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In this issue

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Improving the preanalytical phase in laboratory medicine

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EDITORIAL

Preanalytical phase is responsible for the most frequent errors in laboratory medicine [1], that represent a major source of result variability.

A constant commitment should thus be given by all clinical laboratory players – physicians, nurses, technicians, *in vitro* diagnostic devices providers, and laboratory professionals – to reduce the clinical laboratory variability [2]. Unfortunately, these professionals subestimate the impact of a single source of laboratory variability. Therefore, lack efforts to control details like: tourniquet application time; patient compliance regarding fasting time, handling and processing of biological materials. The most important question when dealing with the laboratory sources of error is 'how huge is the error if all sources of variability impact together on a single laboratory outcome?'. Keeping this question in mind we can progressively work to improve laboratory quality then guarantee patient safety.

This Special Issue from the electronic Journal of the International Federation of Clinical Chemistry and Laboratory Medicine (eJIFCC) entitled ***Improving the preanalytical phase in laboratory medicine*** selected

nine manuscripts: five original articles, two case reports, and two critical reviews.

Some authors have assessed various pre-analytical problems at the hospital. Tóth et al. thoroughly evaluated the hemolysis problem in samples from inpatients (newborns and adults); Barbato et al. present a case report where the rejection of a hemolyzed sample caused failure to confirm hypokalemia by albuterol sulphate (salbutamol); whereas Alavi et al. worked on sample management showing the rate of blood sample nonconformities.

Concerning patient instructions, Stonys et al. provided proper evidence that in fasting patients chewing sugar-free gum could jeopardize laboratory testing [6]. Regarding sample management, Flores et al. determined the stability of K_3EDTA-plasma and serum on different storage conditions; and Salazar-García et al. showed the impact of chemical preservative in urine samples.

Abal et al., presented a case report about pseudothrombocytopenia by ethylenediaminetetraacetic acid (EDTA), drawing attention to the need for an intense communication between the laboratory and the clinician aiming to avoid misinterpreting the laboratory report. A truthfully non-systematic critical review by Caruso et al., highlighted the preanalytical interferences on laboratory immunoassays and appropriately showed the difficulty in performing properly venous blood sampling at high altitude environments. A further non-systematic critical review by Marques-Garcia deals with the main methods thus far developed for assessing the impact of hemolysis on laboratory testing.

On behalf of the eJIFCC, I congratulate all authors for their work on preanalytical phase and express my gratitude to the referees for their efforts to show the authors the best way to improve their manuscript.

REFERENCES

1. Simundic AM, Bolenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov A, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, Salle B, Seipelt C, Sumarac Z, Vermeersch P. Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56:2015-38.

2. Lima-Oliveira G, Volanski W, Lippi G, Picheth G, Guidi GC. Pre-analytical phase management: a review of the procedures from patient preparation to laboratory analysis. Scand J Clin Lab Invest 2017;77:153-63.

In this issue: Improving the preanalytical phase in laboratory medicine

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Detection of haemolysis, a frequent preanalytical problem in the serum of newborns and adults

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Key words:
clinical laboratory techniques, reproducibility of results, blood chemical analysis, specimen handling, total quality management

ABSTRACT

Background

Preanalytical problems can be more frequent in case of preterm and term newborns as compared to the general patient population. Here we present the leading preanalytical errors in our laboratory, the prevalence of haemolysis and its impact on laboratory test results, and our efforts to improve the diagnostic work-up of newborns' samples.

Methods

Preanalytical quality indicators were analysed in all samples in 2018. The haemolysis index was measured spectrophotometrically in serum samples in the period of 2012-2018, and the ratio of haemolysed samples and the test rejection rates were analysed. The data of newborns and other patients were analysed separately.

Results

During the tested year, the leading preanalytical errors were haemolysis in serum samples, inadequate

sample identification and clotting of anticoagulated blood regarding all samples or newborns. In this seven-year period the ratio of haemolysed serum samples was 4.00% in all patients and 46.4% in newborns, while the test rejection rates due to haemolysis were 0.57% and 3.71%, respectively. Haemolysis indices were significantly higher in case of newborns than in patients with documented severe intravascular haemolysis which suggests that the major reason of elevated haemolysis indices in newborns was *in vitro* haemolysis. Accordingly, all C-reactive protein (CRP) results which were rejected by severe haemolysis became reliable after repeating blood sampling.

Conclusion

Haemolysis is the leading preanalytical problem not only in newborns but also in the general patient population. Our study highlights the importance of automated assessment of serum indices and continuous monitoring of the preanalytical quality indicators and suggests the need for education and blood collection trainings.

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INTRODUCTION

In laboratory medicine, the result of an examination procedure is influenced by the correctness of the preanalytical activities [1]. During the total testing process, preanalytical phase has the highest error rate, since preanalytical errors are estimated to account for up to 70% of all errors in laboratory diagnostics [2].

Problems in the pre-preanalytical phase are particularly relevant when several steps are not performed, and are not under the control of the laboratory staff. The quality of the total testing process can be improved by continuous monitoring of quality indicators; and in clinical laboratories it is also a requirement by the International Organization for Standardization (ISO) 15189: 2012 [2, 3].

On the Consensus Conference entitled „Harmonization of Quality Indicators in Laboratory Medicine: 4 years later" organised in Padova, in 2016, a new version of the model of quality indicators was released, and more than half of the quality indicators of this model, monitor the most vulnerable part, the preanalytical phase [4]. Haemolysis was recognized as the most frequent preanalytical error and *in vitro* haemolysis is the leading cause of test or sample rejection in clinical laboratories [5].

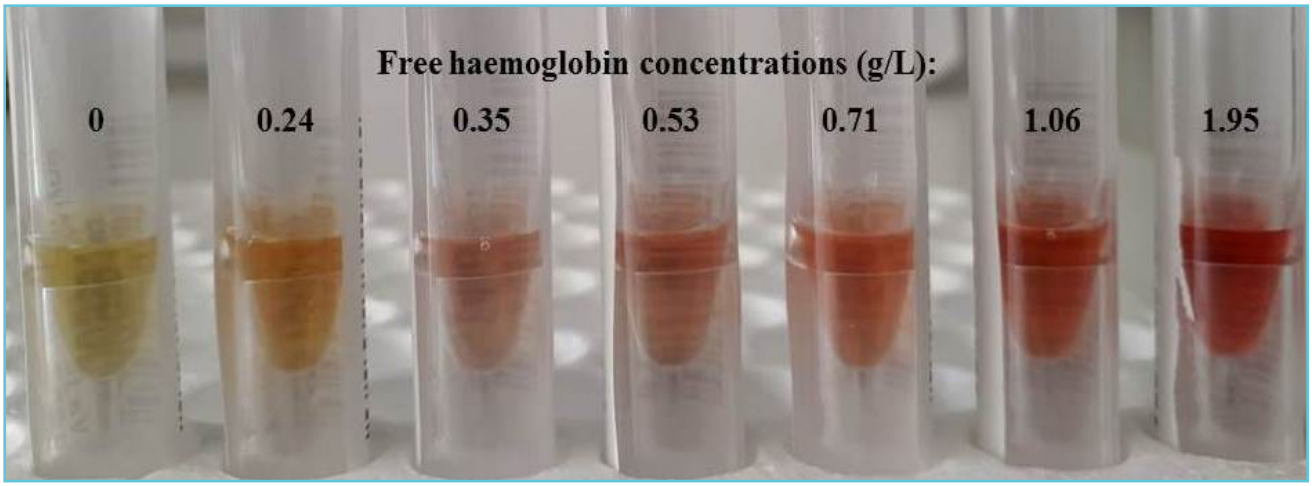
The prevalence of haemolysis can be as high as 3.3% of all routine samples, accounting for up to 40-70% of all unsuitable specimens identified, nearly five times higher than other causes, such as incorrect and clotted samples [6]. Only less than 2% of samples with detectable haemolysis are due to *in vivo* haemolysis [7].

In vitro haemolysis mostly occurs during blood collection and transportation, and it generates biological and analytical interferences [5, 8]. The cut-off of clinically significant interference was defined as 0.5 g/L for cell-free haemoglobin [9]. Although the assessment of sample quality was historically based on visual inspection of the specimen before and after centrifugation, visual detection of haemolysis is inaccurate [5]. After separation of the serum, haemolysis can be detected visually, when the free haemoglobin concentration is 0.2-0.3 g/L (Figure 1) [6, 10].

Clinical chemistry analysers are capable of automated assessment of serum indices including haemolysis index and they provide quantitative measurement with high reproducibility. Presently systematic automated measurement of haemolysis index is strongly recommended [8].

Preanalytical problems can be more frequent in case of preterm and term newborns, and analysis of their samples has special aspects compared to the general patient population. Every year, approximately 15 million babies are born preterm globally and the number is rising [11].

Figure 1 **Comparison of visual and automated detection of haemolysis in serum samples**



Haemolysis indices were measured spectrophotometrically by Roche COBAS analysers. According to the literature, haemolysis can be visible as a pink to red colour of the serum, when the free haemoglobin concentration is 0.2-0.3 g/L.

The World Health Organisation (WHO) defines preterm birth as any birth before 37 completed weeks of gestation, or fewer than 259 days since the first day of the woman's last menstrual period. This is further subdivided on the basis of gestational age: extremely preterm (<28 weeks), very preterm (28-32 weeks) and moderate or late preterm (32-37 completed weeks of gestation).

Our aim was to detect and analyse the leading preanalytical errors in our laboratory; and to monitor the prevalence of haemolysis and its impact on laboratory test results in case of all samples and in preterm and term newborns. We also present here the efforts to improve the diagnostic workup of newborns' samples.

MATERIALS AND METHODS

Preanalytical quality indicators (e.g. misidentified samples and requests, test transcription errors, clotted samples, incorrect sample type or fill level, inappropriate time and temperature of transport and storage) were analysed in all samples analyzed at the Department of Laboratory Medicine, University of Debrecen in the period of January 2018 to December 2018. Most of these indicators were recorded in the General Laboratory Information Management System (GLIMS, Medical Information for Professional Systems, Gent, Belgium) by the laboratory staff. Unidentifiable samples or samples without test requests were noted in a printed register. Serum indices (haemolysis, icterus, lipaemia) were detected automatically and they were stored in the laboratory information system.

In all serum samples that arrived for routine and STAT clinical chemistry and immunochemical assays between the period of January 2012 to December 2018, haemolysis index was measured spectrophotometrically by COBAS-8000, -6000 and 501 analysers (Roche Ltd, Basel, Switzerland), and the ratio of haemolysed samples and the test rejection rates were analysed. A sample was identified as haemolysed when the free haemoglobin concentration of the sample was higher than 0.5 g/L (the haemolysis index was higher than 31 mmol/L). The test rejection ratio was calculated as the number of tests rejected due to haemolysis divided by the total number of tests requested. Data of newborns and other patients were analysed separately.

In order to study whether preterm and term newborns have dominantly *in vitro* or *in vivo* haemolysis, their haemolysis indices were compared to the haemolysis indices of those patients who had severe intravascular haemolysis (serum haptoglobin concentration <0.1 g/L). Haptoglobin concentration was measured by an immunoturbidimetric assay on COBAS-8000, 6000 and 501 analysers (Roche Ltd, Basel, Switzerland). The CRP concentration of the newborns – as a marker of perinatal infections – was measured by a latex-sensitized immunoturbidimetric assay (Roche COBAS-501 analyser).

RESULTS

Among the 868 441 samples that were received by our laboratory in 2018, 11 379 preanalytical errors were registered by the laboratory staff in the laboratory information system. In that year the haemolysis index was measured in 295 130 serum samples.

The most frequent preanalytical error was haemolysis: 4.34% of the serum samples were haemolysed. Sample identification error was the second most frequent cause of preanalytical problems: 0.81% of all samples had some kind of identification error, most of them had less than two identifiers.

The third most frequent preanalytical error was the presence of fibrin clot in anticoagulated samples: the ratio of clotted samples was 0.36%. During the tested year, 9 017 samples were collected from preterm and term newborns, among these the ratio of haemolysed serum was 53.4%. Overall, 8.28% of all samples from newborns had identification errors (93.4% of sample identification problems were caused by less than two identifiers) and among their anticoagulated blood samples, 6.67% were clotted.

In case of all requests, the ratio of test transcription errors (e.g., absent or erroneous barcode is assigned to the request, inadequate test is requested, one or more test request(s) is/are missed) was 0.257 %. Incorrect fill level of evacuated tubes was found in case of 0.066 % of all samples, most of them had inappropriate sample-anticoagulant volume ratio. Inappropriate time and temperature of transport and storage were registered in case of 0.005 % of all samples, the main problem was excessive transportation time. Incorrect sample type and the incidence of unidentifiable samples or samples without test requests were infrequent (0.004 %).

In a second series of studies we checked for haemolysed serum samples that arrived for routine and STAT clinical chemistry and immunochemical

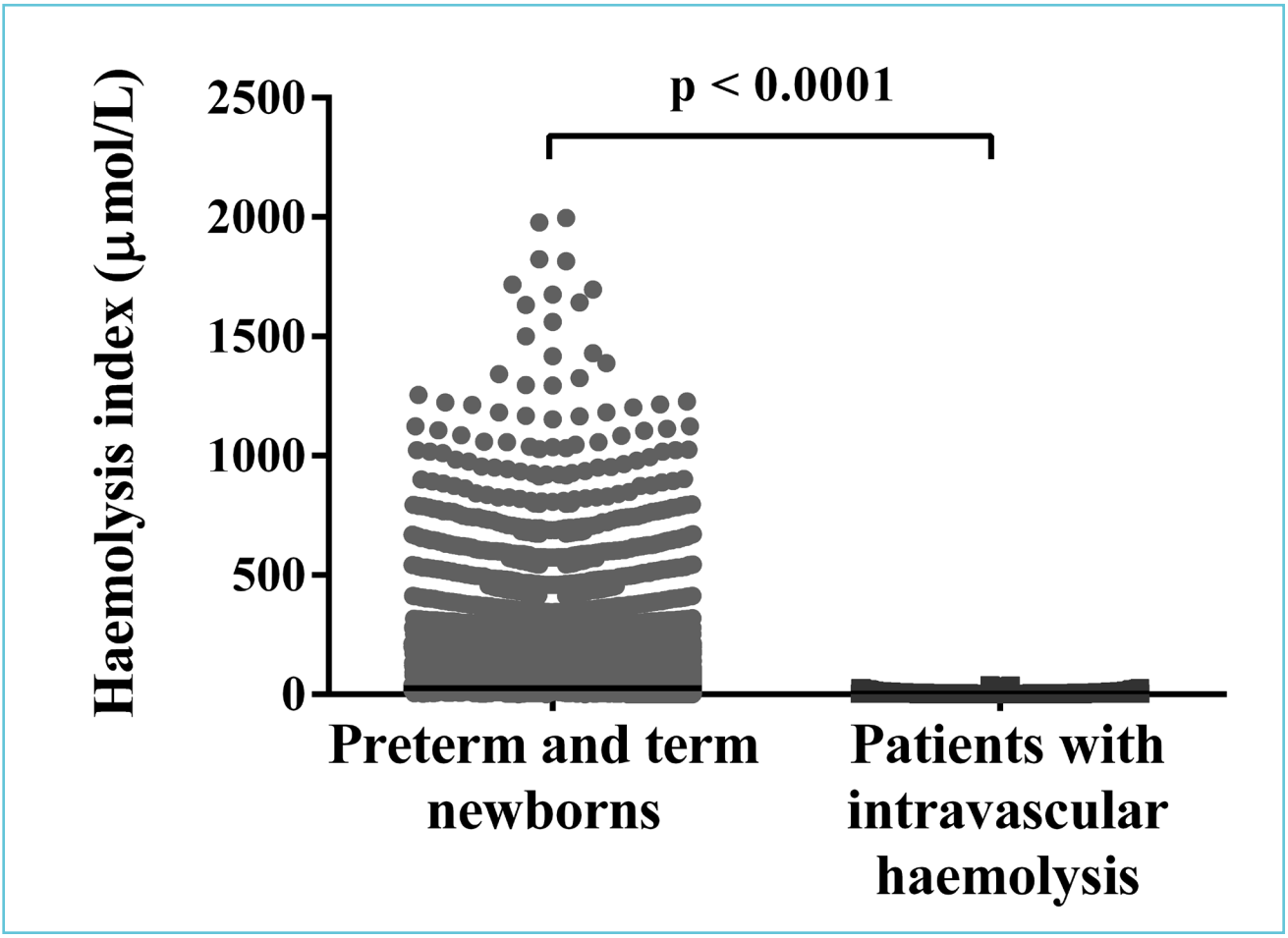
Table 1 Ratio of haemolysed samples and test rejection ratio due to haemolysis in general patient population and in preterm and term newborns in a seven-year period (2012-2018)

Serum samples	Ratio of haemolysed samples	Ratio of rejected tests due to haemolysis
All patients	4.00%	0.57%
General patients	3.33%	0.56%
Newborns	46.4%	3.71%

assays during a seven-year period (from January 2012 to December 2018). Haemolysis index was measured in case of 1 882 721 serum samples. 98.4% of the samples was collected from general patient population and 1.60% from preterm and term newborns. The ratio of haemolysed samples was 14 times higher in preterm and term newborns compared to the general patient population, while their test rejection ratio due to haemolysis was 7 times higher, respectively (Table 1). 7.88% of the serum samples arrived at the laboratory from intensive care units and 8.05% from the adult emergency department. In case of these departments, haemolysis was more frequent compared to the other blood collection sites of the general patient population. The ratio of haemolysed samples was 5.93% in case of intensive care units and 6.81% in emergency department, while the test rejection rates due to haemolysis were 0.97% and 1.73%, respectively.

We hypothesized that in the case of preterm and term newborns – although they might also have *in vivo* haemolysis – the major reason of elevated haemolysis indices was the *in vitro* haemolysis. In order to prove our hypothesis, their

Figure 2 **Haemolysis index of preterm and term newborns and patients with documented severe intravascular haemolysis**



Haemolysis indices were significantly higher in case of preterm and term newborns (median: 27 mmol/L, 25 percentile: 11 mmol/L, 75 percentile: 75 mmol/L, maximum: 1997 mmol/L) than in patients with documented severe intravascular haemolysis (median: 4 mmol/L, 25 percentile: 2 mmol/L, 75 percentile: 7 mmol/L, maximum: 39 mmol/L).

haemolysis indices were compared to the haemolysis indices of those patients who had documented severe intravascular haemolysis (serum haptoglobin concentration <0.1 g/L).

Haptoglobin concentration was measured in case of 2530 serum samples during the seven-year period, and in 557 samples haptoglobin was lower than 0.1 g/L, which refers *in vivo* hemolysis. In patients with documented severe intravascular haemolysis, haemolysis indices were significantly lower compared to the group of preterm and term newborns (Figure 2).

The measurement of specific proteins is less sensitive to haemolysis than several frequent clinical chemistry tests, but severe *in vitro* haemolysis may interfere with some methods. For example, haemolysis index >622 mmol/L may cause underestimation of CRP levels.

In case of preterm and term newborns, CRP measurement is very important to monitor perinatal infections. In 199 samples of newborn patients, the CRP test was rejected because of severe haemolysis, the highest haemolysis index was 1997 mmol/L.

Repeating the blood sampling, the haemolysis index decreased significantly and all CRP results became reliable and were reportable (Table 2).

DISCUSSION

In our laboratory the leading preanalytical errors are haemolysis in serum samples, inadequate sample identification and clotting of anticoagulated blood regarding all samples or from newborns. Preanalytical quality indicator data collected from all participating laboratories of the IFCC „Laboratory Errors and Patient Safety" project on Quality Indicators between 2009 and 2013 showed that the ratio of haemolysed samples was maximum 3% [2].

Howanitz et al found a haemolysis rate less than 3% in 71% of the studied 772 laboratories, and a rate between 3-6% in 15% of the laboratories [12]. Their haemolysis rates were the highest in case of samples from emergency departments.

Heireman et al also found that haemolysis was more often observed in samples received from the emergency department, affecting as much as 10-30% of emergency department samples [13].

Our ratio of haemolysed samples (4% for all samples) is in the higher range of the published values. One possible reason can be the presence of Perinatal Intensive Center where a lot of extremely and very preterm babies are treated and the ratio of haemolysed samples was 46.4% due to the complicated blood collection.

Table 2 Representative cases of severe in vitro haemolysis in newborn patients

Cases	First blood sampling (1-2 days after birth)		Repeated blood sampling (2-5 hours later)	
	Haemolysis index (µmol/L)	CRP (mg/L)	Haemolysis index (µmol/L)	CRP (mg/L)
Case 1	1997	haemolysed	118	0.61
Case 2	1677	haemolysed	23	1.36
Case 3	875	haemolysed	16	4.02

The reference range of CRP is <2.2 mg/L (1-2 days after birth).

Furthermore at the University of Debrecen there is a busy emergency department with a significant sample number, and there are several intensive care units. The higher ratio of haemolysis at the departments where patients are in critical condition suggests the need for education and blood collection trainings. In the literature limited information is available for haemolysis rates detected in neonatal units. Another problem is that many laboratories do not use automated, objective assessment of haemolysis, lipaemia and icterus. In neonatal samples where elevated bilirubin concentration is common, the ability to detect haemolysis by visual inspection may be further biased by underestimation of haemolysis [14]. Khedr et al published that in 2012 the haemolysis rate of the Neonatal Unit in Baystate Medical Center was near or over 40%, and as a consequence of changes (e.g., use of heel warmers to get a more consistent warming prior to drawing, increased education of correct blood withdrawal techniques) it was reduced to 28.6% within a few months [15]. This study also highlights the importance of monitoring and feedback to collecting personnel in improving and maintaining correct blood sampling method.

Among the most frequent clinical chemistry tests, lactate dehydrogenase (LDH), creatine kinase (CK), MB isoenzyme of creatine kinase (CK-MB), potassium, conjugated bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and iron are the most sensitive analytes to haemolysis. Even the measurement of specific proteins e.g. CRP, haptoglobin may have significant interference with free haemoglobin in serum. The interference may be optical, chemical or haemoglobin may interact with antigen or antibody in immunoassays. For this reason, the upper limit of haemoglobin in a serum or plasma sample is test- and method-dependent. The method description of reagents should refer to limitations such as serum indices (haemolysis, icterus, lipaemia).

The most frequent reason of test rejection is haemolysis. It is critical in case of premature babies receiving oxygen supplementary respiratory treatment who are monitored by laboratory tests (Blood gas analysis, LDH). LDH is a basic monitoring test and very sensitive to haemolysis, therefore decreasing *in vitro* haemolysis is essential especially in premature babies. As the haemolysis is often observed in preterm and term newborns [16], we may be more permissive in these cases: we can sligthly elevate the borderline of haemolysis and report the approximate LDH result, with a note that the result is affected by moderate haemolysis. This is in accordance with the result of a survey [10] in which neonatologists preferred to receive the test results with a qualitative comment according to the interference of haemolysis; rather than the rejection of LDH result at moderate haemolysis.

The second most frequent preanalytical error in our laboratory was the identification error, 0.81% of the total samples had less than two identifiers and this ratio was 10 times higher in newborns, simply because there is not enough place to write two identifiers on the label of these special small tubes. Plebani et al published that during their project the frequency of misidentification errors was not more than 0.3% [2], while in a Canadian study the majority of reported errors concerned patient or sample misidentification [17, 18].

