technique requires fulfilment of rigorous and standardized criteria. Taken together, results of the present investigation demonstrate that variations of hematologic testing arising from inappropriate standardization of the preanalytical phase, such as a prolonged venous stasis, may be clinically meaningful. Most of these effects appear analyte specific and depend upon the length of the stasis applied during venipuncture. Nevertheless, these preanalytical biases can be easily prevented and anticipated, adopting the most appropriate preventive measures for minimizing the influence of the tourniquet placing, which include nonapplication in patients with large or prominent veins, standardization of the external pressure by easy-to-apply re/de-inflatable device, early release after needle insertion in the vein.

Interferences from poorly standardized procedures for blood collection represent challenges to clinical laboratories. Thus, a standardized sequence for ordered specimen collection is advisable to ensure reliable results of laboratory testing and meet with increasing quality requirements. According to the most recent H3-A5 document issued by the National Committee for Clinical Laboratory Standards (NCCLS), a specific sequence is suggested: blood culture tubes should be collected first, followed by nonadditive tubes, coagulation tubes and additive tubes in the following order: tubes containing a gel separator and clot activator, sodium heparin anticoagulant tubes with or without a gel separator, EDTA anticoagulant tubes, acid citrate dextrose containing tubes and oxalate/fluoride tubes (Ernst & Calam, 2004). When many tubes are to be collected, the specimen for hematologic testing would hence be placed at the end of this sequence. The NCCLS order of draw has gone through several revisions dictated by technology, publication and common sense. According to our results, we suggest that the currently recommended order of draw may be further amended, placing first those tubes designed to the measurement of analytes which are less influenced by a prolonged venous stasis.

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