A recent survey of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM), carried out in 12 European countries, reported that compliance of phlebotomy procedures with the Clinical and Laboratory Standards Institute (CLSI) H3-A6 guidelines was unacceptably low, accurate patient identification and tube labelling were the most vulnerable steps in blood sampling [19]. Although correct use of barcode may reduce identification errors.

Among anticoagulated blood samples, 0.36% were clotted in our laboratory, while in case of preterm and term newborns this ratio was 6.67% according to blood collection difficulties. Clotting in blood tubes containing anticoagulants mostly occurs for challenging/prolonged venipuncture or failing to appropriately mix the tube after collection [20]. Plebani et al found that the median values for the ratio of clotted samples were between 0.05% and 0.21% in their study [2].

Twenty-six percent of laboratory errors can affect outcome of patient's care, errors result in further inappropriate investigations, patient discomfort, increased costs and/or modification of the therapy [17, 21]. Monitoring of preanalytical quality indicators is a valuable tool to guarantee and improve the quality of the preanalytical phase. Automated assessment of serum indices characterizes the quality of sera, therefore it is recommended for all laboratories - it provides more objective evaluation of the sample and assists to obtain reliable results. Application of serum indices described in each test method should be applied in the analysers when a new test is introduced, and checking the test-specific serum index cut-off values for the most sensitive tests is recommended.

In conclusion, we determined the leading preanalytical errors and their frequency both in case of general patients and newborns. We have to reduce them and therefore the implementation of the joint EFLM-COLABIOCLI recommendation for venous blood sampling is needed [22]. This recommendation covers all steps of the venous blood collection procedure using closed system in case of in- and outpatients except for children and unconscious patients. Implementation of the guidelines, systematic theoretical education and practical trainings of the medical staff, periodical audits of phlebotomy, continuous monitoring of the preanalytical quality indicators and automated assessment of serum indices are recommended to improve the quality of the total testing process.

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REFERENCES

1. Aita A, Sciacovelli L, Plebani M. Extra-analytical quality indicators - where to now? Clin Chem Lab Med 2018;57(1): 127-133.

2. Plebani M, Sciacovelli L, Aita A, Pelloso M, Chiozza ML. Performance criteria and quality indicators for the preanalytical phase. Clin Chem Lab Med 2015;53(6):943-948.

3. Aita A, Sciacovelli L, Plebani M. The silk road to total quality in Laboratory Medicine. Clin Chem Lab Med 2019; 57(6):769-772.

4. Sciacovelli L, Panteghini M, Lippi G, Sumarac Z, Cadamuro J, Galoro CAO, et al. Defining a roadmap for harmonizing quality indicators in Laboratory Medicine: a consensus statement on behalf of the IFCC Working Group "Laboratory Error and Patient Safety" and EFLM Task and Finish Group "Performance specifications for the extra-analytical phases". Clin Chem Lab Med 2017;55(10): 1478-1488.

5. Wan Azman WN, Omar J, Koon TS, Tuan Ismail TS. Hemolyzed Specimens: Major Challenge for Identifying and Rejecting Specimens in Clinical Laboratories. Oman Med J 2019;34(2):94-98.

6. Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, et al. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med 2008;46(6):764-772.

7. Carraro P, Servidio G, Plebani M. Hemolyzed specimens: a reason for rejection or a clinical challenge? Clin Chem 2000; 46(2):306-307.

8. Lippi G, Cadamuro J, von Meyer A, Simundic AM, European Federation of Clinical C, Laboratory Medicine Working Group for Preanalytical P. Practical recommendations for managing hemolyzed samples in clinical chemistry testing. Clin Chem Lab Med 2018;56(5):718-727.

9. Lippi G. Systematic Assessment of the Hemolysis Index: Pros and Cons. Adv Clin Chem 2015;71:157-170.

10. Joshi S, Vaitkute R, Jeffery J, Ayling RM. Haemolysis in neonatal blood samples: a survey of practice. Ann Clin Biochem 2007;44(Pt 2):178-181.

11. Quinn JA, Munoz FM, Gonik B, Frau L, Cutland C, Mallett-Moore T, et al. Preterm birth: Case definition & guidelines for data collection, analysis, and presentation of immunisation safety data. Vaccine 2016;34(49):6047-6056.

12. Howanitz PJ, Lehman CM, Jones BA, Meier FA, Horowitz GL. Practices for Identifying and Rejecting Hemolyzed Specimens Are Highly Variable in Clinical Laboratories. Arch Pathol Lab Med 2015;139(8):1014-1019.

13. Heireman L, Van Geel P, Musger L, Heylen E, Uyttenbroeck W, Mahieu B. Causes, consequences and management of sample hemolysis in the clinical laboratory. Clin Biochem 2017;50(18):1317-1322.

14. Jeffery J, Sharma A, Ayling RM. Detection of haemolysis and reporting of potassium results in samples from neonates. Ann Clin Biochem 2009;46(Pt 3):222-225.

15. Khedr S, Blake V, Erdogan E. Neonatal Unit Hemolysis Rates From an Academic Medical Center: A Quality Improvement Project. Arch Pathol Lab Med 2016;140(6): 502-503.

16. Christensen RD, Yaish HM. Hemolysis in Preterm Neonates. Clin Perinatol 2016;43(2):233-240.

17. Aita A, Sciacovelli L, Plebani M. Laboratory-related errors: you cannot manage what you don't measure. You manage what you know and measure. Diagnosis (Berl) 2017;4(4):193-195.

18. Restelli V, Taylor A, Cochrane D, Noble MA. Medical laboratory associated errors: the 33-month experience of an on-line volunteer Canadian province wide error reporting system. Diagnosis (Berl) 2017;4(2):79-86.

19. Simundic AM, Church S, Cornes MP, Grankvist K, Lippi G, Nybo M, et al. Compliance of blood sampling procedures with the CLSI H3-A6 guidelines: An observational study by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) working group for the preanalytical phase (WG-PRE). Clin Chem Lab Med 2015;53(9):1321-1331.

20. Giavarina D, Lippi G. Blood venous sample collection: Recommendations overview and a checklist to improve quality. Clin Biochem 2017;50(10-11):568-573.

21. Plebani M, Carraro P. Mistakes in a stat laboratory: types and frequency. Clin Chem 1997;43(8 Pt 1): 1348-1351.

22. Simundic AM, Bolenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, et al. Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56(12):2015-2038.

In this issue: Improving the preanalytical phase in laboratory medicine

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Rejection of hemolyzed samples can jeopardize patient safety

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*These graduation-students contributed equally to this work. Therefore, their names are listed in alphabetical order.

ABSTRACT

Introduction

In vitro hemolysis is the primary cause of sample/test rejection by the laboratory.

Case report

A 10-year-old, admitted with an asthma attack in the emergency-room, medicated with albuterol sulphate (intravenous bronchodilator that could induce hypokalemia), needed laboratory test monitoring. The physician prescribed the technical-nurse to perform blood sampling for: complete blood count, electrolytes, glucose, and blood gas analysis–within 30min after therapy. Samples were delivered to laboratory with a note “I had difficult to locate an appropriate access to perform the blood collection”.

Laboratory results

Glucose: 4.77 mmol/L. Complete blood count revealed discreet eosinophilia 0.13x10^9/L, and thrombocytopenia 18x10^9/L. However, platelet clumps were observed in peripheral blood smear. Blood gas analysis was unreported, laboratory informed that sample had micro clots.

Electrolytes: laboratory did not report the results; *sample hemolyzed*. 0.9 g/L of free hemoglobin is the cut-off defined by the laboratory; the sample presented 2.3 g/L of free hemoglobin. 3.9 mmol/L of potassium was the unreported result vs 2.1 mmol/L in the new sample.

Briefly, the laboratory technician was trained to hide potassium results on hemolyzed sample due to the potential overestimation. Even if the hemolyzed sample presented a potassium value close to the lower reference range value (3.5-5.1 mmol/L), reporting the potassium result could allow the physician starting proper therapy to revert the hypokalemia by albuterol sulfate.

Conclusion

The laboratory should be aware of the clinical patient conditions and of the related physician needs, before hiding results. Therefore, both the laboratory and the clinic personnel should communicate in order to guarantee the patient safety.

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INTRODUCTION

Briefly hemolysis is due to leakage of the red blood cells membrane with the release of the erythrocyte-cytoplasm in the fluid (plasma or serum) [1]. We can classify hemolysis in two major categories:

i) *in vitro* because of improperly blood sample collection procedure [2, 3], venous stasis [4-6], unnecessary or excessive sample mix [7, 8], improper temperature maintenance [9], etc.; and

ii) *in vivo* due to iatrogenic conditions, acquired, or hereditary; unrelated with any laboratory technique.

Hemolysis is the most frequent pre-analytical source of variability, and *in vitro* hemolysis is the primary cause of sample/test rejection by laboratory professional [10]. However, Cadamuro et al., properly evidenced that laboratory professionals need deeply understand the pre-analytical interference (i.e., hemolysis) then to establish own laboratory criteria about when and how to report laboratory results in hemolysed samples [11]. The aim of this pre-analytical case report is to show that hemolysis due to inadequate phlebotomy procedure masked hypokalemia by albuterol sulfate (salbutamol).

CASE REPORT

A 10-year-old boy, admitted with asthma attack in the emergency room from a Brazilian general hospital, and properly medicated with intravenous infusion of salbuterol sulfate–4 µg/Kg/min–needed laboratory test monitoring [12, 13]. Briefly, albuterol sulfate (salbutamol - a sympathomimetic amine), is a beta-adrenergic agonist that selectively acts on the beta (2)-adrenergic receptors of intracellular adenyl cyclase, the catalyst for the conversion of adenosine triphosphate (ATP) to cyclic-3', 5'-adenosine monophosphate (cyclic AMP) [14]. This action increases cyclic AMP levels resulting in bronchial smooth muscle relaxation (bronchial dilatation), and inhibition of release of immediate hypersensitivity mediators from mast cells [15].

The physician prescribed the technical nurse to perform blood sampling for laboratory testing–complete blood count, electrolytes (sodium, potassium, chloride, calcium, and magnesium),

glucose, and blood gas analysis–within half an hour after albuterol sulfate infusion.

Samples were delivered to STAT laboratory by pneumatic tube system with a note in the test-order "I had difficulty to locate an appropriate venous access to perform the blood collection".

LABORATORY TESTING

Complete blood count was performed on Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan); clinical biochemistry testing – glucose, sodium, potassium, chloride, calcium, and magnesium – were performed on cobas 8000 c501 (Roche Diagnostics GmbH, Penzberg, Germany); whereas blood gas analysis was performed on GEM Premier 3000® (Instrumentation Laboratory a Werfen Company, Bedford, USA).

RESULTS

The laboratory present complete blood count and glucose results; whereas unreported results of electrolytes, and blood gas analysis (Table 1).

Table 1 Laboratory testing results*

Instruments	Tests	Results	Units
Sysmex XN-1000 Sysmex	Red blood cells	4.86	10^{12}/L
	Haemoglobin	141	g/L
	Hematocrit	40.5	%
	Mean corpuscular volume	83.3	fL
	Red blood cell distribution width	12.6	%
	White blood cells	6.12	10^9/L
	Neutrophils	3.24	10^9/L
	Lymphocytes	2.32	10^9/L
	Monocytes	0.43	10^9/L
	Eosinophils	0.13	10^9/L
	Platelets *1	18	10^9/L
Cobas 8000 c501 Roche	Glucose	4.77	mmol/L
	Sodium	new sample required *2	
	Potassium		
	Chloride		
	Calcium		
	Magnesium		
GEM Premier 3000 Werfen	Blood gas analysis	new sample required *3	

Laboratory notes:
1. platelet clumps were observed in peripheral blood smear from the blood sample collected in ethylenediaminetetraacetic acid (K_2EDTA) tube.
2. sample hemolyzed +++
3. sample had micro clots being a possible analytical problem for the blood gas analyser.

DISCUSSION

The worried physician called the laboratory about the patient showing signs and symptoms compatible with hypokalemia: therefore, the potassium laboratory result was absolutely needed. In reply, the laboratory technician verbally (by phone) informed that result reporting was not allowed, because the sample was hemolyzed.

Hypokalemia should be expected immediately after salbutamol intravenous infusion for severe asthma treatment, with potassium values mainly between 2.7 mmol/L and 3.4 mmol/L, which generally return to normality within half an hour [16]; whereas, inhalation of salbutamol in children can cause hypokalemia–30 min after inhalation–with potassium levels between 2.5 mmol/L and 4.2 mmol/L [17, 18]. This hypokalemic effect of catecholamines is mediated by the B_2-receptor linked to the Na/K ATPase in skeletal muscle [19, 20]. Therefore, the inpatient treatment with salbutamol requires blood gas analysis and potassium monitoring. Measuring potassium in pediatric patients is essential, since these patients frequently have diarrhea, vomiting or are following therapy with diuretics and digitalis too [21].

Cadamuro et al., had shown an impressive heterogeneity from European laboratories on management of hemolyzed samples [22]. Briefly, some laboratories used a color scale for visual hemolysis detection (434/1160); whereas others used hemolysis cut-offs declared by the *in vitro* diagnostic device-manufacturers' (624/1160). However, only 246/1160 verified these cut-offs. The general cut-off that defined a sample as hemolytic, lacks harmonization; i.e., same laboratories have rigorous cut-off of 0.1 g/L of free hemoglobin, whereas others have permissive cut-off of 1 g/L of free hemoglobin [22].

Why did the laboratory not report the results?

0.9 g/L of free hemoglobin is the cut-off defined and verified by the accredited laboratories by International Organization for Standardization 15189 standard [23] for rejecting samples; the present sample had 2.3 g/L of free hemoglobin. 3.9 mmol/L of potassium was the unreported result on the hemolyzed sample. A newly collected non-hemolyzed sample had shown a potassium of 2.1mmol/L; with a turnaround time of 1h45min for reporting the proper result, having the whole course considered; whereas the mean turnaround for potassium report (from collection to verification) should be less than 36 min [24]. The reason: the laboratory technician was trained to hide potassium results on hemolyzed sample due to the potential overestimation (release of potassium from red blood cells). Though, the hemolyzed serum sample presented potassium near the lower reference range value (3.5-5.1 mmol/L), reporting the first potassium result could allow the physician starting the proper pharmacological therapy to revert immediately the hypokalemia by salbutamol. Reports support the importance to communicate potassium results with a comment on hemolyzed samples instead of suppressing it [25, 26].

The blood gas analysis revealed normal results with an abnormal flag on potassium results. The arterial blood sample had shown micro clots. Therefore, the laboratory technician supposed that the wrong result derived from the clot presence. D'Orazio accurately reported the impact of clots on blood gas analyses including potassium [27]. The laboratory technician performed the proper maintenance on the blood gas analyzer to eliminate the potential micro clot from the analyzer system; then verified the analyzer performance using a third-party control materials-independent from calibrator materials, as recommended [28]. Thus, laboratory technician required new arterial sample. The laboratory instruments can provide the concentration of potassium in a few seconds, since several blood gas analyzers are incorporating the electrode [29]. However, clinicians should be aware about the

specific reference range for potassium determination on a different sample matrix (relatively lower K^+ on plasma sample than serum sample) [30]. Hence, divergent potassium results could be reported for the same patient's samples, respectively collected as lithium heparin anticoagulated plasma for blood gas analysis or as serum for clinical biochemistry.

In conclusion, the laboratory should be aware of the clinical patient conditions and of the related physician needs, before hiding results. Therefore, both the laboratory and the clinic personnel should communicate in order to guarantee the patient safety.

REFERENCES

1. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood 2013;121:1276-84.

2. Simundic AM, Bolenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov A, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, Salle B, Seipelt C, Sumarac Z, Vermeersch P. Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56:2015-38.

3. Mrazek C, Simundic AM, Wiedemann H, Krahmer F, Felder TK, Kipman U, Hoppe U, Haschke-Becher E, Cadamuro J. The relationship between vacuum and hemolysis during catheter blood collection: a retrospective analysis of six large cohorts. Clin Chem Lab Med 2017;55:1129-34.

4. Cadamuro J, von Meyer A, Wiedemann H, Klaus Felder T, Moser F, Kipman U, Haschke-Becher E, Mrazek C, Simundic AM. Hemolysis rates in blood samples: differences between blood collected by clinicians and nurses and the effect of phlebotomy training. Clin Chem Lab Med 2016;54: 1987-92.

5. Lima-Oliveira G, Lippi G, Salvagno GL, Gaino S, Poli G, Gelati M, Picheth G, Guidi GC. Venous stasis and whole blood platelet aggregometry: a question of data reliability and patient safety. Blood Coagul Fibrinolysis 2015;26: 665-8.

6. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Scartezini M, Guidi GC, Picheth G. Transillumination: a new tool to eliminate the impact of venous stasis during the procedure for the collection of diagnostic blood specimens for routine haematological testing. Int J Lab Hematol 2011;33:457-62.

7. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Gelati M, Volanski W, Boritiza KC, Picheth G, Guidi GC. Effects of vigorous mixing of blood vacuum tubes on laboratory test results. Clin Biochem 2013;46:250-4.

8. Lima-Oliveira G, Lippi G, Salvagno GL, Brocco G, Gaino S, Dima F, Volaski W, Rego FG, Picheth G, Guidi GC. Processing of diagnostic blood specimens: is it really necessary to mix primary blood tubes after collection with evacuated tube system? Biopreserv Biobank 2014;12:53-9.

9. Lippi G, Lima-Oliveira G, Nazer SC, Moreira ML, Souza RF, Salvagno GL, Montagnana M, Scartezini M, Picheth G, Guidi GC. Suitability of a transport box for blood sample shipment over a long period. Clin Biochem 2011;44:1028-9.

10. Wan Azman WN, Omar J, Koon TS, Tuan Ismail TS. Hemolyzed Specimens: Major Challenge for Identifying and Rejecting Specimens in Clinical Laboratories. Oman Med J 2019;34:94-8.

11. Cadamuro J, Simundic AM, Ajzner E, Sandberg S. A pragmatic approach to sample acceptance and rejection. Clin Biochem 2017;50:579-81.

12. Clayton S, Gilchrist F, Alexander J, Enberby B, Lenney W. An algorithm for the use of intravenous salbutamol in children. Br J Nurs 2012;21:S30-4.

13. Babl FE, Sheriff N, Borland M, Acworth J, Neutze J, Krieser D, Ngo P, Schutz J, Thomson F, Cotterell E, Jamison S, Francis P. Paediatric acute asthma management in Australia and New Zealand: practice patterns in the context of clinical practice guidelines. Arch Dis Child 2008;93: 307-12.

14. Johnson M. Beta2-adrenoceptors: mechanisms of action of beta2-agonists. Paediatr Respir Rev 2001;2:57-62.

15. Neame M, Aragon O, Fernandes RM, Sinha I. Salbutamol or aminophylline for acute severe asthma: how to choose which one, when and why? Arch Dis Child Educ Pract Ed 2015;100:215-22.

16. Leitch AG, Clancy LJ, Costello JF, Flenley DC. Effect of intravenous infusion of salbutamol on ventilatory response to carbon dioxide and hypoxia and on heart rate and plasma potassium in normal men. Br Med J 1976;1: 365-7.

17. Hung CH, Chu DM, Wang CL, Yang KD. Hypokalemia and salbutamol therapy in asthma. Pediatr Pulmonol 1999;27: 27-31.

18. Burgess CD, Flatt A, Siebers R, Crane J, Beasley R, Purdie G. A comparison of the extent and duration of hypokalaemia following three nebulized beta 2-adrenoceptor agonists. Eur J Clin Pharmacol 1989;36:415-7.

19. Gelmont DM, Balmes JR, Yee A. Hypokalemia induced by inhaled bronchodilators. Chest 1988;94:763-6.

20. Brown MJ, Brown DC, Murphy MB. Hypokalemia from beta2-receptor stimulation by circulating epinephrine. N Engl J Med 1983;309:1414-9.

21. Kerem E, Levison H, Schuh S, O'Brodovich H, Reisman J, Bentur L, Canny GJ. Efficacy of albuterol administered by nebulizer versus spacer device in children with acute asthma. J Pediatr 1993;123:313-7.

22. Cadamuro J, Lippi G, von Meyer A, Ibarz M, van Dongen E, Lases, Cornes M, Nybo M, Vermeersch P, Grankvist K, Guimaraes JT, Kristensen GBB, de la Salle B, Simundic AM. European survey on preanalytical sample handling - Part 2: Practices of European laboratories on monitoring and processing haemolytic, icteric and lipemic samples. On behalf of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group for the Preanalytical Phase (WG-PRE). Biochem Med (Zagreb) 2019;29:020705.

23. International Organization for Standardization. Medical laboratories - Requirements for quality and competence ISO document 15189. Geneva, Switzerland 2012:International Organization for Standardization.

24. Valenstein P. Laboratory turnaround time. Am J Clin Pathol 1996;105:676-88.

25. Carraro P. Potassium report of hemolyzed serum samples. Clin Chem Lab Med 2008;46:425-6.

26. Ismail A, Shingler W, Seneviratne J, Burrows G. In vitro and in vivo haemolysis and potassium measurement. BMJ 2005;330:949.

27. D'Orazio P. Effects of Blood Clots on Measurements of pH and Blood Gases in Critical Care Analyzers. Point of Care: The Journal of Near-Patient Testing & Technology 2011;10: 186-8.

28. Lima-Oliveira G, Lippi G, Salvagno GL, Brocco G, Guidi GC. In vitro diagnostic company recalls and medical laboratory practices: an Italian case. Biochem Med (Zagreb) 2015; 25:273-8.

29. Beneteau-Burnat B, Pernet P, Pilon A, Latour D, Goujon S, Feuillu A, Vaubourdolle M. Evaluation of the GEM Premier 4000: a compact blood gas CO-Oximeter and electrolyte analyzer for point-of-care and laboratory testing. Clin Chem Lab Med 2008;46:271-9.

30. Lima-Oliveira G, Monneret D, Guerber F, Guidi GC. Sample management for clinical biochemistry assays: are serum and plasma interchangeable specimens? Crit Rev Clin Lab Sci 2018;55:480-500.

In this issue: Improving the preanalytical phase in laboratory medicine

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Challenges in preanalytical phase of laboratory medicine: rate of blood sample nonconformity in a tertiary care hospital

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ABSTRACT

Objective

To evaluate the major causes of preanalytical errors in medical laboratory of a tertiary care hospital.

Methods

It was a retrospective study in which we analyzed the sample rejection data of hematology and chemical pathology sections from January to December 2018. Number of rejected samples, reason for rejection and type of test ordered on monthly basis were recorded on a platform.

Results

A total of 113,817 samples were received during the study period. Preanalytical errors were found in 1,688 samples, which constitute approximately 1.48% of the total number of samples received.

Conclusion

Our study highlights the magnitude of preanalytical errors in our setup. Preanalytical errors can lead to loss of patient trust in diagnostic services, can dent the

laboratory's reputation, and lead to an increase in the overall operating expenses, both for laboratories as well as the hospitals. Compliance with good laboratory practices can significantly reduce the frequency of pre analytical errors.

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INTRODUCTION

Quality in medical diagnostics is essential to the goal of providing safe health care to patients. Among other clinical disciplines, laboratory medicine assumes a vital role in patient safety (1).

Conventionally, laboratory practice can be divided into three phases, i.e., preanalytical, analytical and post-analytical phase. Preanalytical phase comprises of test selection, patient identification, collection of the sample, handling of the sample, sorting out, pipetting and centrifugation (2, 3). Negligence in any of these steps can lead to erroneous results attributed to preanalytical phase.

Although all three phases are equally important for improving total quality management and should be targeted individually for improving standards of the laboratory, preanalytical phase is considered as the most error prone part of the total testing process. Preanalytical issues have been included in the list of biggest challenges faced by the laboratory professionals in the last two decades (4).

Lippi and colleagues reported that total error rate in laboratory medicine is 0.1% to 3.0% (5). Analytical errors which have been focus of research in the past, account for less than 10% of all the diagnostic mistakes, whereas preanalytical errors are reported to be accounting for 46 to 68.2% (6). Moreover, preanalytical errors constitute 18.5 to 47% of the laboratory errors. Missing patient's identification, inappropriate containers, missing samples are most commonly encountered preanalytical errors (7).

Worldwide standards relating to blood sampling and standardization are available but compliance to guidelines is very low especially in the background where sampling is done by the nurses/junior doctors without involvement of the laboratory staff (8). Furthermore, there is heterogeneity in criteria for sample rejection from one laboratory to another. Alongside the long road of patient safety, preanalytical phase of laboratory medicine offers a wide room for improvement (9).

There is scarcity of the local data regarding documentation, root cause analysis and preventive strategies for laboratory errors (10). Our study aims to evaluate the major causes of pre analytical errors in medical laboratory of a tertiary care hospital.

MATERIAL AND METHODS

Shalamar Hospital is a teaching hospital in Lahore, Pakistan specializing in various fields like: Surgery, Gynecology & Obstetrics, Cardiology, Gastroenterology, and Psychiatry. It is a 430 bedded hospital providing services to about 376,000 patients per year. Here, phlebotomies of the inpatients are performed by the clinical staff (nurses and junior doctors), whereas blood samples from the outpatients are collected by laboratory personnel.

The samples are collected in evacuated tubes (BD vacutainer), that include purple top ethylenediaminetetraacetic acid di/trisodium salt (EDTA) tubes, blue top sodium citrate tubes, yellow top gel separation tubes, and syringes for arterial blood gas analysis. Upon receiving the samples, they are sorted out for any problem at the reception desk before transporting to concerned sections. In case, any problem exists, it is manually registered in the logbook.

Samples are rejected on the basis of preset rejection criteria, as follows: unlabeled specimen container, specimen without request form, incorrect

tube (wrong choice of tube), wrong label/wrong medical record number, incorrect quantity or insufficient sample, hemolysed sample, anticoagulated sample (EDTA and citrated) with clots, improper sample transport, improper container closure, specimen delayed in transit making results invalid, diluted sample. The data generated is viewed periodically (monthly).

It was a retrospective study; we analyzed the sample rejection data of hematology and chemical pathology sections from January to December 2018. Number of rejected samples, and reason for rejection of tests ordered on monthly basis were recorded on a proforma. Data were analyzed on Statistical Package for the Social Sciences version 20 (SPSS V 20). Frequency of each type of preanalytical error was assessed.

RESULTS

A total of 113,817 samples were received during the study period in hematology and chemical pathology sections. Out of these samples, pre analytical errors were found in 1,688 samples, which constituted approximately 1.48% of the total number of samples received. The frequency of errors are show in Table 1.

The most frequent error was "unlabeled samples" which was responsible for 36% of the total preanalytical errors. The total preanalytical errors in the period divided by month is show in Figure 1. Monthly breakup of the pre analytical errors showed that maximum number of errors occurred in the month of October.

DISCUSSION

Our results are comparable to studies that were carried out in other developing countries. In an Indian study by Chawala et al, preanalytical error rate was reported to be 1.52% (11), which is analogous to our result. In another 5 years Spanish study, overall rate of pre analytical errors was reported at 0.047% (12). In previous studies, the variable receiving highest frequency was "hemolysed" sample (11-13). The most frequent pre analytical error in our setting was "unlabeled specimen" (Table 1). Proper labeling

Table 1 Frequency of errors on blood samples*

Type of error	N	Frequency (%)
Unlabeled sample	604	35.8
Sample clotted (EDTA and sodium citrate)	252	14.9
Sample diluted	200	11.8
Wrong medical record number	172	10.2
Sample hemolysed	164	9.7
Incorrect tube	148	8.8
Incorrect quantity or insufficient sample	148	8.8

**Frequency = N / 1688 (total pre analytical errors in a year) ×100.*

of the specimen cannot be overemphasized, as this step is the backbone of a good sampling process (14). Missing slips, wrong labeling/wrong medical record numbers and most important unlabeled specimens can jeopardize the patient outcomes.

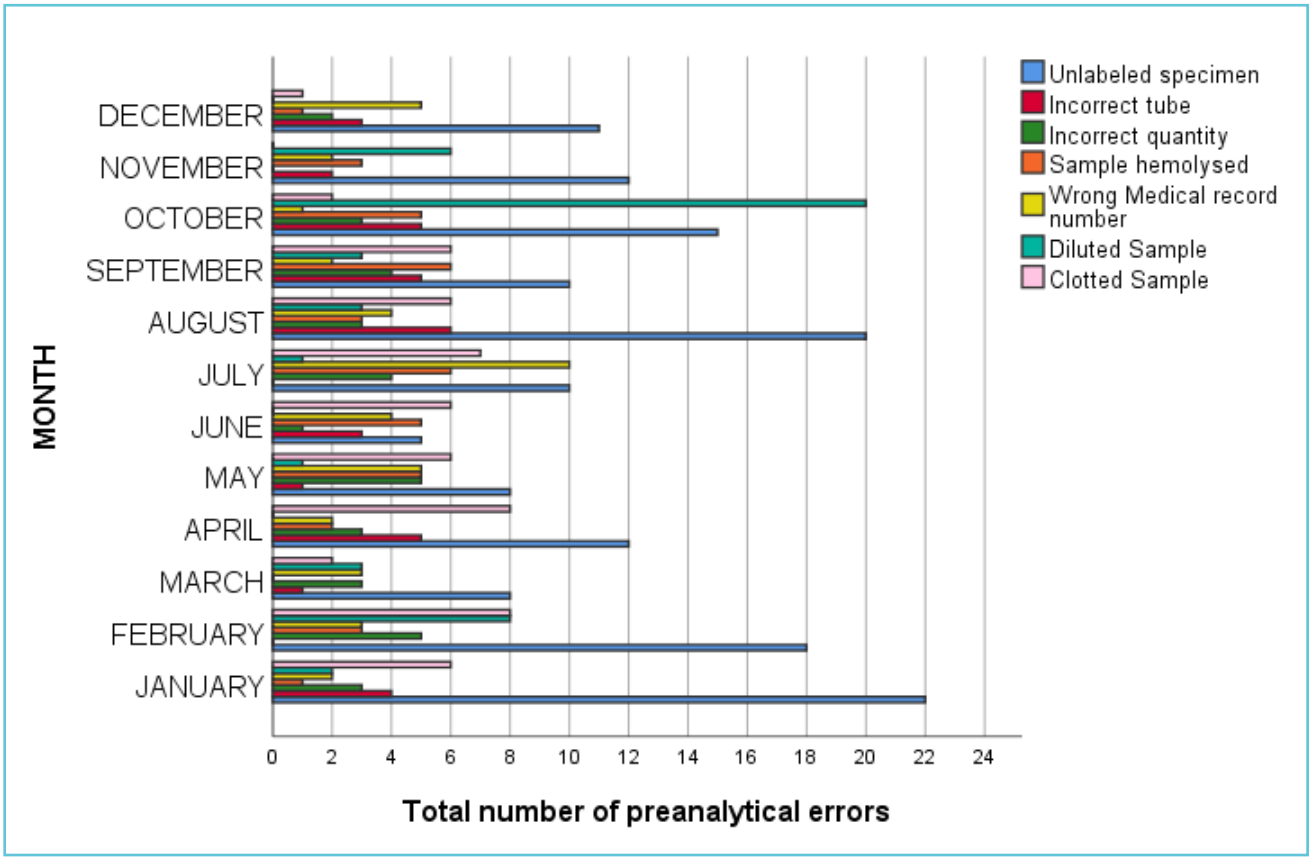
Negligence in this domain can lead to delayed diagnosis, additional laboratory testing, or treatment of a patient for a wrong medical condition. Sometimes, these errors may even be fatal (e.g., acute hemolytic reaction after incompatible blood transfusion because of wrong labeling of the EDTA/serum evacuated tubes in which the samples are sent for blood grouping and cross match).

An interesting fact in our study was that majority of the unlabeled samples were sent for analysis of arterial blood gases (ABG). As arterial-blood specimen is used for ABG analysis, the sampling process is relatively cumbersome for the patient as well as for the doctors. However, due to negligence in labeling, it results in unnecessary delays and recollecting of the arterial-blood. Mishandling of the specimen at this step may be attributed to excessive patient workload/ shortage of time in the clinical departments, lack of awareness of the doctors/nursing staff regarding the patient information and lack of bar code system in our setup.

As modern-day laboratory practice is not just confined to "report making", it is also involved in disseminating this important information to the clinicians. Labeling errors can make this practice delayed and redundant.

After "labeling" the next big problem in our setup was "diluted" samples (Table 1). Majority of the workload of our laboratory comprises of samples from inpatient department, where sampling is

Figure 1 **Preanalytical errors per month**



being performed by nurses/junior doctors. They sometimes underestimate the importance of drawing blood from the vein without intravenous lines. If intravenous fluid is being given in a patient's arm, sample should be drawn from the opposite arm (14). If both arms are occupied, blood may be taken after intravenous infusion is turned off for at least two min and tourniquet should be applied below the infusion site before sampling (15).

Anticoagulated samples (EDTA and sodium citrate) with clots constituted around 14% in the present study (Table 1). Gross clots can be easily detected by visual inspection of the sample, however micro clots are sometimes difficult to detect. The presence of clots can be attributed to increased blood to anticoagulant ratio and improper mixing of the blood after dispensing in the tube with anticoagulant (15). In this study, this error could be due to improper mixing of the blood sample and overfilling of the EDTA/ citrated vials.

Another significant finding in our study was that maximum number of preanalytical errors occurred in the month of October, and out of them most of the samples were diluted. When we performed the root cause analysis of this issue, we found that neophyte nursing staff was inducted in the month of October. So, this issue can be explained on the basis of inadequate training, poor skills and improper sampling technique of the staff.

Therefore, we conducted in house training sessions for our nursing staff to get them familiarized with the proper phelobotomy technique. After the training session, number of preanalytical errors in the subsequent months had declined to average which was comparable with that of the other months (Figure 1).

Errors in the laboratory are directly proportional to financial constraints and lead to decreased patient satisfaction. Laboratory errors not only affect patient care, including delay in turnaround time, unnecessary redraws, wrong diagnosis, inappropriate treatment but also damages reputation of a laboratory and diminishes confidence of the patient on diagnostic services. Negative impact of laboratory error on patient outcomes is reported to be as high as 24.4% (16). On a broader horizon, they are also adding fuel to the financial constraints of the health system.

A study by Green shows that preanalytical error costs represent between 0.23% and 1.2% of total hospital running budget (17). North American hospitals reported the costs of $337.05 for outpatient (which includes emergency department patients), $162.18 for inpatient (critical), $357.15 for inpatient (other) (17). We lack a comprehensive local data about the effect of preanalytical error on hospital budgets, but for sure we are in a more troublesome situation because of the errors in this phase.

Though improvement in laboratory workflow has significantly reduced the error rate during the analytical phase, preanalytical phase remains the most vulnerable part of laboratory testing, due to the presence of many steps that occur both before and after the specimen reaches the laboratory.

It is evident from the above issues that incorrect sampling technique is the main reason behind the pre analytical errors. This can be attributed to lack of proper training about standard operating procedures of sampling, underestimating the importance of sampling and heavy workloads at the clinical sites. Appropriate training of the staff and proper quality control procedures can help to reduce the pre analytical errors (18,19).

Phlebotomy is considered as a separate domain in most of the developed countries, we could also adopt similar approach for the improvement in quality of our laboratory work.

This whole process demands a holistic approach, including good liaison among the members of the specimen management team, ordering clinicians, phlebotomists, courier who transports the specimen, as well as the laboratory personnel who processes the specimen for testing. Moreover, laboratories should keep a strict record of all the errors observed in the preanalytical phase. Strict compliance to the corrective strategies can gradually reduce the error rates.

CONCLUSION

Compliance with good laboratory practices can significantly reduce the occurrence of preanalytical errors. Management of preanalytical errors needs involvement of the clinical domain for proper patient identification and test requisition, completely filling accompanying slips and sending proper samples for laboratory analysis, since many of the errors fall outside the physical boundaries of the laboratory. We recommend that there should be laboratory policy for error record keeping so that there should be a settlement in “laboratory sentinel events” covering the total testing process.

REFERENCES

1. Saurav Patra M, Mukherjee B and Das AK. Preanalytical errors in the clinical laboratory and how to minimize them. Int J Bioassays 2013;02:551-553.

2. Lima-Oliveira G, Volanski W, Lippi G, Picheth G and Guidi GC. Preanalytical phase management: a review of the procedures from patient preparation to laboratory analysis. Scand J Clin Lab Invest 2017;77:153–163.

3. Lippi G, Lima-Oliveira G, Brocco G, Bassi A and Salvagno GL. Estimating the intra- and inter-individual imprecision of manual pipetting. Clin Chem Lab Med 2017;55: 962–966.

4. Simundic AM and Lippi G. Preanalytical phase-a continuous challenge for laboratory professionals. Biochem Med 2012;22:145-149.

5. Lippi G, Plebani M and Simundic AM. Quality in laboratory diagnostics: From theory to practice. Biochem Med 2010;20:126–130.

6. Hammerling JA. A Review of Medical Errors in Laboratory Diagnostics and Where We Are Today. Lab Med 2012;43: 41–44.

7. Almatrafi AA. Preanalytical Errors: A Major Issue in Medical Laboratory. Acta Sci Med Sci 2019;3:93-95.

8. Giavarina D and Lippi G. Blood venous sample collection: Recommendations overview and a checklist to improve quality. Clin Biochem 2017;50:568-573.

9. Lima-Oliveira G, Lippi G, Salvagno GL, Picheth G and Guidi GC. Laboratory Diagnostics and Quality of Blood Collection. J Med Biochem 2015;34:288–294.

10. Lima-Oliveira G, Cesare Guidi G, Guimaraes AVP, Abol Correa J and Lippi G. Preanalytical Nonconformity Management Regarding Primary Tube Mixing in Brazil. J Med Biochem 2017;36:39–43.

11. Chawla R, Goswami B, Tayal D and Mallika V. Identification of the types of preanalytical errors in the clinical chemistry laboratory: 1 year study at GB Pant hospital. Lab Med 2010;41:89–92.

12. Gimenez-Marin A, Rivas-Ruiz F, Perez-Hidalgo Mdel M and Molina-Mendoza P. Preanalytical errors management in the clinical laboratory: a five year study. Biochem Med 2014;24:248-257.

13. Najat D. Prevalence of Preanalytical Errors in Clinical Chemistry Diagnostic Labs in Sulaimani City of Iraqi Kurdistan. PLoS ONE 2017;12:e0170211.

14. Simundic AM, Bölenius K, Cadamuro J,Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov H, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, De la Salle B, Seipelt C, Sumarac Z, Vermeersch P; Working Group for Preanalytical Phase (WG-PRE), of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin American Working Group for Preanalytical Phase (WG-PRE-LATAM) of the Latin America Confederation of Clinical Biochemistry (COLABIOCLI). Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56:2015-2038.

15. Arul P, Pushparaj M, Pandian K, Chennimalai L, Rajendran K, Selvaraj E and Masilamani S. Prevelence and types of preanalytical error in hematology laboratory of a tertiary care hospital in South India. J Lab Physicians 2018;10:237-240.

16. Lippi G, Bassi A, Brocco G, Montagnana M, Salvagno GL and Guidi GC. Preanalytic error tracking in a laboratory medicine department: results of a 1-year experience. Clin Chem 2006;52:1442-1443.

17. Green SF. The cost of poor blood specimen quality and errors in preanalytical processes. Clin Biochem 2013;46:1175-1179.

18. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G and Guidi GC. The effective reduction of tourniquet application time after minor modification of the CLSI H03-A6 blood collection procedure. Biochem Med 2013;23:308–315.

19. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G and Guidi GC. Impact of the phlebotomy training based on CLSI/NCCLS H03-a6-procedures for the collection of diagnostic blood specimens by venipuncture. Biochem Med 2012;22:342–351.

In this issue: Improving the preanalytical phase in laboratory medicine

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Can chewing gum be another source of preanalytical variability in fasting outpatients?

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ABSTRACT

Introduction

In the daily laboratory practice, there are patients coming to blood collection sites chewing sugar-free gum, considering it irrelevant to laboratory tests. The aim of this study was to evaluate whether a sugar-free chewing gum can interfere with laboratory tests.

Methods

We studied 22 healthy volunteers. After a 12-hour overnight fasting, the first blood sample was collected between 8:00 and 8:30 a.m. Then, immediately after the first venous blood collection, the subjects started chewing the gum (declared sugar-free) for 20 min. Subsequent venous blood samples were collected at 1, 2, and 4 hours after chewing the gum. Significant differences between samples were assessed by the Wilcoxon ranked-pairs test.

Results

Among all the results, statistically significant differences ($p < 0.05$) between basal and x hours after chewing

sugar-free gum were observed for the following parameters: cortisol, insulin, C-peptide, triglycerides, uric acid, urea, amylase, alanine aminotransferase, lipase, creatine kinase, total bilirubin, direct bilirubin, phosphate, iron, potassium, thyroid stimulating hormone, red blood cell count, hematocrit, hemoglobin, mean cell volume, red cell distribution width, white blood cell count, lymphocytes, neutrophils, and eosinophils; whereas, coagulation tests were not impacted by chewing sugar-free gum.

Conclusions

We recommend instructing the patients to avoid the use of chewing gum before blood collection for laboratory tests.

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INTRODUCTION

Chewing is essential during food consumption since it helps swallowing and food digestion. Moreover, Hirano et al. have shown that chewing is linked with cognitive functions (i.e., learning, memory and attention) [1]. Regarding the prolonged chewing consequent to chewing gum habit, it has some favorable outcomes such as: i) removal of food debris and plaque from teeth; ii) stimulation of saliva flow; and iii) reduction of gingivitis [2]. Moreover, chewing gum has been proven to stimulate the cephalic phase of gastric secretion on par with food chewing. The amount of acid output stimulated after 15 min of chewing the gum was very similar to the acid output after a cheeseburger meal [3]. It is well known that the hydrochloric acid secretion into the stomach is linked to:

1) extraction from blood plasma of Cl^-, Na^+, CO_2, and H_2O;

2) maintenance of blood electrolyte equilibrium through the release of HCO_3^- into the blood leaving the stomach (alkaline tide) [4].

Therefore, the fake feeding-induced gastric secretion due to chewing gum should be considered carefully when laboratory tests needing patient fasting are to be performed.

Currently, the management of patient preparation before laboratory testing requires attention since the evidence has shown that fasting time is required before blood sampling [5-7], except for lipid profile assessment [8, 9]. However, the GP 41, a standard for global application developed through the Clinical and Laboratory Standards Institute (CLSI) consensus process, recommends *"no chewing gum, or other objects (eg. thermometer) should be in the patient's mouth at the time the specimen is collected"* although no information is provided about allowance or denial regarding chewing gum habit before blood specimen collection [10]; whereas, a joint document from the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin America Confederation of Clinical Biochemistry (COLABIOCLI) states that "chewing gum should not be used" without any scientific evidence that it could be a source of laboratory variability [11].

In the daily laboratory practice, there are patients coming to blood collection sites chewing sugar-free gum, considering it irrelevant with respect to fasting status. Moreover, laboratory professionals lack evidence to define patient preparation regarding this issue. The aim of this study was to evaluate whether chewing sugar-free gum can interfere with laboratory tests.

MATERIALS AND METHODS

A total of 22 healthy volunteers (13 women and 9 men; average age was 31 (22-52) years) were selected from the personnel of the Center of Laboratory Medicine of the Vilnius University Hospital Santaros Klinikos and included in the study. Informed consent was obtained from all study subjects according to the 2013 Declaration

of Helsinki and the protocol was approved by the Ethic Committee.

After a 12-hour overnight fast, the first blood sample was collected between 8:00 and 8:30 a.m. Then, immediately after the first venous blood collection, the subjects started chewing 2.8 g of sugar-free chewing gum (Orbit Spearmint, Wrigley company, Plymouth, USA) for 20 minutes. The composition of the chewing gum is declared in Table 1. Subsequent venous blood samples were collected at 1, 2, and 4 hours after chewing sugar-free gum.

Table 1 Nutritional composition of sugar-free chewing gum

Nutritional composition	
overall weight (g)	2.8
KJ	17.8
Kcal	4.3
total carbohydrates (g)	1.8
proteins (g)	0
total lipids (g)	0

According to the CLSI GP 41 and the EFLM-COLABIOCLI recommendations [10, 11], all venous blood sampling procedures were carried out by a single phlebotomist. In order to eliminate possible blood distribution interferences, all volunteers were kept in an upright sitting position for 15 min [12-14]. Then, a vein was located on the forearm using a subcutaneous tissue transilluminator device (Venoscópio IV plus; Duan do Brasil, Brazil), in order to prevent venous stasis interference through tourniquet [15-18] and to avoid clenching [19].

All blood samples were collected directly into one 1.8 mL evacuated tube containing 3.2% sodium citrate 9N, one 3.5 mL evacuated tube containing gel separator and clot activator for serum samples, and one 4.0 mL evacuated tube containing K_2EDTA (Vacumed®, FL Medical, Torreglia, Italy) using a 20 gauge needle in a closed evacuated system (FL Medical, Torreglia, Italy). To eliminate any possible interference due to either the contact phase or tissue factor, approximately 2 mL of blood were preliminarily collected in a discard tube without additives (Vacumed®, FL Medical, Torreglia, Italy). The blood collection procedure was appropriately standardized in each phase, as already reported [20, 21], particularly regarding the sample processing, centrifugation and serum/plasma separation.

All the samples were assayed in a single analytical run in the same instrument according to the manufacturer's specifications and using proprietary reagents. The panel of tests that were performed and the instruments used by the Center of Laboratory Medicine of the Vilnius University Hospital Santaros Klinikos are shown in Table 2.

The instruments were calibrated against appropriate proprietary reference standard materials and verified with independent third-party control materials (Liquid Assayed Multiqual® Level 1 for routine biochemistry tests (Bio-Rad, California, USA) and Multichem IA Plus® Level 1 for immunochemistry assays (Technopath Clinical Diagnostics, Ballina, Ireland)) [22].

The evaluation of the within-run precision by the internal quality control of the instruments used in this study showed low coefficients of variation (Table 2).

Table 2 Results of within-run precision by the internal quality control used on the instruments

Part I: Clinical chemistry				
Instrument	**Test**	**Method**	**IQC assigned value**	**CVa (%)**
Arhitect ci8200, Abbott	Glc	enzymatic, Hexokinase / G-6-PDH, UV	3.13 mmol/L	1.2
	Cortisol	CMIA	127 nmol/L	7.4
	Ins	CMIA	17.1 pmol/L	6.7
	C peptide	CMIA	39.1 nmol/L	4.6
	CHOL	enzymatic, cholesterol oxidase / cholesterol esterase	2.90 mmol/L	0.5
	HDL	accelerator selective detergent, cholesterol oxidase / cholesterol esterase	0.74 mmol/L	2.5
	TG	enzymatic, glycerol phosphate oxidase	0.91 mmol/L	1.4
	TP	biuret	38.1 g/L	0.5
	Alb	bromocresol green, colorimetric	0.36 mmol/L	0.4
	UA	enzymatic, uricase	0.21 mol/L	6.8
	Urea	UV, urease	2.46 mmol/L	4.0
	CREA	enzimatic	0.08 mmol/L	4.1
	CRP	latex immunoturbidimetric	8.10 nmol/L	2.6
	ALP	p-nitrophenyl phosphate	34.2 U/L	2.8
	AMY-P	enzymatic, colorimetric	23.3 U/L	4.3
	AMY	CNPG3 substrate	45.4 U/L	1.6

	AST	IFCC, NADH, with P5P	40.6 U/L	7.3
	ALT	IFCC, NADH, with P5P	24.5 U/L	1.2
	GGT	L-Gamma-glutamyl-3-carboxy-4-nitroanilide substrate	29.5 U/L	3.2
	LD	IFCC, UV lactate-pyruvate	116 U/L	0.6
	Lip	quinone dye	20.6 U/L	8.2
	CK	N-acetyl-L-cysteine, NAC	83.5 U/L	4.8
	TBIL	diazonium salt	11.8 µmol/L	1.0
	DBIL	diazo reaction	5.82 µmol/L	7.8
	Phos	UV, phosphomolybdate	0.61 mmol/L	0.3
	Ca	arsenazo III, colorimetric	1.54 mmol/L	0.4
	Mg	enzymatic, isocitrate dehydrogenase	0.77 mmol/L	4.9
	Fe	ferene, colorimetric	12.2 µmol/L	4.0
	Na	ion-selective electrode, indirect	117 mmol/L	0.9
	K	ion-selective electrode, indirect	2.59 mmol/L	1.1
	Cl	ion-selective electrode, indirect	78.9 mmol/L	1.0
	TSH	CMIA	0.048 µIU/L	11.0
	fT4	CMIA	11.2 pmol/L	3.3
Part II: Hematology				
XN-1000, Sysmex	RBC	impedance	4.50 10^{12}/L	8.0
	Hct	calculated	0.47 L/L	0.3
	Hb	photometric	155 g/L	0.7
	MCV	calculated	88.5 fL	0.1

	RDW	calculated	15.2 %	0.5
	WBC	flourescent flow citometry	9.10 10^9/L	1.5
	Lympho	flourescent flow citometry	2.4 10^9/L	3.7
	Mono	flourescent flow citometry	0.8 10^9/L	4.1
	Neu	flourescent flow citometry	5.3 10^9/L	2.7
	Eos	flourescent flow citometry	0.6 10^9/L	17.3
	Baso	flourescent flow citometry	0.33 10^9/L	11.8
	IG	flourescent flow citometry	0.75 10^9/L	16.1
	Plt	impedance	245 10^9/L	2.2
	PDW	calculated	9.2 %	2.8
	MPV	calculated	9.8 fL	0.8
Part III: Coagulation				
STA Compact Max, Diagnostica Stago	PT	coagulometric, Owren	14.7 s	6.5
	APTT	coagulometric, silica	35.5 s	7.7
	Fbg	coagulometric, Clauss	2.9 g/L	11.1
	PC	chromogenic, Agkistrodon c. contortrix venom	96 %	7.3
	PS	latex immunoturbidimetric	62 %	12.6

IQC – internal quality control; CVa – analytical coefficient of variation; Glc – glucose; Ins – insulin; CHOL – cholesterol; HDL – high density lipoprotein; TG – triglycerides; TP – total protein; Alb – albumin; UA – uric acid; CREA – creatinine; CRP – C reactive protein; ALP – alkaline phosphatase; AMY-P – pancreatic amylase; AMY – amylase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; LD – lactate dehydrogenase; Lip – lipase; CK – creatine kinase; TBIL – total bilirubin; DBIL – direct bilirubin; Phos – phosphate; Ca – calcium; Mg – magnesium; Fe – iron; Na – sodium; K – potassium; Cl – chloride; TSH – thyroid stimulating hormone; fT4 – free thyroxin; RBC – red blood cells; Hct – haematocrit; Hb – haemoglobin; MCV – mean cell volume; RDW – red cell distribution width; WBC – white blood cells; Lympho – lymphocytes; Mono – monocytes; Neu – neutrophils; Eos – eosinophils; Baso – basophils; IG – immature granulocytes; Plt – platelets; PDW – platelet distribution width; MPV – mean platelet volume; PT – prothrombin time; APTT – activated partial thromboplastin time; Fbg – fibrinogen; PC – protein C; PS – protein S; UV – ultraviolet; CMIA – chemiluminescent microparticle immunoassay; NADPH - nicotinamide adenine dinucleotide phosphate; NAC – N-acetyl cysteine; IFCC – international federation of clinical chemistry and laboratory medicine.

For assessing the statistical differences between samples, the Wilcoxon ranked-pairs test was used in agreement with Simundic's [23] recommendations regarding sample size (i.e. less than 30), with a licensed statistical software (GraphPad Prism® version 5.01, La Jolla, CA, USA). The level of statistical significance was set at $p < 0.05$.

Presently there is a lack of harmonization on the preanalytical methodology for evaluating a single source of laboratory variability (e.g., impact of chewing gum). Researchers use different statistical tools to estimate bias and clinical significance: (i) bland-Altman analysis [24]; (ii) percentage mean difference [25] – which represents the difference between baseline and treatment values divided by the treatment value – the method we chose; and iii) Passing-Bablok regression with 95% confidence intervals [26].

To calculate the percentage mean difference, we decided to use the following formula:

mean % difference =
[(× h after chewing sugar-free gum − basal)/× h after chewing sugar-free gum] × 100%.

Using it we could avoid the possible "false positive source of variability" since it is more sensitive than:

mean % difference =
[(basal – x h after chewing sugar-free gum) / basal] × 100%.

In this way, only strong sources of preanalytical variability can be identified.

Finally, the mean % differences between blood samples taken 1, 2 and 4 hours after chewing sugar-free gum, were compared with the desirable specification for imprecision (DSI) derived from biologic variation [27]. We used DSI as criterium of acceptable level of interference by chewing gum in the laboratory tests; then interferograms were provided for each laboratory parameters with significant difference between h after chewing sugar-free gum and basal.

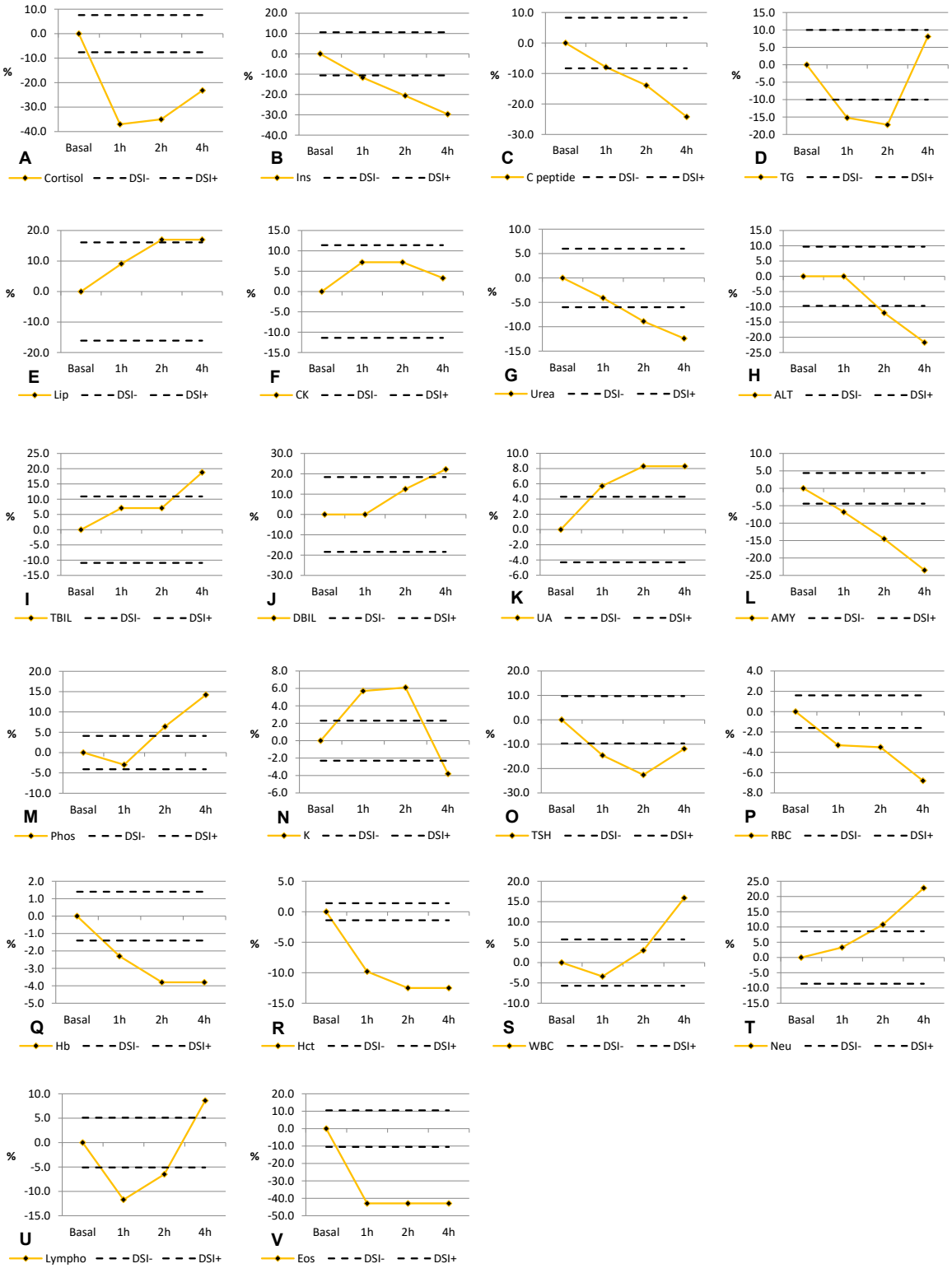
Briefly, in our study design, each volunteer was devised to be her/his own control (i.e. the results from 1, 2 and 4 hours after chewing sugar-free gum were compared with basal-results of the same individual). Indeed, this kind of study design – a case-crossover study – is most suitable for outcomes where the induction time is short [28], like our evaluation of impact of chewing gum on laboratory test results. In fact, in a case-crossover study, only cases showing discordant exposure status in the case/control window-of-time contribute to the effect and thus to the measure estimation. Because cases and controls are the same individuals, the problem of between-person confounders—that anyway exists with a control group—being constant for the characteristics, do not occur [29]. Therefore, our design minimizes the variability that could jeopardize the preanalytical evaluation.

RESULTS

The results of the laboratory tests are presented as median [interquartile range] in Table 3.

Among all the results, statistically significant differences between basal and x h after chewing sugar-free gum were observed for the following parameters: cortisol, insulin, C peptide, triglycerides, uric acid, urea, amylase, alanine aminotransferase, lipase, creatine kinase, total bilirubin, direct bilirubin, phosphate, iron, potassium, thyroid stimulating hormone, red blood cell count, hematocrit, hemoglobin, mean cell volume, red cell distribution width, white blood cell count, lymphocytes, neutrophils, and eosinophils; whereas, coagulation tests were not impacted by chewing sugar-free gum (Figure 1).

Figure 1 **Interferograms**



Legend follows on next page.

Legend of Figure 1:
A, cortisol; B, Ins – insulin; C, C-peptide; D, TG – triglycerides; E, Lip – lipase; F, CK – creatine kinase; G, urea; H, ALT – alanine aminotransferase; I, TBIL – total bilirubin; J, DBIL – direct bilirubin; K, UA – uric acid; L, MY – amylase; M, Phos – phosphate; N, K – potassium; O, TSH – thyroid stimulating hormone; P, RBC – red blood cells; Q, Hb – haemoglobin; R, Hct – haematocrit; S, WBC – white blood cells; T, Neu – neutrophils; U, Lympho – lymphocytes; and V, Eos – eosinophils
Hours after the chewing the gum (x-axis) are plotted against bias values (y-axis). Solid line – bias. Dashed lines - acceptable criteria based on desirable specification for imprecision (DSI) derived from biologic variation.

Table 3 **Laboratory test results variation after chewing sugar-free gum**

Part I: Clinical chemistry					
Test	**Unit**	**Basal**	**1h**	**2h**	**4h**
Glc	mmol/L	4.61 [4.30 - 4.78]	4.60 [4.31 - 4.77] 0.758	4.51 [4.43 - 4.84] 0.646	4.49 [4.32 - 4.71] 0.268
Cortisol	nmol/L	290 [219 - 383]	212 [149 - 279] **0.002**	215 [183 - 290] **0.003**	235 [172 - 292] **0.018**
Ins	pmol/L	41.0 [30.6 - 67.9]	36.7 [24.9 - 51.8] **0.002**	34.0 [24.5 - 55.1] **0.004**	31.6 [22.0 - 42.9] **0.001**
C peptide	nmol/L	0.41 [0.36 - 0.62]	0.38 [0.33 - 0.56] **0.003**	0.36 [0.30 - 0.53] **0.002**	0.33 [0.26 - 0.46] **<0.001**
CHOL	mmol/L	4.68 [4.34 - 5.58]	5.59 [4.32 - 4.46] 0.641	5.60 [4.24 - 5.48] 0.673	4.68 [4.37 - 5.48] 0.962
HDL	mmol/L	1.59 [1.38 - 1.96]	1.58 [1.35 - 1.92] 0.972	1.59 [1.33 - 1.90] 0.822	1.57 [1.32 - 1.88] 0.511
TG	mmol/L	0.68 [0.54 - 0.83]	0.59 [0.48 - 0.87] **0.017**	0.58 [0.50 - 0.86] **0.005**	0.74 [0.57 - 0.88] **0.001**
TP	g/L	76.3 [71.8 - 77.9]	75.4 [72.0 - 77.7] 0.788	75.3 [71.8 - 78.1] 0.714	75.5 [72.8 - 80.4] 0.754

Alb	g/L	46.3 [44.5 - 48.0]	46.5 [44.7 - 47.3] 0.651	46.4 [43.6 - 49.8] 0.642	46.5 [45.7 - 48.0] 0.653
UA	mmol/L	0.33 [0.25 - 0.39]	0.35 [0.27 - 0.41] **0.009**	0.36 [0.27 - 0.42] **0.005**	0.36 [0.27 - 0.42] **0.002**
Urea	mmol/L	4.68 [3.75 - 5.79]	4.50 [3.70 - 5.64] **0.001**	4.30 [3.66 - 5.51] **0.001**	4.16 [3.68 - 5.46] **0.001**
CREA	mmol/L	0.08 [0.06 - 0.09]	0.08 [0.07 - 0.09] 0.876	0.08 [0.07 - 0.09] 0.829	0.08 [0.07 - 0.09] 0.798
CRP	nmol/L	4.76 [2.86 - 5.81]	4.67 [2.76 - 6.19] 0.729	4.86 [2.76 - 6.67] 0.851	4.76 [2.86 - 6.19] 0.892
ALP	U/L	56 [48 - 66]	57 [47 - 62] 0.876	57 [49 - 62] 0.841	57 [47 - 61] 0.865
AMY-P	U/L	27 [21 - 35]	27 [21 - 30] 0.823	27 [20 - 31] 0.888	27 [21 - 31] 0.891
AMY	U/L	63 [55 - 80]	59 [49 - 78] **0.002**	55 [45 - 78] **0.001**	51 [40 - 68] **0.001**
AST	U/L	23 [17 - 29]	22 [20 - 28] 0.751	23 [19 - 29] 0.704	22 [19 - 30] 0.781
ALT	U/L	28 [17 - 38]	28 [16 - 37] 0.979	25 [16 - 32] **0.021**	23 [15 - 30] **0.002**
GGT	U/L	17 [10 - 22]	17 [10 - 23] 0.978	17 [10 - 24] 0.813	17 [10 - 24] 0.838

LD	U/L	185 [161 - 200]	187 [163 - 201] 0.439	186 [160 - 203] 0.483	186 [160 - 199] 0.481
Lip	U/L	20 [17 - 23]	22 [16 - 22] **0.002**	24 [18 - 26] **0.001**	24 [18 - 27] **0.001**
CK	U/L	116 [71 - 130]	125 [79 - 145] **0.002**	125 [78 - 146] **0.001**	120 [80 -138] **0.021**
TBIL	µmol/L	11.1 [8.55 - 12.3]	12.0 [8.38 - 13.7] 0.091	12.0 [8.21 - 14.0] 0.083	13.7 [10.6 - 16.2] **0.002**
DBIL	µmol/L	4.79 [3.59 - 5.64]	4.79 [3.76 - 5.64] 0.784	5.47 [4.10 - 6.16] **0.002**	6.17 [4.28 - 6.84] **0.001**
Phos	mmol/L	1.03 [0.94 - 1.23]	1.00 [0.97 - 1.17] **0.035**	1.10 [1.00 - 1.21] **0.036**	1.20 [1.10 - 1.23] **0.002**
Ca	mmol/L	2.43 [2.42 - 2.46]	2.44 [2.40 - 2.47] 0.829	2.44 [2.39 - 2.47] 0.794	2.44 [2.37 - 2.48] 0.817
Mg	mmol/L	0.83 [0.79 - 0.86]	0.83 [0.79 - 0.84] 0.912	0.83 [0.79 - 0.84] 0.897	0.83 [0.80 - 0.84] 0.849
Fe	µmol/L	18.1 [12.4 - 23.3]	18.3 [12.2 - 23.1] **0.046**	18.3 [12.5 - 23.5] **0.047**	19.5 [14.7 - 23.3] **0.005**
Na	mmol/L	140 [139 - 141]	140 [140 - 141] 0.887	140 [138 - 140] 0.732	141 [140 - 143] 0.481
K	mmol/L	4.15 [3.91 - 4.22]	4.40 [4.21 - 4.48] **0.003**	4.42 [4.20 - 4.40] **0.004**	4.00 [3.89 - 4.18] **0.023**

Cl	mmol/L	106 [104 - 106]	106 [104 - 107] 0.910	106 [105 - 107] 0.931	106 [104 - 107] 0.914
TSH	µIU/L	1.41 [0.94 - 2.10]	1.23 [0.84 - 1.81] **<0.001**	1.15 [0.93 - 1.71] **<0.001**	1.26 [0.95 - 1.85] **<0.001**
fT4	pmol/L	12.9 [11.7 - 13.5]	12.9 [12.0 - 13.8] 0.752	12.7 [11.7 - 13.6] 0.801	12.7 [11.9 - 13.9] 0.842
Part II: Hematology					
RBC	10^{12}/L	4.70 [4.33 - 5.20]	4.55 [4.29 - 5.03] **0.001**	4.54 [4.20 - 5.02] **0.001**	4.40 [4.25 - 4.90] **0.001**
Hct	L/L	0.45 [0.40 - 0.46]	0.41 [0.37 - 0.46] **0.001**	0.40 [0.38 - 0.45] **0.001**	0.40 [0.36 - 0.45] **0.001**
Hb	g/L	135 [126 - 149]	132 [122 - 147] **0.001**	130 [122 - 148] **0.001**	130 [121 - 147] **0.001**
MCV	fL	90.8 [87.3 - 92.0]	90.0 [86.8 - 91.4] **0.001**	89.1 [86.0 - 90.6] **0.001**	87.9 [85.0 - 90.1] **0.001**
RDW	%	44.0 [41.0 - 46.0]	43.0 [40.2 - 45.9] **0.001**	42.1 [40.0 - 43.9] **<0.001**	41.0 [39.3 - 44.2] **<0.001**
WBC	10^{9}/L	5.20 [4.01 - 5.74]	5.03 [4.18 - 5.72] 0.548	5.36 [4.42 - 6.01] 0.062	6.18 [5.11 - 6.98] **0.007**
Lympho	10^{9}/L	1.81 [1.40 - 1.93]	1.62 [1.51 - 1.80] **<0.001**	1.70 [1.52 - 2.03] **<0.001**	1.98 [1.78 - 2.31] **<0.001**

Mono	10^9/L	0.41 [0.28 - 0.54]	0.41 [0.29 - 0.50] 0.618	0.38 [0.29 - 0.47] 0.714	0.45 [0.31 - 0.53] 0.839
Neu	10^9/L	2.64 [1.98 - 3.10]	2.73 [2.20 - 3.32] **0.041**	2.96 [2.44 - 3.41] **0.031**	3.42 [2.63 - 4.10] **0.001**
Eos	10^9/L	0.10 [0.06 - 0.14]	0.07 [0.05 - 0.12] **0.001**	0.07 [0.05 - 0.10] **0.001**	0.07 [0.04 - 0.10] **0.001**
Baso	10^9/L	0.04 [0.02 - 0.04]	0.04 [0.02 - 0.04] 0.792	0.04 [0.02 - 0.04] 0.859	0.04 [0.03 - 0.04] 0.817
IG	10^9/L	0.01 [0.00 - 0.01]	0.01 [0.01 - 0.01] 0.759	0.01 [0.00 - 0.02] 0.893	0.01 [0.01 - 0.02] 0.848
Plt	10^9/L	228 [204 - 269]	226 [204 - 268] 0.818	225 [204 - 267] 0.836	229 [206 - 269] 0.891
PDW	%	11.2 [10.0 - 12.0]	11.5 [9.74 - 12.1] 0.737	11.7 [9.98 - 12.4] 0.761	11.4 [10.0 - 12.1] 0.729
MPV	fL	10.0 [9.28 - 10.3]	10.0 [9.35 - 10.2] 0.751	10.1 [9.50 - 10.1] 0.849	10.1 [9.51 - 10.2] 0.772
Part III: Coagulation					
PT	s	13.0 [12.6 - 13.2]	13.0 [12.7 - 13.3] 0.618	13.1 [12.8 - 13.4] 0.684	13.1 [12.7 - 13. 3] 0.679
APTT	s	33.1 [32.2 - 36.0]	33.2 [32.1 - 35.9] 0.841	33.0 [31.6 - 36.5] 0.973	33.1 [32.0 - 35.2] 0.897

Fbg	g/L	2.94 [2.53 - 3.12]	2.88 [2.50 - 3.15] 0.529	2.89 [2.41 - 3.22] 0.671	2.89 [2.42 - 3.16] 0.541
PC	% activity	102 [89.3 - 120]	100 [89.7 - 119] 0.638	100 [89.9 - 119] 0.699	101 [90.3 - 117] 0.594
PS	% activity	88.3 [75.5 - 111]	87.8 [75.2 - 111] 0.482	88.2 [76.2 - 112] 0.875	88.7 [76.1 - 111] 0.897

Results are presented as median [interquartile range]. Statistically significant differences (P < 0.05) are presented in bold. Glc – glucose; Ins – insulin; CHOL – cholesterol; HDL – high density lipoprotein; TG – triglycerides; TP – total protein; Alb – albumin; UA – uric acid; CREA – creatinine; CRP – C reactive protein; ALP – alkaline phosphatase; AMY-P – pancreatic amylase; AMY – amylase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; LD – lactate dehydrogenase; Lip – lipase; CK – creatine kinase; TBIL – total bilirubin; DBIL – direct bilirubin; Phos – phosphate; Ca – calcium; Mg – magnesium; Fe – iron; Na – sodium; K – potassium; Cl – chloride; TSH – thyroid stimulating hormone; fT4 – free thyroxin; RBC – red blood cells; Hct – haematocrit; Hb – haemoglobin; MCV – mean cell volume; RDW – red cell distribution width; WBC – white blood cells; Lympho – lymphocytes; Mono – monocytes; Neu – neutrophils; Eos – eosinophils; Baso – basophils; IG – immature granulocytes; Plt – platelets; PDW – platelet distribution width; MPV – mean platelet volume; PT – prothrombin time; APTT – activated partial thromboplastin time; Fbg – fibrinogen; PC – protein C; PS – protein S.

DISCUSSION

Our primary hypothesis was that chewing gum – even sugar free – could impact the glucose metabolism. However, the results of glucose, cortisol, insulin and C peptide levels properly evidenced that chewing gum lacks influence on glucose results (Table 3); the variability observed in cortisol, insulin and C peptide (Table 3, Figure 1a, 1b, 1c) could mirror cortisol circadian cycle [30] (since the first blood collection was at 8 am), and life cycle of these hormones [31]. Studies have shown that the insulin half-life varies between 4.3 and 9.8 min, whereas for C peptide the half-life could vary between 11.1 and 33.5 min [32-35]. Therefore, our insulin and C peptide results could mirror the prolonged fasting.

The chewing gum producer declares the following chemical constituents of the product on the pack label: a) sweeteners: E967 – xylitol, E420 – sorbitol, E951 – aspartame, E421 – mannitol, E950 – acesulfame potassium, and E955 – sucralose; b) moisture additive: E422 – glycerol; c) thickener: E414 – gum Arabic; d) emulsifier: soy lecithin; e) food colouring: E171 – titanium dioxide; f) filler: E903 – carnauba wax; g) antioxidant: E320 – butylated hydroxyanisole. However, the chewing gum producer do not specify the quantities of mentioned constituents, furthermore, they do not specify the base of the gum (i.e., it is not declared on the gum package). Probably it is a mixture of resins, elastomers, fillers, and plasticizers [2].

Regarding lipids profile, total cholesterol and HDL cholesterol were not affected neither by prolonged fasting time [8] nor chewing gum (Table 3). However, the decrease in triglycerides one and two hours after chewing gum can mirror the gluconeogenesis and beta oxidation process, since volunteers had fasted for 13 and

14 hours prior to blood collections, respectively. However, four hours after chewing gum the triglycerides concentration increased and returned almost up to the basal levels (Table 3, Figure 1d). Since glycerol is the moisture additive declared by the chewing gum producer (Table 1), it could be absorbed and provide positive bias in our enzymatic, glycerol phosphate oxidase method for triglyceride test. Moreover, the methodology of *quinone dye* is used on Architect to determine lipase activity. Briefly, lipase acts on a natural substrate, 1,2-diglyceride, to liberate 2-monoglyceride. This is hydrolyzed by monoglyceride lipase into glycerol and free fatty acid. Glycerol kinase acts on glycerol to form glycerol-3-phosphate which is in turn acted on by glycerol-3-phosphate oxidase to generate hydrogen peroxide. Peroxidase converts the hydrogen peroxide, 4-aminoantipyrine, and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) into a quinone dye. Thus, the rate of formation of the dye is measured as an increase in absorbance at 548 nm and is proportional to the lipase activity in the sample. Therefore, the glycerol from sugar-free chewing gum could potentially jeopardize lipase results (Table 3, Figure 1e).

The oxidation of NADPH by the radical of butylated hydroxyanisole (BHA, present in the chewing gum) was previously demonstrated [36, 37]. The short decryptions of the laboratory methods below could explain the variability observed in CK, urea, and ALT (Table 3, Figure 1f, 1g, 1h).

- CK: in N-acetyl-L-cysteine (NAC) methodology, the creatine kinase, present in the sample, catalyzes the transfer of a high energy phosphate group from creatine phosphate to ADP. The ATP produced in this reaction is subsequently used to phosphorylate glucose to produce glucose-6-phosphate (G-6-P) in the presence of hexokinase. G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with the concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduced (NADPH). The rate of formation of NADPH is monitored at 340 nm and is proportional to the activity of CK in the sample. These reactions occur in the presence of N-acetyl-L-cysteine (NAC) which is present as an enzyme reactivator;
- Urea: in urease methodology, two moles of NADH are oxidized for each mole of urea present. The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample;
- ALT: in IFCC methodology, the pyruvate in the presence of NADH and lactate dehydrogenase is reduced to L-lactate. In this reaction NADH is oxidized to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD.

Moreover, Vandghanooni et al. experimentally demonstrated that slight concentration of butylated hydroxyanisole – lower than expected from human exposure to antioxidants in food products, i.e. chewing gum – inhibits the growth rate of cells by inducing apoptosis via chromatin and DNA fragmentation [38]. Furthermore, the xylitol in the sugar-free chewing gum could be responsible for the increase observed in bilirubin, and uric acid [39, 40], and decrease in amylase [41] (Table 3, Figure 1i, 1j, 1k, 1l).

Similar results on phosphate, potassium and TSH (Table 3, Figure 1m, 1n, 1o) were demonstrated by Bajaña et al. [5] on volunteers after breakfast. Therefore, a question remains partially unanswered “Is the chewing gum able to activate digestive pathway?”. Regarding Smith et al. [4] chewing gum is, at least partially, able to stimulate the initial phases of gastric secretion that involve essentially water and ions’ movements. Our results seem to support the relative effect of haemoconcentration as deduced by red blood cell count, haemoglobin and

hematocrit decrease, an effect that appears reversible with time according to the physiological process of digestion (Table 3, Figure 1p, 1q, 1r).

Moreover, fake feeding-induced gastric secretion due to chewing gum is probably responsible for the white blood cell count and neutrophils increase, with lymphocytes and eosinophils decrease observed in our study (Table 3, Figure 1s, 1t, 1u, 1v) as reported by Koscielniak et al. after a normal meal [42]. On the contrary the sugar-free chewing gum does not have impact on routine coagulation tests (Table 3).

In conclusion, based on the variability evidenced above, we recommend instructing the patients to avoid the use of chewing gum before blood collection for laboratory tests.

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REFERENCES

1. Hirano Y, Onozuka M. Chewing and attention: a positive effect on sustained attention. Biomed Res Int 2015;2015: 367026.

2. Imfeld T. Chewing gum--facts and fiction: a review of gum-chewing and oral health. Crit Rev Oral Biol Med 1999;10: 405-19.

3. Helman CA. Chewing gum is as effective as food in stimulating cephalic phase gastric secretion. Am J Gastroenterol 1988;83:640-2.

4. Smith ME, Morton DG. The Digestive System: Systems of the Body Series. UK: Elsevier Health Sciences; 2011.

5. Bajaña W, Aranda E, Arredondo ME, Brennan-Bourdon LM, Campelo MD, Espinoza E, Flores S, Ochoa P, Vega V, Varela B, Lima-Oliveira G. Impact of an Andean breakfast on biochemistry and immunochemistry laboratory tests: an evaluation on behalf COLABIOCLI WG-PRE-LATAM. Biochem Med (Zagreb) 2019;19:020702.

6. Guidi GC, Simundic AM, Salvagno GL, Aquino JL, Lima-Oliveira G. To avoid fasting time, more risk than benefits. Clin Chem Lab Med 2015;53:e261-4.

7. Arredondo ME, Aranda E, Astorga R, Brennan-Bourdon LM, Campelo MD, Flores S, Medel C, Manriquez I, Ochoa P, Varela B, Salinas CV, Lima-Oliveira G. Breakfast can Affect Routine Hematology and Coagulation Laboratory Testing: An Evaluation on Behalf of COLABIOCLI WG-PRE-LATAM. TH Open 2019;3:e367-e76.

8. Nordestgaard BG, Langsted A, Mora S, Kolovou G, Baum H, Bruckert E, Watts GF, Sypniewska G, Wiklund O, Boren J, Chapman MJ, Cobbaert C, Descamps OS, von Eckardstein A, Kamstrup PR, Pulkki K, Kronenberg F, Remaley AT, Rifai N, Ros E, Langlois M, European Atherosclerosis S, the European Federation of Clinical C, Laboratory Medicine Joint Consensus I. Fasting Is Not Routinely Required for Determination of a Lipid Profile: Clinical and Laboratory Implications Including Flagging at Desirable Concentration Cutpoints-A Joint Consensus Statement from the European Atherosclerosis Society and European Federation of Clinical Chemistry and Laboratory Medicine. Clin Chem 2016;62:930-46.

9. Lima-Oliveira G, Valentim CD, Guidi GC. Lipid profile, changes in laboratory prescriptions are necessary. J Clin Lipidol 2017;11:768-9.

10. Clinical Laboratory Standards Institute. Procedures for collection of diagnostic blood specimens by venipuncture. CLSI GP41. Wayne, PA.: Clinical Laboratory Standards Institute.

11. Simundic AM, Bolenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov A, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, Salle B, Seipelt C, Sumarac Z, Vermeersch P. Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56: 2015-38.

12. Lippi G, Salvagno GL, Lima-Oliveira G, Brocco G, Danese E, Guidi GC. Postural change during venous blood collection is a major source of bias in clinical chemistry testing. Clin Chim Acta 2015;440:164-8.

13. Lippi G, Salvagno GL, Lima-Oliveira G, Danese E, Favaloro EJ, Guidi GC. Influence of posture on routine hemostasis testing. Blood Coagul Fibrinolysis 2015;26:716-9.

14. Lima-Oliveira G, Guidi GC, Salvagno GL, Danese E, Montagnana M, Lippi G. Patient posture for blood collection by venipuncture: recall for standardization after 28 years. Rev Bras Hematol Hemoter 2017;39:127-32.

15. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Manguera CL, Sumita NM, Picheth G, Guidi GC, Scartezini M. New ways to deal with known preanalytical issues: use of transilluminator instead of tourniquet for easing vein access and eliminating stasis on clinical biochemistry. Biochem Med (Zagreb) 2011;21:152-9.

16. Lima-Oliveira G, Lippi G, Salvagno GL, Gaino S, Poli G, Gelati M, Picheth G, Guidi GC. Venous stasis and whole blood platelet aggregometry: a question of data reliability and patient safety. Blood Coagul Fibrinolysis 2015;26: 665-8.

17. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Scartezini M, Guidi GC, Picheth G. Transillumination: a new tool to eliminate the impact of venous stasis during the procedure for the collection of diagnostic blood specimens for routine haematological testing. Int J Lab Hematol 2011;33: 457-62.

18. Lima-Oliveira G, Salvagno GL, Lippi G, Montagnana M, Scartezini M, Picheth G, Guidi GC. Elimination of the venous stasis error for routine coagulation testing by transillumination. Clin Chim Acta 2011;412:1482-4.

19. Lima-Oliveira G, Guidi GC, Salvagno GL, Brocco G, Danese E, Lippi G. Estimation of the imprecision on clinical chemistry testing due to fist clenching and maintenance during venipuncture. Clin Biochem 2016;49:1364-7.

20. Lima-Oliveira G, Salvagno GL, Lippi G, Gelati M, Montagnana M, Danese E, Picheth G, Guidi GC. Influence of a regular, standardized meal on clinical chemistry analytes. Ann Lab Med 2012;32:250-6.

21. Lima-Oliveira G, Salvagno GL, Lippi G, Danese E, Gelati M, Montagnana M, Picheth G, Guidi GC. Could light meal jeopardize laboratory coagulation tests? Biochem Med (Zagreb) 2014;24:343-9.

22. Lima-Oliveira G, Lippi G, Salvagno G, Brocco G, Guidi GC. In vitro diagnostic company recalls and medical laboratory practices: an Italian case. Biochem Med (Zagreb) 2015;25: 273-8.

23. Simundic AM. Practical recommendations for statistical analysis and data presentation in Biochemia Medica journal. Biochem Med (Zagreb) 2012;22:15-23.

24. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307-10.

25. Ialongo C, Bernardini S. Preanalytical investigations of phlebotomy: methodological aspects, pitfalls and recommendations. Biochem Med (Zagreb) 2017;27:177-91.

26. Passing H, Bablok. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. J Clin Chem Clin Biochem 1983;21:709-20.

27. Westgard J. Desirable Biological Variation Database Specifications http://www.westgard.com/biodatabase1.htm 2014 [Accessed June 12th 2019].

28. Nazari SSH, Mansournia MA. Case-crossover and case-time-control studies: concepts, design, and analysis. J Biostat Epidemiol 2015;1:59-69.

29. Jensen AK, Gerds TA, Weeke P, Torp-Pedersen C, Andersen PK. On the validity of the case-time-control design for autocorrelated exposure histories. Epidemiology 2014;25:110-3.

30. Rose AJ, Herzig S. Metabolic control through glucocorticoid hormones: an update. Mol Cell Endocrinol 2013;380:65-78.

31. Hoekstra JB, van Rijn HJ, Erkelens DW, Thijssen JH. C-peptide. Diabetes Care 1982;5:438-46.

32. Faber OK, Hagen C, Binder C, Markussen J, Naithani VK, Blix PM, Kuzuya H, Horwitz DL, Rubenstein AH, Rossing N. Kinetics of human connecting peptide in normal and diabetic subjects. J Clin Invest 1978;62:197-203.

33. Kuzuya T, Matsuda A. Disappearance rate of endogenous human C-peptide from blood. Diabetologia 1976;12:519-21.

34. Sonksen PH, Tompkins CV, Srivastava MC, Nabarro JD. A comparative study on the metabolism of human insulin and porcine proinsulin in man. Clin Sci Mol Med 1973;45:633-54.

35. Starr JI, Rubenstein AH. Metabolism of endogenous proinsulin and insulin in man. J Clin Endocrinol Metab 1974;38:305-8.

36. Castor LR, Locatelli KA, Ximenes VF. Pro-oxidant activity of apocynin radical. Free Radic Biol Med 2010;48:1636-43.

37. Babu S, Uppu S, Claville MO, Uppu RM. Prooxidant actions of bisphenol A (BPA) phenoxyl radicals: implications to BPA-related oxidative stress and toxicity. Toxicol Mech Methods 2013;23:273-80.

38. Vandghanooni S, Forouharmehr A, Eskandani M, Barzegari A, Kafil V, Kashanian S, Ezzati Nazhad Dolatabadi J. Cytotoxicity and DNA fragmentation properties

of butylated hydroxyanisole. DNA Cell Biol 2013;32: 98-103.

39. Korttila K, Mattila MA. Increased serum concentrations of lactic, pyruvic and uric acid and bilibubin after postoperative xylitol infusion. Acta Anaesthesiol Scand 1979;23:273-7.

40. Schumer W. Adverse effects of xylitol in parenteral alimentation. Metabolism 1971;20:345-7.

41. Chukwuma CI, Islam MS. Effects of xylitol on carbohydrate digesting enzymes activity, intestinal glucose absorption and muscle glucose uptake: a multi-mode study. Food Funct 2015;6:955-62.

42. Koscielniak BK, Charchut A, Wojcik M, Sztefko K, Tomasik PJ. Impact of Fasting on Complete Blood Count Assayed in Capillary Blood Samples. Lab Med 2017; 48: 357-61.

In this issue: Improving the preanalytical phase in laboratory medicine

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Sample management: stability of plasma and serum on different storage conditions

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ABSTRACT

Background and objective

The analytes stability on serum and plasma are critical for clinical laboratory, especially if there is a delay in their processing or if they need to be stored for future research. The objective of this research was to determine the stability of K_3EDTA-plasma and serum on different storage conditions.

Materials and methods

A total of thirty healthy adults were studied. The serum/plasma samples were centrifuged at 2000g for 10 minutes. Immediately after centrifugation, the serum/plasma analytes were assayed in primary tubes using a Cobas c501 analyzer (T0); the residual serum/plasma was stored at either 2-8°C or -20°C for 15 (T15) and 30 days (T30).

Mean concentrations changes in respect of initial concentrations (T0) and the reference change values were calculated. For assessing statistical difference between samples, the Wilcoxon ranked-pairs test was applied.

Results

We evidenced instability for total bilirubin, uric acid, creatinine and glucose at T15 and T30 and stored at -20°C ($p<0.05$). However, potential clinical impact significance were observed only for total bilirrubin T30 at -20°C, and creatinine T30 at 2-8°C.

Conclusions

Our results had shown that storage samples at -20°C is a better way to preserve glucose, creatinine, and uric acid. Therefore, laboratories should freeze their samples as soon as possible to guarantee proper stability when there is need to repeat analysis, verify a result, or add a laboratory testing.

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INTRODUCTION

Since the 1960s, the study of analyte stability has been considered a critical aspect for laboratory medicine, since variations due to lack or reduced stability reflect on the results of laboratory tests employed for taking clinical or therapeutic decisions for patients (1,2).

The stability of an analyte in clinical biochemistry can be defined as "the space of time in which it maintains its value within established limits, by storing the sample in which the analytes are analyzed under certain specific conditions" (3,4). Currently, between 60-80% of medical decisions are based on the results obtained by the laboratory (5).

Moreover, it is well known that the pre-analytical phase is most susceptible to errors possibly leading to the unexpected variations of laboratory testing in biological samples; indeed, it is considered responsible for approximately 70% of the total errors in clinical laboratories (6).

Laboratories should use to quantify the analytes fresh serum or plasma in order to avoid degradation. However, the use or re-use of primary samples previously obtained from patients can be needed in the following situations:

i) due to delay in the analysis procedure;

ii) to confirm or to check a previously obtained value;

iii) to add new quantifications of missing analytes.

Therefore, this may lead to false concentration measurements (7), in case of uncontrolled storage conditions. On other occasions, the samples are transferred from more or less distant places, and under different storage conditions until the moment of the analysis; which several times do not correspond to quality standards (i.e., temperature control) (8,9). All of this causes the matrix analysis to undergo changes or degradation processes, which can be cumulative over time and possibly leading to alterations of original sample results (10).

Prolonged contact of plasma or serum with cells is a common cause of variability on test results (11). Plasma and serum should preferably be separated from cells as quickly as possible to avoid the ongoing metabolism of cellular constituents, as well as the active and passive movements of analytes between plasma or serum and cell compartments (12) This study was planned to verify the stability of K_3EDTA-plasma and serum on different storage conditions.

MATERIALS AND METHODS

Blood samples were obtained from 30 healthy adult laboratory workers (20 women and 10 men; average age was 33 [21-50] years), after signing informed consent. The inclusion criteria were not to suffer from infectious or chronic diseases, not to be on antibiotic treatment or any other medication for any acute illness. Moreover, 12 hour overnight fasting was required (13, 14). Blood samples where obtained in the morning

Table 1 Results of within-run precision by internal quality control

Tests	Method	IQC assigned values	CVa (%)	CVi (%)
Glucose	Enzymatic Colorimetric – Hexokinase	5.78 mmol/L	2.2	5.6
Creatinine	Kinetic Colorimetric Assay - Jaffé (2-compensated method)	90.5 µmol/L	4.6	5.9
Uric acid	Enzymatic colorimetric test - Uricase –Peroxidase	269 mmol/L	1.5	8.6
Total Bilirubin	Colorimetric assay – DPD (Diazonium salt)	17.8 µmol/L	4.0	21.8
Direct Bilirubin	Colorimetric assay – DPD (Diazo reaction)	17.1 µmol/L	3.2	36.8

by two phlebotomists with a puncture of the anterior bend of the elbow using the multiple extraction device (Vacuette®), Vacuette® 21G needle: 2 evacuated tubes with K3EDTA (4 mL), and 2 with clot activator and gel separator (8 mL), for plasma and serum collection respectively; samples were mixed by gently inversion (15). The tubes were placed vertically in a rack for 30 minutes at room temperature (24 °C) and then centrifuged at 2000 g for 10 minutes. All samples were not hemolyzed, icteric or lipemic.

In the primary serum and K_3EDTA-plasma tubes (T0, used for reference measurement), five clinical chemistry analytes – glucose, uric acid, creatinine, direct bilirubin and total bilirubin – were quantified in duplicate on Cobas® c501 autoanalyzer from Roche Diagnostics (Germany). The equipment was previously calibrated and controlled according to our own analytical quality assurance procedure established by the laboratory's management system in NetLab (Synlab Ecuador) Accredited Specialty Laboratory under ISO 15189 standards (Table 1) (16).

Serum and plasma samples were aliquoted in sterile plastic tubes with lids. Two aliquots of serum and two aliquots of plasma were stored at 2-8°C and two aliquots of serum and two aliquots of plasma were stored at -20°C. Therefore, four aliquots per patient and per matrix were stored for 15 days (T15) and 30 days (T30), this process prevents freeze-thawing of the samples in each analysis. Moreover, all aliquots were protected from light. Prior to the experimental stability measurements, the aliquots were placed at room temperature (24 °C) for 45 minutes to thaw and then the five biochemical parameters in serum and plasma were analyzed at the same time, with the same methodology and under the same conditions as the reference measurement (T0).

The mean and standard deviation were calculated for each analyte. The coefficient of variation (CVa%) of each analyte was the cumulative of the internal quality control data (last six months) (17,18), using third-party control materials-independent from calibrator materials-either (19). For assessing statistical difference between samples, the Wilcoxon ranked-pairs test was used. Difference % mean was calculated using the formula:

$$(T_x - T_0)/(T_0)\,x100\ \%$$

T_0: Initial median value

T_x: Median value of the measured values at T15 or T30

Finally, the reference change value (RCV) was applied for the analysis of the potential clinical impact.

The following formula was used for its calculation:

$$RCV = 2^{1/2} \times Z \times \left(CVa^2 + CVi^2\right)^{1/2}$$

It corresponds to the square root of 2, the statistic Z = 1.9 (obtained from the normal distribution table, with a 95% confidence) and the coefficient of intra-individual biological variation (CVi) of the analyte being assayed (20). The last factor is the laboratory's own coefficient of analytical variation (CVa%). When the mean percentage difference of an analyte is less than the RCV calculated, one infers that there are no errors in the last quantification; whereas if mean percentage difference is bigger than the RCV calculated, this mirror potential clinical impact; in our study it refers to instability of the analyte evaluated.

The difference between serum and K_3EDTA-plasma were evaluated by paired t-test on T0. The collected data were processed in a data matrix in Excel (Microsoft) and statistical analysis were performed with the program SPSS v.18 (SPSS Inc.).

RESULTS

We evidenced instability for total bilirubin, uric acid, creatinine and glucose at T15 and T30 and stored at -20°C ($p<0.05$). However, potential clinical impact significance were observed only for total bilirubin T30 at -20°C, and creatinine T30 at 2-8°C. Statistical analysis of serum versus K_3EDTA-plasma did not show significant difference ($p>0.05$); Table 2.

DISCUSSION

The results of this study showed that the concentrations of the analytes in serum and K3EDTA-plasma were equivalent. Our results regarding differences between serum and plasma were similar with other studies (21,22), also if they used heparinized plasma instead of K3EDTA-plasma. In the past, uncertainty regarding the stability of serum analytes was a major concern because serum has been the preferred specimen by most laboratories. However, the serum specimens have several inherent problems, such as the time necessary for clot formation: at least 30 minutes (12, 23, 24). Several studies prefer lithium heparin plasma for the analysis of routine clinical chemistry (11, 25), particularly for the analysis of certain analytes (i.e., phenytoin) (21).

The inclusion of K3EDTA-plasma in our study is due to the observed need to obtain data in this matrix. Inpatients with hypovolemic shock generally require biochemical and hematological tests, however is difficult to obtain the number of samples and volume necessary for these analytical tests (whole blood for hematology and serum for chemistry).

Therefore, in these clinical conditions, it could be enough to take a single tube of blood sample (i.e., K_3EDTA) for the required tests. Obviously, electrolytes cannot be measured in this sample matrix, since EDTA is an important chelate (26).

The World Health Organization further states that heparin plasma samples either are not recommended for all analytical methods that include glucose and others (27). Over time, several studies support the fact that serum is preferred as the matrix analysis by most clinical laboratories (28).

Boyanton & Blick evaluated the stability of 24 analytes in serum and plasma where several analytes remained stable in both matrices for a period of 56 hours; the greatest change was observed for direct bilirubin (12).

Table 2 Analyte concentration by sample type (serum or plasma EDTA), storage time (T0, T15 and T30) and temperature of conservation

Analyte	Serum						K_3EDTA Plasma					
	Stability	median [interquartile range]	mean % difference	p	RCV	Potential clinical impact	Stability	median [interquartile range]	mean % difference	p**	RCV	Potential clinical impact
Glucose 2-8°C *(mmol/L)*	T0	4.86 [4.32–5.23]	-	-	-	-	T0	5.02 [4.48–5.35]	-	-	-	-
	T15	5.22 [4.66–5.73]	7.41	<0.001	16.4	**No**	T15	5.19 [4.70–5.62]	3.38	<0.001	16.4	No
	T30	5.05 [4.36–5.33]	3.91	<0.001	-	No	T30	4.97 [4.33–5.40]	-0.99	0.202	-	No
Glucose -20°C *(mmol/L)*	T0	4.86 [4.32–5.23]	-	-	-	-	T0	5.02 [4.48–5.35]	-	-	-	-
	T15	4.96 [4.37–5.23]	2.06	0.005	16.4	No	T15	5.16 [4.69–5.44]	2.78	0.010	16.4	No
	T30	4.72 [4.14–5.11]	2.88	<0.001	-	No	T30	4.97 [4.44–5.26]	-0.99	0.037	-	No
p-Value serum vs. plasma EDTA*	0.304											

Creatinine 2-8°C *(µmol/L)*	T0	64.1 [53.0–79.8]	-	-	-	-	T0	63.7 [53.9–78.2]	-	-	-	-
	T15	75.1 [61.0–92.4]	17.2	<0.001	20.9	**No**	T15	71.6 [58.3–85.3]	12.4	<0.001	20.9	**No**
	T30	78.2 [63.4–92.2]	22.0	<0.001	-	Yes	T30	72.1 [59.2–87.7]	13.2	<0.001		**No**
Creatinine -20°C *(µmol/L)*	T0	64.1 [53.0–79.8]	-	-	-	-	T0	63.7 [53.9–78.2]	-	-	-	-
	T15	68.9 [56.6–84.2]	7.49	<0.001	20.9	No	T15	66.7 [54.6–83.1]	4.70	<0.001	20.9	No
	T30	70.7 [59.0–84.2]	10.3	<0.001	-	No	T30	71.2 [57.2–86.9]	11.8	<0.001	-	**No**
p-Value serum vs. plasma EDTA*						0.844						
Uric acid 2-8°C *(mmol/L)*	T0	0.24 [0.22–0.29]	-	-	-	-	T0	0.23 [0.20–0.26]	-	-	-	-
	T15	0.28 [0.24–0.32]	16.7	<0.001	24.2	**No**	T15	0.24 [0.22–0.30]	4.3	<0.001	24.2	No
	T30	0.28 [0.24–0.32]	16.7	<0.001	-	No	T30	0.24 [0.22–0.29]	4.3	<0.001	-	**No**
Uric acid -20°C *(mmol/L)*	T0	0.24 [0.22–0.29]	-	-	-	-	T0	0.23 [0.20–0.26]	-	-	-	-
	T15	0.25 [0.23–0.30]	4.16	<0.001	24.2	No	T15	0.24 [0.21–0.28]	4.3	<0.001	24.2	No
	T30	0.26 [0.23–0.30]	8.33	<0.001	-	No	T30	0.25 [0.21–0.29]	8.7	<0.001	-	No
p-Value serum vs. plasma EDTA*						0.212						

Total Bilirubin 2-8°C *(µmol/L)*	T0	7.27 [5.09–12.7]	-	-	-	-	T0	7.35 [4.92–12.1]	-	-	-	-
	T15	4.79 [3.08–9.45]	-34.1	<0.001	61.5	**No**	T15	3.42 [2.27–6.58]	-53.5	<0.001	61.5	No
	T30	2.57 [1.33–4.66]	-64.6	<0.001	-	No	T30	3.93 [1.88–6.84]	-46.5	<0.001	-	No
Total Bilirubin -20°C *(µmol/L)*	T0	7.27 [5.09–12.7]	-	-	-	-	T0	7.35 [4.92–12.1]	-	-	-	-
	T15	5.05 [3.38–11.4]	-30.5	<0.001	61.5	No	T15	6.24 [4.02–11.4]	-15.1	<0.001	61.5	No
	T30	1.45 [0.68–4.53]	-80.1	<0.001		Yes	T30	1.54 [0.86–3.51]	-79.0	<0.001		Yes
p-Value serum vs. plasma EDTA*						0.759						
Direct Bilirubin 2-8°C *(µmol/L)*	T0	2.99 [2.57–5.13]	-	-	-	-	T0	2.48 [2.01–3.81]	-	-	-	-
	T15	2.05 [1.45–3.63]	-31.4	<0.001	102.4	**No**	T15	1.37 [0.94–2.22]	-44.8	<0.001	102.4	No
	T30	0.86 [0.34–1.71]	-71.2	<0.001	-	No	T30	0.86[0.51–1.24]	-65.3	<0.001	-	**No**
Direct Bilirubin -20°C *(µmol/L)*	T0	2.99 [2.57–5.13]	-	-	-	-	T0	2.48 [2.01–3.81]	-	-	-	-
	T15	2.65 [2.05–4.32]	-11.4	0.057	102.4	No	T15	2.39 [1.84–3.76]	-3.62	0.057	102.4	No
	T30	1.80 [0.98–2.74]	-39.8	<0.001	-	No	T30	1.80 [1.37–2.82]	-27.4	<0.001	-	No
p-Value serum vs. plasma EDTA*						0.078						

** = Student T test; ** = Wilcoxon ranked-pairs test.*

Our results had shown concentrations decrease on total bilirubin and direct bilirubin. However, this change could be produced by photo degradation (29).

Similar data were found by Tambse et al., who reported 10% variation for bilirubin in samples stored for 18-20 days at 4-8°C; whereas glucose, uric acid and creatinine showed an increase concentration over time (30).

A further study by Heins et al. reports that, from 22 analytes studied in serum, bilirubin stored at room temperature for 7 days had the greatest variation in concentration (31). Despite this, they conclude that all concentrations of the analytes studied including uric acid, creatinine and bilirubin are stable for four days in serum samples that were separated from the cells and stored at 9°C (31); whereas our results were different (Table 2).

Probably the increased concentrations observed in our study is due to evaporation of the samples stored. Similar results were evidenced by Alcaraz et al., after stored serum samples at 4°C for 5 days (32).

Only creatinine presented a potential clinical impact in T30 at 2-8°C (Table 2). In contrast with previous study described by Kachhawa et al., who report no clinical differences in creatinine, and uric acid concentrations between the mean values of day 1 and T7, T15 and T30 stored at -20°C (33). Thus demonstrating that the optimal storage condition is obtained by freezing the biological samples, since it could decrease the activity of some proteolytic enzymes that can alter the structure of the analytes (34).

Monneret et al. had shown good samples stability for 3, 4, 5, and 6 hours at room temperature in total blood and plasma (lithium heparin) for creatinine, total bilirubin and uric acid testing (25).

The analysis of the potential clinical impact in plasma at T15 and T30 stored at -20°C showed no significant difference for glucose, uric acid and direct bilirubin; demonstrating that the variations in concentrations are equivalent to the concentrations found in serum.

These results are consistent with those reported by other researchers (2, 21, 35, 36). Therefore, its necessary for each laboratory to evaluate the specific conditions and to define the storage time for any analyte to guarantee the stability. This will properly lead physicians to perform diagnostic, follow up, or therapeutic management.

In conclusion, our results had shown that storage samples at -20°C is a better way to preserve glucose, creatinine, and uric acid. Therefore, laboratories should freeze their samples as soon as possible to guarantee properly stability when there is a need to repeat analysis, verify a result, or add a laboratory testing.

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REFERENCES

1. Kreutzer HH, Fennis WHS. Lactic dehydrogenase isoenzyme in blood serum after storage at different temperatures. Clin Chim Acta. 1964;9:64–8.

2. Donnelly JG, Soldin SJ, Nealon DA, Hicks JM. Stability of twenty-five analytes in human serum at 22°C, 4°C, and -20°C. Pediatr Pathol Lab Med. 1995;15(6):869–74.

3. Cruz LM, Monge N, Valero J, Fuentes X. Estabilidad de las magnitudes bioquímicas. Química Clínica. 2002;21(2): 52–61.

4. Alsina MJ, Gonzales Oller J. Protocolo para el estudio de la estabilidad de las magnitudes biológicas. Química Clinica. 2006;25(2):86–9.

5. Nikolac N, Supak-Smolcic V, Simundic A-M, Celap I. Croatian Society of Medical Biochemistry and Laboratory

Medicine: national recommendations for venous blood sampling. Biochem medica. 2013;23(3):242–54.

6. Giménez Marín A, Molina Mendoza P, Ruiz Arredondo JJ Acosta González F, López Pérez M, Jiménez Cueva M, Cueto Santamaría T, Olmedo Sanchez R, López Somosierras M, Rivas Ruiz F, Perez Hidalgo M. Application of failure mode and effects analysis of the pre-analytical phase in a clinical laboratory. Rev del Lab Clin. 2010;3(4):161–70.

7. Cuhadar S, Atay A, Koseoglu M, Dirican A, Hur A. Stability studies of common biochemical analytes in serum separator tubes with or without gel barrier subjected to various storage conditions. Biochem Medica. 2012;22(2):202–14.

8. Simundic A, Lippi G. Preanalytical phase – a continuous challenge for laboratory professionals. Biochem Medica. 2012;22(2):145–9.

9. Recio Quijano F. Estudio multicéntrico de la estabilidad de las muestras de Serum mediante un protocolo de degradación acelerada. Rev del Lab Clin. 2008;1(3):84–93.

10. Lippi G, Lima-Oliveira G, Nazer SC, Moreira ML, Souza RF, Salvagno GL, Montagnana M, Scartezini M, Picheth G, Guidi GC. Suitability of a transport box for blood sample shipment over a long period. Clin Biochem. 2011 Aug;44(12):1028-9.

11. Lippi G, Salvagno GL, Danese E, Lima-Oliveira G, Brocco G, Guidi GC. Inversion of lithium heparin gel tubes after centrifugation is a significant source of bias in clinical chemistry testing. Clin Chim Acta. 2014;436:183-7.

12. Boyanton BL, Blick KE. Stability studies of twenty-four analytes in human plasma and serum. Clin Chem. 2002;48(12):2242–7.

13. Guidi GC, Simundic AM, Salvagno GL, Aquino JL, Lima-Oliveira G. To avoid fasting time, more risk than benefits. Clin Chem Lab Med. 2015;53(10):e261-4

14. Bajaña W, Aranda E, Arredondo ME, Brennan-Bourdon LM, Campelo MD, Espinoza E, Flores S, Ochoa P, Vega V, Varela B, Lima-Oliveira G. Impact of an Andean breakfast on biochemistry and immunochemistry laboratory tests: an evaluation on behalf COLABIOCLI WG-PRE-LATAM. Biochem Med (Zagreb). 2019;29(2):020702

15. Simundic AM, Bölenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov H, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, De la Salle B, Seipelt C, Sumarac Z, Vermeersch P; Working Group for Preanalytical Phase (WG-PRE), of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin American Working Group for Preanalytical Phase (WG-PRE-LATAM) of the Latin America Confederation of Clinical Biochemistry (COLABIOCLI). Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med. 2018;56(12):2015-2038.

16. SInternational Organization for Standardization. Medical Laboratories - Requirements for Quality and Competence ISO Document 15189 Geneva, Switzerland: International Organization for Standardization; 2012.

17. SRicós C, Perich C, Doménech M, Fernández P, Biosca C, Minchinela J, Simón M, Cava F, Álvarez V, Jiménez CV, García Lario JV. Biological variation. A review from a practical perspective. Rev del Lab Clin. 2010;3(4):192–200.

18. Corte Arboleya Z, Cándenas M, Venta R. Valor de referencia del cambio del PSA en la evaluación del riesgo de cáncer de próstata. Rev del Lab Clínico. 2011;4(3):115–20.

19. Lima-Oliveira G, Lippi G, Salvagno GL, Brocco G, Guidi GC. In vitro diagnostic company recalls and medical laboratory practices: an Italian case. Biochem Med (Zagreb). 2015;25(2):273–278.

20. Westgard J. Desirable Biological Variation Database Specifications. 2014; Available at: http://www.westgard.com/biodatabase1.htm. Accessed: February15th 2018.

21. O´Keane MP, Cunningham SK. Evaluation of three different specimen types (serum, plasma lithium heparin and serum gel separator) for analysis of certain analytes: Clinical significance of differences in results and efficiency in use. Clin Chem Lab Med. 2006;44(5):662–8.

22. SLima-Oliveira G, Monneret D, Guerber F, Guidi GC. Sample management for clinical biochemistry assays: Are serum and plasma interchangeable specimens? Crit Rev Clin Lab Sci. 2018 Nov;55(7):480-500.

23. Oddoze C, Lombard E, Portugal H. Stability study of 81 analytes in human whole blood, in serum and in plasma. Clin Biochem. 2012;45(6):464–9.

24. Leino A, Koivula MK. Stability of chemical and immunochemical analytes in uncentrifuged plasma samples. Ann Clin Biochem. 2009;46(2):159–61.

25. Monneret D, Godmer A, Le Guen R, Bravetti C, Emeraud C, Marteau A, Alkouri J, Mestari F, Dever S, Imbert-Bismut F, Bonnefon-Rousselot D. Stability of Routine Biochemical Analytes in Whole Blood and Plasma From Lithium Heparin Gel Tubes During 6-hr Storage. J Clin Lab Anal. 2016;30(5): 602-9.

26. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G, Guidi GC. Incorrect order of draw could be mitigate the patient safety: a phlebotomy management case report. Biochem Med (Zagreb). 2013;23(2):218-23.))

27. Banfi G, Bauer K, Brand W, Buchberger M, Deom A, Ehret W, Engel WD, da Fonseca-Wollheim F, Fraser CG, Friemert VJ, Golf S, Gross H, GruderWG, Gunzer G, Hagemann P, HeilW, Henny J, Hinzmann R, Hyltoft Persen P, Hoffman G, Kallner A, Karallus A, Kitta H, KlahrD, KolpeD,

Kukuk J, Kunert-Latus T, Lammers M, Leppa¨nen EA, Mikulcik P, Narayanan S, Neumaier M, Pec¸a Amaral Gomes MA, Probst R, Schmitt Y, Sonntag O, To¨pfer G, Weisheit R, WisserH, Zawta B, Zinck R:Use of Anticoagulants in Diagnostic Laboratory Investigations and Stability of Blood, Plasma and Serum Samples.WHO/DIL/LAB/99.1 Rev 2, Geneva,Switzerland, World Health Organization, 2002

28. Grankvist K, Gomez R, Nybo M, Lima-Oliveira G, von Meyer A. Preanalytical aspects on short- and long-term storage of serum and plasma. Diagnosis (Berl). 2019;6(1): 51-56.

29. Baer DM, Krause RB. Spurious laboratory values resulting from simulated mailing conditions. A study of time and temperature variables. Am J Clin Pathol 1968;50: 111–9

30. Tambse V, Manoorkar GS, Banik M, Tambse M. Study of the stability of various biochemical analytes in pooled sera preserved at 4-8 o C. Asian J Biomed Pharm Sci. 2015;5(48):38–9.

31. Heins M, Heil W, Withold W. Storage of Serum or Whole Blood Samples? Effects of Time and Temperature on 22 Serum Analytes. Eur J Clin Chem Clin Biochem. 1995;33(4): 231–8.

32. Alcaraz Quiles J, Rico Santana N, Bedini Chesa JL. Estabilidad de 27 magnitudes bioquímicas en muestras de Serum conservadas en refrigeración. Rev del Lab Clin. 2014;7(1): 9–16.

33. Kachhawa K, Kachhawa P, Varma M, Behera R, Agrawal D, Kumar S. Study of the stability of various biochemical analytes in samples stored at different predefined storage conditions at an accredited laboratory of India. J Lab Physicians. 2017;9(1):11.

34. Mechanic L, Mendez A, Merrill L, Rogers J, Layton M, Varanasi A, O´Brien B, Meyer WA, Zhang M, Schleicher RL, Moye Jr J. Planned variation in preanalytical conditions to evaluate biospecimen stability in the National Children's Study (NCS). Clin Chem Lab Med. 2013;51(12):2287–94.

35. Cuhadar S, Koseoglu M, Atay A, Dirican A. The effect of storage time and freeze-thaw cycles on the stability of serum samples. Biochem Medica. 2013;23(1):70–7.

36. Brinc D, Chan MK, Venner AA, Pasic MD, Colantonio D, Kyriakopolou L, Adeli K. Long-term stability of biochemical markers in pediatric serum specimens stored at -80°C: A CALIPER Substudy. Clin Biochem. 2012;45(10–11): 816–26.

In this issue: Improving the preanalytical phase in laboratory medicine

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Impact of chemical preservative in urine samples

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ABSTRACT

Urinalysis is one of the most important tests in the clinical laboratory. In this study we assessed the use of chemical preservative in urinalysis during preanalytical phase. Fifty first morning urine samples from medical laboratory patients were collected and stored with and without chemical preservative.

Difference between medians were analyzed using Wilcoxon signed rank test for glucose, bilirubin, ketones, specific gravity, erythrocytes, pH, proteins, nitrites, leukocytes using urine strips; and on leukocytes, erythrocytes, epithelial cells, and bacteria in the urinary sediment, at 90 minutes after sampling.

Our results showed that the specific gravity and the pH values increased in samples with chemical preservative in urine strip tests.

Concerning urinary sediment analysis no differences were observed in the studied parameters between samples with and without chemical preservative. We suggest that the effect on urine pH is due to the chemical nature of the substances in the preservative.

Thus, we caution about the use of chemical preservatives in samples to be analyzed within short time (i.e. less than 1.5 - 2 hours) after sample collection. Avoid chemical preservatives, in this situation, could help avoid changes in the pH and specific gravity, which could eventually help in maintaining quality in the preanalytical phase of urinalysis.

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INTRODUCTION

Urinalysis is one of the most important tests in the clinical laboratory [1, 2]. Urine contains an enormous amount of information linked to patient's health [3, 4]. The laboratory process consists of three phases: the pre-analytical, analytical and post-analytical phases [5].

The preanalytical phase covers all the procedures before the sample reaches the laboratory to be examined [6, 7]. It encompasses all the steps from sample collection to sample delivery for analysis. Moreover, a pre-preanalytical phase is to be considered mainly related to patient preparation before sample collection [8].

Although most efforts to improve the efficiency in urinalysis have been focused on the analytical phase thus underestimating the preanalytical one, it has been evidenced that up to 60% of errors still occur in the preanalytical phase [9, 10]. In any case the improvements of the analytical phase through automation have led to reduction of up to 10 times the error rate [11,12].

When dealing with urinalysis, both pre-preanalytical and preanalytical phase are to be regarded as critical for the appropriateness of the whole analytical process [3].

As for patient preparation there are several conditions to satisfy that include information on diet, exercise, possible contamination to be avoided by genital cleaning prior to specimen collection, sample collection time (first or second in the morning or casual collection), stream portion of collected sample (first, mid, last), type of sample whether spontaneously collected or by catheterization [1].

One major issue with urine specimens is contamination and consequent microbial growth when sample analysis is delayed due to transportation time. This problem is dealt with by sample refrigeration [13] or by adding chemical preservatives to the urine sample [14].

The use of chemical preservatives has been recommended due to its ability to keep bacterial populations stable for up to 24 hours [9, 10]. In this sense, the aim of this study is to determine the impact of chemical preservative in urine samples.

METHODS

Study design

Fifty urine samples (first morning urine) from the Autonomous University of San Luis Potosi's medical laboratory were included in the analytical study. The BD Vacutainer® Urinalysis Cup Kit was used to collect the sample, which includes a cup to obtain the sample, and a conical tube with sodium propionate 94%, ethyl paraben 5.6% and chlorhexidine 0.4% as preservative.

Patients were asked to transfer urine to the tube with a preservative after collecting the urine sample, and the remaining sample from the cup was also delivered to the laboratory, which was considered as a urine sample without preservative.

This work was performed following the current international ethical guidelines involving human beings for research purposes, adopted by the Declaration of Helsinki [15]. Also, all the participants gave informed consent approved by the Research Ethics Committee from Faculty of Chemistry Science of the Autonomy University of San Luis Potosí (CEID2019-011).

Analysis of urine strips

The standardized chemical analysis for the following parameters: glucose, bilirubin, ketones, specific gravity, erythrocytes, pH, proteins, nitrites, leukocytes was performed on the urine samples using Multistix® 10 SG test strips, and the automated reading system CLINITEK Advantus, both from SIEMENS.

The methods of analysis for each parameter evaluated with urine test strips are shown in Table 1. The analytical performance was verified with SIEMENS CheK-Stix® Combo Pak Control strips for Urinalysis.

Analysis of urinary sediment

The parameters observed in the urinary sediment were leukocytes, erythrocytes, epithelial cells, mucin filaments, crystals and bacteria.

The procedure for urinary sediment analysis was: 8 mL of first morning urine was placed in a conical tube and centrifuged at 400 *g* for 5 minutes [16, 17]. After discarded 7.5 mL of the supernaµL of this re-suspended pellet was placed on a slide, covered with a coverslip (18 x 18 mm2) then observed under the microscope using 100X and 400X objective [18].

Table 1 Methods of analyses

Parameter	Method
Glucose	Glucose oxidase
Bilirubin	Union of bilirubin with dichloroaniline diazotized in an acidic medium
Ketones	Colorimetric reaction between acetoacetic acid and nitroprusside
Specific gravity	Change of pKa in polyelectroliters in relation to ionic concentration
Blood	Hemoglobin pseudoperoxidase activity that catalyzes the reaction of diisopropylbenzene dihydroperoxide with 3, 3', 5, 5'-tetramethylbenzidine
pH	Combination of methyl red and blue bromothymol that react with hydrogen ions
Protein	Protein error of indicators
Urobilinogen	Reaction of ρ-diethylaminobenzaldehyde with urobilinogen in acidic medium
Nitrites	Griess assay principle. The reaction reveals the presence of nitrite and therefore, indirectly, the existence of bacteria forming it in the urine
Leukocytes	Esterases that catalyze the hydrolysis of the pyrrolic amino acid ester

Statistical analysis

For the statistical analysis the Wilcoxon signed rank test was used to evaluate the difference between urine samples with and without chemical preservatives; the established statistical significance was set at $p \leq 0.05$. The statistical package used was the software SPSS Statistics® 20.

RESULTS

We found that the urine samples with chemical preservative showed higher values of specific gravity and pH in comparison with the samples without chemical preservative. As for the remaining parameters no differences were observed between the medians of samples with and without chemical preservative. And in addition to this, we did not observe significant differences between the figurate elements of the urinary sediment in the presence or absence of chemical preservative (Table 2).

DISCUSSION

In this work we evaluated the effect of chemical preservative in the preanalytical phase and we found that the specific gravity and the pH were both lowered in the absence of chemical preservative at 90 minutes after sample collection. Chemical preservatives are available to maintain sample integrity without cell lysis, to avoid bacterial growth, or *in vitro* crystal formation [9,14,19]. Moreover, preservatives can affect some parameters such as leukocyte esterase, glucose and proteins [20, 21, 22].

According to the increase of the pH and specific gravity values in the samples with chemical preservative (Table 2), the question arises if such changes regarding pH and specific gravity are clinically relevant, thus being able to influence the interpretation of urinalysis. For example, one possible implication of increased specific gravity due to preservative could jeopardize the diagnosis of pseudohyposthenuria in the pediatric population, whose values of specific gravity are lower than normal children [23, 24]. In addition, the pH may affect the concentration of certain urinary parameters [21].

Although samples without chemical preservative showed a reduced specific gravity in respect of added preservative, when evaluating the figurated elements such as cells and microorganisms, we did not find any differences between the samples with or without chemical preservative (Table 2).

Therefore, we can say that although the chemical preservative increased specific gravity, this did not affect the microscopic analysis of urine.

It has been reported that most of the parameters evaluated during urinalysis depend strongly on the time window between sampling and analysis. Moreover, morphological studies showed a higher reproducibility when time was between 1 and 2 hours [25]. Thus, the use of chemical preservative is recommended for those samples that will be processed after two hours of collection [21,22].

The optimal time for performing the urinalysis and the impact of the chemical preservative on urine samples has been previously studied by Dolscheid-Pommerich *et al*. [26]. They found a significant decrease in concentrations of erythrocytes and leukocytes between 90 and 120 minutes after sample collection, in samples stored at room temperature [26]. However, they did not find changes in pH and specific gravity before 120 minutes of collection; the authors recommend 90 minutes, as an optimal time for the urinalysis to be performed after the collection of the sample [26].

On the other hand, the changes that chemical preservatives can cause on the different parameters of urinalysis have also been studied; Delanghe *et al*., described that chlorhexidine can causes an alteration in glucose and pH parameters [21].

We suggest that the effect on urine pH (Table 2) is due to the chemical nature of the substances in the preservative and that this change in pH is related to the increase in specific gravity, thus explaining possible changes in these parameters even in short time windows.

In conclusion, given the unclear consensus on the optimal time for the use of chemical preservatives, and based on our findings, we caution about the use of chemical preservatives in samples to be analyzed within short time (i.e. less than 1.5 - 2 hours) after sample collection.

Avoid chemical preservatives, in this situation, could help avoid changes in the pH and specific gravity, which could eventually help in maintaining quality in the preanalytical phase of urinalysis.

Table 2 Impact of chemical preservative in urine samples

Parameter	Frequency (N = 50)		*p*
	Samples with chemical preservative	Samples without chemical preservative	
Urine strip			
Glucose			
Absent	49/50	49/50	> 0.05
100	1/50	1/50	
Bilirubin			
Absent	50/50	50/50	> 0.05
Ketones			
Absent	50/50	50/50	> 0.05
Specific gravity			
1.005	1/50	4/50	**0.001**
1.010	7/50	4/50	
1.015	3/50	7/50	
1.020	11/50	13/50	
1.025	13/50	9/50	

1.030	15/50	13/50	
Blood			
Absent	39/50	37/50	> 0.05
Erythrocytes			
10	6/50	9/50	
Free hemoglobin			
10	1/50	0/50	
25	2/50	2/50	
80	1/50	0/50	
200	1/50	2/50	
pH			
5.0	1/50	17/50	**0.001**
6.0	39/50	26/50	
6.5	9/50	5/50	
7.0	1/50	2/50	
Proteins			
Absent	49/50	50/50	> 0.05
< 30	1/50	-	
Urobilinogen			
0.2	50/50	50/50	> 0.05
Nitrites			
Absent	45/50	45/50	> 0.05
Present	5/50	5/50	
Leukocytes			
Absent	42/50	44/50	

15	2/50	1/50	> 0.05
70	5/50	4/50	
125	1/50	1/50	
Microscopic analysis			
Leukocytes/high power field			
0-5	44/50	44/50	> 0.05
6-10	5/50	5/50	
11-25	1/50	1/50	
Erythrocytes/high power field			
0-2	49/50	49/50	> 0.05
6-10	1/50	1/50	
Epithelial cells			
Absent	20/50	20/50	> 0.05
Low	24/50	25/50	
Moderate	5/50	4/50	
Abundant	1/50	1/50	
Mucin filament			
Absent	15/50	15/50	> 0.05
Low	24/50	24/50	
Moderate	9/50	9/50	
Abundant	2/50	2/50	
Bacteria			
Low	38/50	38/50	> 0.05
Moderate	6/50	6/50	

Abundant	6/50	6/50	
Crystals			
Absent	37/50	36/50	> 0.05
Low	9/50	10/50	
Moderate	3/50	3/50	
Abundant	1/50	1/50	

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REFERENCES

1. Coppens A, Speeckaert M and Delanghe J. The pre-analytical challenges of routine urinalysis. Acta Clin Belg 2010; 65:182-189.

2. Arinzon Z, Peisakh A, Shuval I, Shabat S and Berner YN. Detection of urinary tract infection (UTI) in long-term care setting: is the multireagent strip an adequate diagnostic tool? Arch Gerontol Geriatr 2009; 48:227-231.

3. Delanghe JR and Speeckaert MM. Preanalytics in urinalysis. Clin Biochem 2016; 49:1346-1350.

4. Simerville JA, Maxted WC and Pahira JJ. Urinalysis: a comprehensive review. Am Fam Physician 2005; 71: 1153-1162.

5. Lima-Oliveira G, Guidi GC, Guimaraes AVP, Correa JA and Lippi G, Preanalytical Nonconformity Management Regarding Primary Tube Mixing in Brazil. J Med Biochem 2017; 36:39-43.

6. Lima-Oliveira G, Volanski W, Lippi G, Picheth G and Guidi GC. Pre-analytical phase management: a review of the procedures from patient preparation to laboratory analysis. Scand J Clin Lab Invest 2017; 77:153-163.

7. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G and Guidi GC. Quality impact on diagnostic blood specimen collection using a new device to relieve venipuncture pain. Indian J Clin Biochem 2013; 28: 235-241.

8. Lima-Oliveira G, Guidi GC, Salvagno GL, Danese E, Montagnana M and Lippi G. Patient posture for blood collection by venipuncture: recall for standardization after 28 years. Rev Bras Hematol Hemoter 2017; 39:127-132.

9. Lauer BA, Reller LB and Mirrett S. Evaluation of preservative fluid for urine collected for culture. J Clin Microbiol 1979; 10:42-45.

10. Guenther KL and Washington JA. Evaluation of the B-D urine culture kit. J Clin Microbiol 1981; 68:628-630.

11. Lippi G, Becan-McBride K, Behúlová D, Bowen RA, Church S, Delanghe J, Grankvist K, Kitchen S, Nybo M, Nauck M, Nikolac N, Palicka V, Plebani M, Sandberg S and Simundic AM. Preanalytical quality improvement: In quality we trust. Clin Chem Lab Med 2013; 51:229-241.

12. Lima-Oliveira G, Lippi G, Salvagno GL, Danese E, Montagnana M, Brocco G, Voi M, Picheth G and Guidi GC. Does laboratory automation for the preanalytical phase improve data quality? J Lab Autom. 2013; 18:375-381.

13. Lippi G, Lima-Oliveira G, Nazer SC, Moreira ML, Souza RF, Salvagno GL, Montagnana M, Scartezini M, Picheth G and Guidi GC. Suitability of a transport box for blood sample shipment over a long period. Clin Biochem 2011; 44: 1028-1029.

14. Eisinger SW, Schwartz M, Dam L and Riedel S. Evaluation of the BD Vacutainer Plus Urine C&S Preservative Tubes compared with nonpreservative urine samples stored at 4°C and room temperature. Am J Clin Pathol 2013; 140: 306-313.

15. Williams JR. The Declaration of Helsinki and public health. Bulletin of the World Health Organization. 2008; 86:650-652.

16. Kouri T, Fogazzi G, Gant V, Hallander H, Hofmann W and Guder WG .European Urinalysis Guidelines. Scand J Clin Lab Invest 2000; 60: Issue sup231.

17. Bunjevac A, Gabaj NN, Miler M and Horvat A. Preanalytics of urine sediment examination: Effect of relative

centrifugal force, tube type, volume of sample and supernatant removal. Biochem Med (Zagreb) 2018; 28:010707.

18. Fernández DJ, Di Chiazza S, Veyretou FP, González LM and Romero MC. Analisis de orina: estandarizacion y control de calidad. Acta Bioquím Clín Latinoam 2014; 48:213-221.

19. Ercan M, Akbulut ED, Abuşoğlu S, Yılmaz FM, Oğuz EF, Topçuoğlu C, Öztekin V and Boğdaycıoğlu N. Stability of urine specimens stored with and without preservatives at room temperature and on ice prior to urinalysis. Clin Biochem 2015; 48: 919-922.

20. Almaani S, Hebert LA, Rovin BH and Birmingham DJ. The urine preservative acetic acid degrades urine protein: Implications for urine biorepositories and the AASK cohort study.J Am Soc Nephrol 2017; 28:1394-1398.

21. Delanghe J and Speeckaert M.Preanalytical requirements of urinalysis. Biochem Med (Zagreb) 2014; 24:89-104.

22. Ekşioğlu MK, Madenci ÖÇ, Yücel N, Elçi A, Turhan B, Orhan G and Orçun A. The effectiveness of BD vacutainer® plus urinalysis preservative tubes in preservation of urine for chemical strip analysis and particle counting. Biochem Med (Zagreb) 2016; 26:224-232.

23. Lozano-Triana CJ. Examen general de orina: una prueba útil en niños. Rev Fac Med 2016; 64:137-147.

24. Vaisbich MH, Carneiro J, Bóson W, Resende B, De Marco L, Honjo RS, Ae Kim C and Koch VH. Nephrogenic diabetes insipidus (NDI): clinical, laboratory and genetic characterization of five Brazilian patients.Clinics 2009; 64:409-414.

25. Langlois MR, Delanghe JR, Steyaert SR, Everaert KC and De Buyzere ML. Automated flow cytometry compared with an automated dipstick reader for urinalysis. Clin Chem 1999; 45:118-122.

26. Dolscheid-Pommerich RC, Klarmann-Schulz U, Conrad R, Stoffel-Wagner B and Zur B. Evaluation of the appropriate time period between sampling and analyzing for automated urinalysis. Biochem Med (Zagreb) 2016; 26: 2-89.

In this issue: Improving the preanalytical phase in laboratory medicine

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Pseudothrombocytopenia by ethylenediaminetetraacetic acid can jeopardize patient safety – a case report

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ABSTRACT

Pseudothrombocytopenia by ethylenediaminetetraacetic acid (EDTA) is an infrequent phenomenon of *in vitro* platelet agglutination due to the presence of antiplatelet autoantibodies. It has no clinical significance, but misdiagnosis may lead to clinical or therapeutic decision-making. In this study, we report a case of an 8-year-old boy with no history of platelet disorder presenting a low platelet count and a peripheral blood smear showing clumping of platelets by EDTA. The initial diagnosis hypothesis was of an idiopathic thrombocytopenic purpura, and an unnecessary bone marrow aspirate was made even though he did not have personal or family history of bleeding. A second sample collected in sodium citrate confirmed the pseudothrombocytopenia by EDTA. In conclusion, the laboratory should enhance a strong relationship with clinicians trying to avoid misunderstandings as that reflected in this case report. It should be reminded that, in those cases where a pseudothrombocytopenia by EDTA is suspected, a blood smear is mandatory

to confirm platelet clumps and blood must be tested anticoagulated with another anticoagulant (i.e., sodium citrate or heparin).

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INTRODUCTION

Several preanalytical factors could impact platelets evaluation and jeopardize patient safety (1). Therefore, laboratory professional should guarantee preanalytical procedures to avoid the following source of platelets variability:

- improperly fasting time (2,3)
- improperly tourniquet application time (4,5)
- unstandardized patient posture (6)
- improper order of evacuated tubes (7,8)
- improper blood venous sampling (9)

According to the World Health Organization (WHO), patient safety is the absence of preventable harm to a patient during the process of health care and reduction of risk of unnecessary harm associated with health care to an acceptable minimum (7). The work in healthcare services should focus in the culture of patient safety, understanding that the problem is often a succession of several oversights or errors, from which we can learn and implement solutions to prevent them in the future.

Ethylenediaminetetraacetic acid (EDTA) is a calcium chelator (10), widely used as an anticoagulant for a complete blood count test because it generally does not distort blood cells. The term pseudothrombocytopenia by EDTA define a low platelet count in patients without any bleeding tendency, and platelet distribution curve that indicates platelet aggregation (11). Moreover, the patient should present a normal platelet count if a different anticoagulant is used (12). The aim of this report is to show that a pseudothrombocytopenia by EDTA can jeopardize the patient safety.

CLINICAL-DIAGNOSTIC CASE

An 8-year-old boy was referred to paediatric onco-haematology for presenting a two-year evolution thrombocytopenia with a platelet count below $20x10^3/\mu L$ (Reference Interval: 130-$400x10^3/\mu L$) with normal values of white blood cells (WBC) and haemoglobin (Hb) (Table 1). He did not have a significant past medical history, besides his familial hypercholesterolemia, and his clinical examination was unremarkable.

The initial diagnosis hypothesis was of an idiopathic thrombocytopenic purpura even though he did not have personal or family history of bleeding. A bone marrow aspirate was made, with no significant anomalies in the sample obtained. It was described as a normocellular marrow, showing trilinear haematopoiesis, with a preserved myeloid/erythroid ratio and a megakaryocytic series without significative dysmorphias. At that moment the clinician realized that there was a commentary on the platelet count remarking the presence of plentiful platelet clumps in all the previous laboratory reports. The complete blood count was repeated in the emergency laboratory. The platelet count was of $16x10^3/\mu L$ together with a platelet clumps alarm in the automated cell counter (Advia 2120, Siemens Medical Solutions Diagnostics, Los Ángeles, CA, USA), without alterations in the other two haematological series (Table 1). Given this result, a peripheral blood smear was made, and multiple platelet clumps were observed (Figure 1). This was remarked in the laboratory report and a new sample was collected using an evacuated tube with 3.2% sodium citrate, as anticoagulant additive (BD Vacutainer). The platelet count in this sample was normal (Table 1) and this result was discussed with the clinician. Physicians decide to perform a new blood collection with both anticoagulants 14 days after these results to confirm the pseudothrombocytopenia by EDTA (Table 1).

Table 1 Laboratory results

Laboratory test (unit)		Anticoagulant	Platelet ($x10^3/\mu L$)	WBC ($x10^3/\mu L$)	Neutrofil ($x10^3/\mu L$)	Lymphocyte ($x10^3/\mu L$)	Monocyte ($x10^3/\mu L$)	Eosinophil ($x10^3/\mu L$)	Basophil ($x10^3/\mu L$)	Hb (g/dL)	RBC ($X10^6/\mu L$)	Hct (%)	MCV (fL)
1st	October 4th	EDTA	20	8.70	4.69	3.00	0.62	0.34	0.05	13.9	4.61	38.1	82.6
2nd	November 6th	EDTA	16	6.15	2.25	2.00	1.49	0.20	0.04	13.5	4.54	38.3	84.4
2nd	November 6th	Na citrate	160	NA									
3rd	November 20th	EDTA	5	7.94	3.69	3.14	0.56	0.23	0.02	13.1	4.58	38.3	83.6
3rd	November 20th	Na citrate	198	NA									

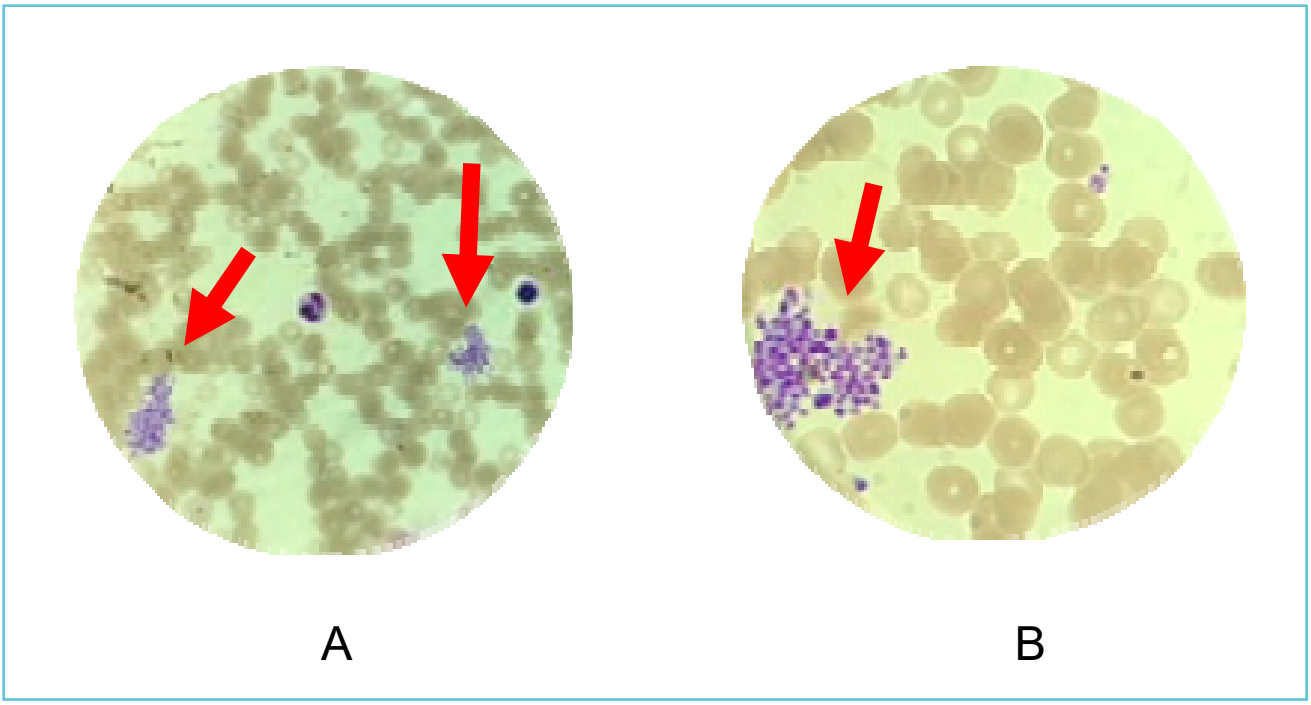
DISCUSSION

EDTA-dependent pseudothrombocytopenia is an infrequent phenomenon of *in vitro* platelet agglutination due to the presence of antiplatelet autoantibodies (13,14); its incidence has been reported to be between 0.10 and 0.29% (15). As described in our case the agglutination *in vitro* results in a decrease in platelet count, typically below $100x10^3/\mu L$ with the presence of a "platelet aggregation" alert flag from the analyser (16), the peripheral blood smear must be observed in order to confirm the presence of platelet clumps (17). In addition, a new blood sample from the same patient using sodium citrate as anticoagulant is the most suitable sample for the platelet count (18). The laboratory professional has the responsibility of detecting these false thrombocytopenias and notifying this information in the laboratory report (19). Likewise, the clinician should consider this condition in order to avoid unnecessary diagnostic tests and therapeutic interventions with the aim of preserving patient safety. Therefore, we would like to highlight the following learning points from this case report:

- The spurious low platelet count must be reported together with a commentary on the presence of platelet clumps that underestimate the real count.
- The clinical laboratory has an essential role over patient safety, but more efforts are required to prevent inappropriate clinical or therapeutic decision-making.

In conclusion, the laboratory should enhance a strong relationship with clinicians trying to avoid misunderstandings as that reflected in this case report. It should be reminded that, in those cases where a pseudothrombocytopenia by EDTA is suspected, a blood smear is mandatory to confirm platelet clumps and blood must be tested anticoagulated with other anticoagulant (i.e., sodium citrate or heparin).

Figure 1 Peripheral blood smear from blood sample collected with EDTA (May-Grünwald-Giemsa stained) observed in optical microscopy



Note: Arrows indicate platelet clumps at 400X (A) and at 1000X magnification (B).

Disclosures and contributions

Cristina Collazo Abal is the main responsible for the study conception design, intellectual content and drafting of this paper. All other authors confirmed they have contributed significantly to the analysis and interpretation of data, revising, and approva the article. Al authors dec are no conflicts of interest.

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REFERENCES

1. Lima-Oliveira G, Volanski W, Lippi G, Picheth G and Guidi GC. Pre-analytical phase management: a review of the procedures from patient preparation to laboratory analysis. Scand J Clin Lab Invest 2017;77:153–163.

2. Arredondo ME, Aranda E, Astorga R, Brennan-Bourdon LM, Campelo MD, Flores S, Medel C, Manríquez I, Ochoa P, Varela B, Salinas CV and Lima-Oliveira G. Breakfast can Affect Routine Hematology and Coagulation Laboratory Testing: An Evaluation on Behalf of COLABIOCLI WG-PRE-LATAM. TH Open 2019;03:e367–376.

3. Guidi GC Simundic AM, Salvagno GL, Aqu no JL and Lima-Oliveira G. To avoid fasting time, more risk than benefits. Clin Chem Lab Med 2015;53:e261–264.

4. Lima-Olivei a G, Lippi G, Salvagno GL, Gaino S, Poli G, Gelati M, Picheth G and Guidi GC. Venous stasis and whole blood platelet aggregometry: A question of data reliability and patient safety. Blood Coagul Fibrinolysis 2015;26: 665–668

5. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Scartezini M, Guidi GC and Picheth G. Transillumination: A new tool to eliminate the impact of venous stasis during the procedure for the collection of diagnostic blood specimens for routine haematological testing. Int J Lab Hematol 2011; 33:457–462.

6. Lima-Oliveira G, Guidi GC, Salvagno GL, Danese E, Montagnana M and Lippi G. Patient posture for blood collection by venipuncture: recall for standardization after 28 years. Rev Bras Hematol Hemoter 2017;39:127–132.

7. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Poli G, Solero G Pietro, Picheth G and Guidi GC. K 3 EDTA Vacuum Tubes Validation for Routine Hematological Testing. ISRN Hematol 2012;2012:875357.

8. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Poli G, Solero GP, Picheth G and Guidi GC. Brand of dipotassium

EDTA vacuum tube as a new source of pre-analytical variability in routine haematology testing. Br J Biomed Sci 2013; 70:6–9.

9. Simundic AM, Bölenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov H, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, De la Salle B, Seipelt C, Sumarac Z, Vermeersch P; Working Group for Preanalytical Phase (WG-PRE), of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin American Working Group for Preanalytical Phase (WG-PRE-LATAM) of the Latin America Confederation of Clinical Biochemistry (COLABIOCLI). Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56:2015-2038.

10. Lima-Oliveira G, Monneret D, Guerber F and Guidi GC. Sample management for clinical biochemistry assays: Are serum and plasma interchangeable specimens? Crit Rev Clin Lab Sci 2018;55:480–500.

11. Gschwandtner ME, Siostrzonek P, Bodinger C, Neunteufl T, Zauner C, Heinz G, Maurer G and Panze S. Documented sudden onset of pseudothrombocytopenia. Ann Hematol 1997;74:283–285.

12. Akyol L, Onem S, Ozgen M and Sayarlioglu M. Ethylenediaminetetraacetic acid-dependent pseudothrombocytopenia in a patient with systemic lupus erythematosus and lupus nephritis. Eur J Rheumatol 2016;3:36–37.

13. Pegels JG, Bruynes EC, Engelfriet CP and von dem Borne AE. Pseudothrombocytopenia: an immunologic study on platelet antibodies dependent on ethylene diamine tetraacetate. Blood 1982;59:157–161.

14. Casonato A, Bertomoro A, Pontara E, Dannhauser D, Lazzaro AR and Girolami A. EDTA dependent pseudothrombocytopenia caused by antibodies against the cytoadhesive receptor of platelet gpIIB-IIIA. J Clin Pathol 1994;47: 625–30.

15. Yoneyama A and Nakahara K. EDTA-dependent pseudothrombocytopenia--differentiation from true thrombocytopenia. Nihon Rinsho 2003;61:569–574.

16. Lin J, Luo Y, Yao S, Yan M, Li J, Ouyang W and Kuang M. Discovery and Correction of Spurious Low Platelet Counts due to EDTA-Dependent Pseudothrombocytopenia. J Clin Lab Anal 2015;29:419–426.

17. Sarasa-Bosque C, Arruga-Mombiela C and Denizon-Arranz S. Aprendiendo de los errores: fenómeno EDTA. Semer-Med Fam. 2009;35:106–107.

18. Lippi G and Plebani M. EDTA-dependent pseudothrombocytopenia: further insights and recommendations for prevention of a clinically threatening artifact. Clin Chem Lab Med 2012;50:1281–1285.

19. Cadamuro J, Simundic AM, Ajzner E and Sandberg S. A pragmatic approach to sample acceptance and rejection. Clin Biochem 2017;50:579–581.

In this issue: Improving the preanalytical phase in laboratory medicine

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Causes of preanalytical interferences on laboratory immunoassays – a critical review

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ABSTRACT

The immunoassays methods need avoiding interferences that can influence result interpretation. Main sources of interference arise from either patient status, preparation and physiology or laboratory process and procedures.

The aim of this non-systematic critical review is to highlight the preanalytical interferences on laboratory immunoassays.

Blood hormone profile changes according with age and depending on sex: these are important variables, mainly in newborn, during both sexual maturation and childbearing. Gonadotropins FSH and LH show a sharp increase with age in females, whereas in males LH appears rather stable. With age both males and females show progressive decay of the hormone profile. Stress causes variations, as it influences GH, prolactin, cortisol and the total/free ratio of thyroid hormone. Diurnal variations, day of cycle, influence by estrogens on thyroid hormone are relevant for result variability. Paraproteins and autoantibodies can interfere in some

assays particularly drug, vitamin D and thyroid hormone. As regards the variables due to sample matrix, and to evacuated tubes components, some additives and anticoagulants have been reported to influence specific assays, e.g. thyroid hormone. Hemolysis, lipemia and bilirubin cause interferences on specific techniques/tests, e.g. ferritin, TSH, Vitamin B12, progesterone and folic acid. Nicotine and cocaine addictions interfere with some hormones. Thus, laboratory professionals should be aware of preanalytical problems particularly important when dealing with the immunoassays, by taking appropriate actions to avoid any relevant interferences.

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INTRODUCTION

The immunoassays methods employ antibodies showing high affinity for epitopes on antigens in order to both detect them with high specificity and make the immune reaction detectable and measurable, using a range of indicator reactions or labels. Due to the nature of immune reactions, well-controlled conditions are needed in order to avoid non-specific binding. When analyzing biological samples such as serum or plasma (usual laboratory matrices), interferences are also possible due to similar compounds, such as other proteins presenting like epitopes (1).

All the above analytical interferences can account for several technical problems. Moreover, pathophysiological variables can also confound the interpretation of e.g. the pituitary-thyroid axis interaction (2). Generally, TSH-independent states are observed when the disorder affects primarily the thyroid gland, but there are non-thyroid conditions that can alter the interaction and confound the diagnosis, such as trophoblastic tumors, struma ovarii, and generalized resistance to thyroid hormone or selective organ resistance to thyroid hormone. Besides, there are TSH-dependent variables due to inappropriate TSH secretion or lack of secretion such as pituitary adenoma, resistance states to thyroid hormone (generalized or pituitary), psychiatric states, smoking, and malabsorption. Besides, there are physiological variables, as follows: circadian rhythm, seasonal influences, environmental influences, exercise, posture, and pregnancy.

Furthermore, dealing with thyroid, Iatrogenic causes should always be considered, taking into account: prior thyroid treatment (surgical or medical), drug therapy (systemic or local), and plasmapheresis. Regarding pituitary function, some physiological variables can induce modifications to the pituitary secretion, as follows: age, travel (that influences both pituitary and adrenal functions), circadian rhythm and pulsatile release, seasonal influences, environmental influences, exercise, stress, and posture (3).

Moreover, there are pathophysiological variables acting on pituitary, such as malnutrition, starvation, lifestyle (e.g. smoking increases secretion; whereas alcohol reduces secretion), blindness (that causes loss of hypothalamic pituitary axis stimulation), and drugs (4).

Adding to the above issues, clinical laboratories should be aware of preanalytical problems that are particularly relevant when dealing with the immunoassays. The preanalytical phase is the major source of laboratory variability.

The most common preanalytical variables (Figure 1) could be classified in:

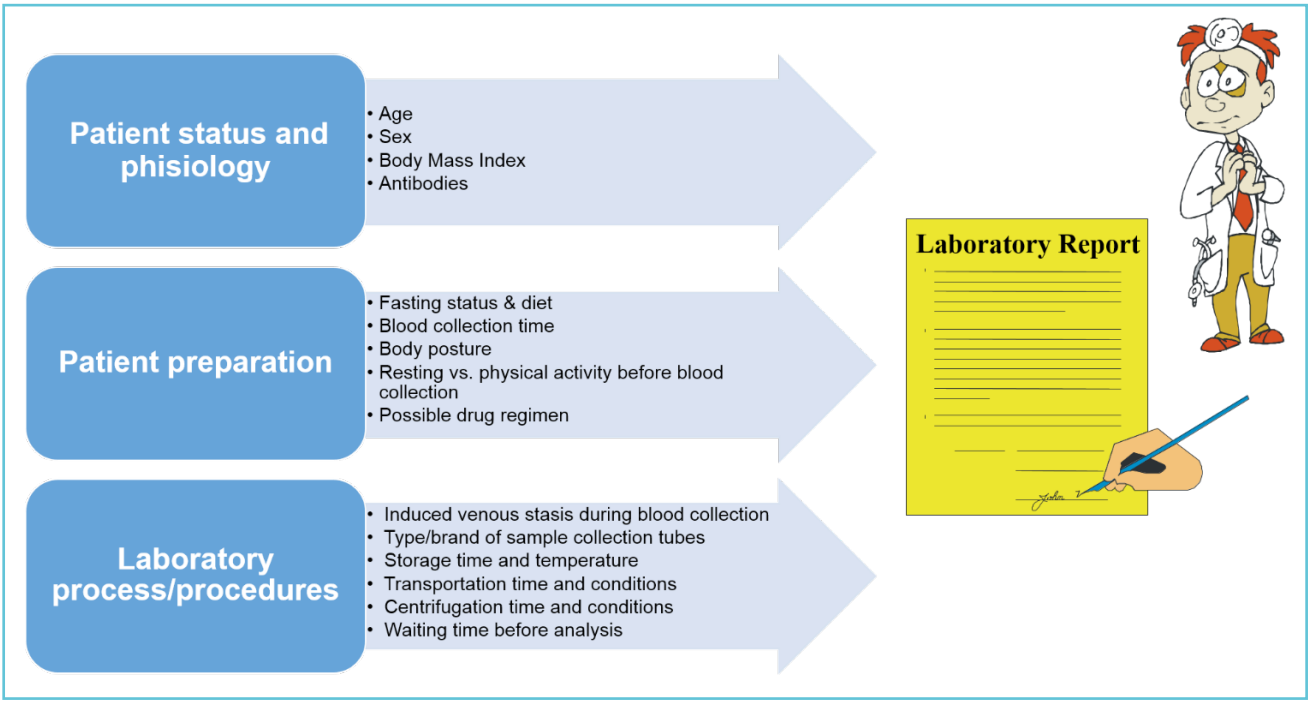
i) patient status and physiology (5);

ii) patient preparation (6-11); and

iii) laboratory process/procedures (12-18)

The collection of liquid biological specimens by evacuated tubes is usual practice in healthcare and in veterinary care settings (19). The aim of this non-systematic critical review is to highlight the preanalytical interferences on laboratory immunoassays. To enrich the argument, we also provide an additional evacuated tube verification.

Figure 1 Preanalytical sources of laboratory interferences



CAUSES OF PREANALYTICAL INTERFERENCES ON LABORATORY ASSAYS

Lipemia, bilirubin, and hemolysis interference

Lipemia may interfere the antigen-antibody precipitation leading to falsely high results, whereas high bilirubin *per se* can cause spectral interferences in immunoassays with absorbance measurement readings at about 450-460 nm.

Hasanato et al., assessed the interference of progressively increasing amounts of hemoglobin, bilirubin and lipids added to sera on a series of immunoassays. The following immunoassays: ferritin, TSH, Vitamin B_{12}, progesterone and folic acid, all showed variable degrees of interference. It was observed that ferritin and TSH levels were overestimated in direct relationship with hemolysis, whereas Vitamin B_{12} progressively decreased. Progesterone levels decreased with increasing lipemia. Folic acid levels decreased with increasing bilirubin (20). Free hemoglobin, at different concentrations (g/L), has shown various levels of interference on different methods for anti-HIV-1 antibody detection, either immunochromatography (at 5.7 g/L), or enzyme-immunoassay (at 86 g/L), or chemiluminescence (at 115 g/L) based methods. Lipemia did not show any interference (21). The effects of hemolysis (H index range 0-1000), icterus (I index range 0-40) and lipemia (L index range 0-1000) were evaluated on three acetaminophen assays, respectively the Syva® EMIT®, the Microgenics DRI® assay, and the Roche assay on a Roche cobas® c501 or an Integra 800 analyzer. No interference was observed due to hemolysis and icterus on Syva® EMIT® and DRI® assay, whereas interference was marked on Roche assays; whereas lipemia was less evident on Roche than on Syva® EMIT® and DRI® assay (22).

Saracevic et al. reported interferences on resistin and myeloperoxidase immunosorbent assays from BioVendor. Both resistin and myeloperoxidase levels showed significant biases at 1 g/L of free hemoglobin (58.7% and 66.7%, respectively) and at 4.66 mmol/L triglycerides (33.8% and 12.2%, respectively) (23).

Variables regarding patient status and physiology

Age and sex are particularly important for interpreting the results of hormones, mostly when considering the ample range of variations shown by several hormones during the life, from newborn to adult age. Each newborn shows the influence of the maternal hormones that mainly pass through placenta during pregnancy and can influence some physiological responses in babies, particularly as regards thyroid function (24). Even during the breast feeding there is passage of hormones from mother to newborn through the milk, particularly of hormones that are needed for regulating infant growth (25, 26).

With age progression the blood hormone profile changes accordingly and depending on sex, showing the effects both of sexual maturation by the relevant hormone levels and of the stature lengthening by the growth hormones. Gonadotropins FSH and LH show sharp increase with age in females, whereas in males LH appears rather stable. FSH is far higher in females than in males, particularly during puberty (27). Moreover, serum concentrations of thyroid hormones are reportedly different between males and females and for age groups in white children/adolescents (28).

During maturity, the main hormone changes are shown at menopause by the sexual hormones in females. The hormone replacement therapy is used as a prevention treatment for women at the onset of menopause (29). The parallel andropause is a condition due to low plasma testosterone in conjunction with symptoms of reduced sexual function (30). With age both males and females are implicated by a progressive decay of the hormone profile, with some aspects regarding particular hormones such as the thyroid ones that should be carefully considered, since critical for several metabolic functions of the body (31).

The female sex hormones show variations during the menstrual cycle depending on the length of the cycle (32). Important information to be gathered before blood collection regards exact day of cycle and/or intake of hormone replacement therapy and/or birth control pill. Moreover, estrogens influence thyroglobulin during the cycle, thus influencing fT4 levels. With menopause the cessation of ovarian production of estrogens and progesterone is preceded by irregular menstrual cycles (33). During this period estrogen, in particular, and progesterone decline, whereas FSH rises and high levels of this hormone are typically observed at the beginning of menopause, although with wide oscillations.

Measurement of serum testosterone level is important for the assessment of hypogonadism in men and androgen excess in women, where it vary mainly according to the phase of the menstrual cycle. Yet serum testosterone levels can vary widely, even between samples from the same patient and among different laboratory assays, due to a multitude of factors, such as diurnal variation, systemic illnesses, and seasonal variation, as well as assay-specific factors (34).

Kisspeptins (Kps) are peptide hormones, generators of both sex steroid negative and positive feedback signals for GnRH secretion in male and female at puberty, preserve male fertility, and govern the pre-ovulatory LH surge in females (35). Kps show very low values (<2 pmol/L) in male and in non-pregnant female; however, increase dramatically during pregnancy. There are some preanalytical issues to be considered: i) rapid degradation is observed in serum, therefore sample must be processed immediately after collection (freezing-thawing does not significantly affect results); ii) either heparin with 2000 U trasylol or EDTA should be used as anticoagulant, whereas citrate causes lowering of results.

The main hormone that determines the height growth at each stage of development is growth

hormone (GH), together with its mediator, insulin-like growth factor 1 (IGF-1). The presence of an alteration in the GH/IGF-1 axis in the pediatric population results in impaired growth (36). The assays of random serum GH concentrations are of no clinical value as GH secretion is pulsatile and most of GH pulses occur overnight, showing very low hormone concentrations between pulses. Thus, provocative tests of GH secretion using appropriate stimuli are employed to test for GH deficiencies (GHD). GH stimulation tests use a defined cut-off concentration for peak GH to distinguish deficient from non-deficient GH subjects. Serum IGF-I is synthesized in the liver under the control of GH and circulates bound to the IGF binding proteins (IGFBPs). There are six known IGFBPs of which IGFBP-3 is the major serum carrier of IGF-I. Unlike GH, serum concentrations of IGF-I and IGFBP-3 are stable. Total IGF-I concentrations in serum are mainly measured using assays that dissociate IGF-I from its binding proteins (37). Serum IGF-I concentrations vary with age and it must be considered that the normal range for serum IGF-I concentrations in young children superimposes on the range observed in children with GH deficiency. Moreover IGF-I concentrations are reduced in malnourished children, and/or with hypothyroidism, chronic disease, renal failure and diabetes (37). They also rise intensely during puberty. IGFBP-3 concentrations were thought to be potentially superior to measurement of IGF-I alone as IGFBP-3 is less nutritionally sensitive than IGF-I. However, multiple studies have found no difference in IGFBP-3 concentrations between GHD and non-GHD subjects (38), with a poor sensitivity at 50% and no advantage over measurement of IGF-I alone (39).

Body mass index increase (BMI), waist circumference and hip circumference have demonstrated association with increased levels of several sex hormones, in particular obese individuals show increased blood concentrations of estrone, estradiol and free estradiol with negative association with Sex Hormone Binding Globulin (SHBG) (40). Moreover, TSH has shown significant direct association with BMI, which has been explained as due to TSH directly stimulating preadipocyte differentiation that results in adipogenesis (41); whereas even leptin can be involved (42). Other studies have shown association between adiposity and prolactin, although more variables might be involved such as alcohol intake and sedentary lifestyle (43). Therefore, each country should define reference intervals and clinical decision limits to minimize the impact of age, sex and BMI on laboratory assays based in their population (44). High concentrations of paraproteins can interfere with various laboratory immunoassays, and laboratory personnel should be aware of this when the laboratory findings are discordant with the clinical findings (45). Possible false negative results have been reported as regards the assay of phenytoin in a patient with high levels of IgM. The interference caused no detection of the drug in a Particle-Enhanced Turbidimetric Inhibition Immunoassay (PETINIA) method notwithstanding, based on dosage, the patient trough plasma concentration was estimated between 5 and 10 mg/L (46). Moreover, false negative results for vancomycin were reported in a patient with high IgM levels, despite assuming appropriate dosage of the drug. The high IgM caused interference on a PETINIA method for vancomycin that was not evident when an enzyme multiplied immunoassay (EMIT) was employed (47). Very high, near to toxicity, 25-OH vitamin D level (327 nmol/L) was assayed on ARCHITECT. After recalling the patient to confirm the result, 49 nmol/L of 25-OH vitamin D was measured by mass spectrometry; whereas 289 nmol/L was measured by ARCHITECT. Briefly, the patient had high circulating IgG paraproteins with a clinical history of rheumatoid arthritis with rheumatoid factor (RF) and myeloma. Thus, the interference on immunoassay was due to IgG paraprotein,

but the contribution of rheumatoid factor could not be ruled out (48). As regards RF, falsely elevated results were reported in serum (but not in plasma) samples spiked with patient RF pool when vancomycin immune assays were performed (49).

The patient's blood itself can cause pre-analytical errors. Briefly, some patients have circulating antibodies that can strongly modify the results of the immunoassays and simulate a disease.

There are reports regarding:

a) the interference of heterophile antibody affecting fT4 Roche immunoassay, indicating a suspect hyperthyroidism which revealed false after retesting on another platform (Centaur, Siemens) (50);

b) cases of anti-streptavidin antibodies mimicking heterophilic antibodies in thyroid tests (51).

c) cases of apparent hyperthyroidism due to Biotin-like interference from IgM anti-streptavidin, a pattern that mimics the excess biotin ingestion (52);

Moreover, drugs over the counter, high-dose biotin supplements are accompanied by observations of analytical interference by exogenous biotin (53) in the immunoassays used to evaluate endocrine function (54). Best practices have been proposed to avoid such interferences, comprising sample dilutions, retesting after biotin clearance, assessment of biotin presence (55).

Variables due to patient preparation

Thyroid hormones appear influenced by fasting status or diet composition (6, 56). On the other hand, either diet composition, meal frequency or eating time, all can influence GH, prolactin, and cortisol levels (57). Moreover, treatments or drugs that delay gastric emptying and motility, such as omeprazole can act on the levels of some hormones. Fasting induces enhancement of GH release with a concomitant reduction of somatomedin C. The GH release is achieved through combined frequency and amplitude modulation (57). Ghrelin is controlled by food intake, being higher during fasting or before meals then decreasing after meal. It is known as the "hunger hormone" since after being released mainly by gastric cells – and also by gut, pancreas and brain cells – it stimulates appetite and consequent food intake and fat accumulation acting on hypothalamus. However, ghrelin is more than that, since it regulates glucose homeostasis by inhibiting insulin secretion. Moreover, it shows a series of positive effects on heart, muscle and bone (58).

To increase accuracy, testosterone in men should be measured with a fasting morning sample and repeated if the level is found to be low; in women, the laboratory must perform the measurements at the follicular phase of the cycle. In both cases, borderline results may be clarified by the assessment of free testosterone. Thus, interpretation of testosterone results is challenging. Presently the reference assay method for measuring blood testosterone levels is based on mass spectrometry (59). Moreover, as avoidance of fasting time could jeopardize result accuracy and thus patient safety (60), 12h overnight fasting before blood sampling for laboratory immunoassays are recommended (6, 8, 61).

Short-term studies have shown that caffeine increases insulin levels, reduces insulin sensitivity and increases cortisol levels (62). Coffee has been considered to influence the energy intake possibly acting on appetite hormones. On this note, it has been shown that although coffee and caffeine ingested 0.5-4 h before a meal can suppress acute energy intake, the influence on appetite hormones appears equivocal (63). Moreover, habitual coffee intake is related to effects on either glycerophospholipid metabolism (64) or several metabolic pathways (65). All the above suggest that before blood collection,

patients should be requested to inform about their drinking coffee habit, either short-term or long-term.

The nicotine from smoking increases cortisol, GH, prolactin, LH, ACTH and DHEA, in males; similar effects are observed with intravenous cocaine abuse (66, 67). On the contrary, nicotine decreases prolactin in pregnant (68) and in lactating women (69) (without effect on fetal prolactin).

Many hormones show diurnal variations (e.g. ACTH, cortisol, GH) and pulsatile secretion is reported for some hormones with short time variations, up to 25%. The pituitary hormones show a circadian cycle that is linked to the sleep-wake rhythm. Cortisol shows diurnal variation, with the peak value at about 8 am in the morning and the lowest levels in the late night until early morning, from 12 pm to 4 am. Individuals affected by Cushing's syndrome show incongruously high ACTH and cortisol levels during late night hours. Thus, the sample collection time should be planned consequently. Cortisol can be assayed either in serum or in saliva (70). The salivary cortisol assay was described several years ago. It has the advantages of being simple to collect, non-invasive and the sample can be easily stored for repetition. The advantages of salivary cortisol assays have been evidenced in patients with Addison disease and appear more adequate than serum in the screening for Cushing disease after assaying salivary samples obtained during night time (71). When collecting saliva samples, it is important to instruct the patient to avoid hydrocortisone containing creams or ointments in order to prevent analytical interference (72). Salivary melatonin assay can be a reliable non-invasive biomarker of the circadian secretive rhythm of the pineal gland (73). Prolactin is another hormone with diurnal variation and with higher levels at night (74). Prolactin levels in samples taken in the morning can sometimes show increased levels thus indicating a hyperprolactinemia status. Since prolactin diurnal variation is associated with night peak that sometimes declines steadily and prolongs in the first morning hours, there are individuals showing late reduction of prolactin to the reference values observed in the morning values. Besides a true hyperprolactinemia, elevated morning levels can be due to stress conditions, medications or spurious hyperprolactinemia. Indeed, in most cases after sample retaking a normal value is observed. For these reasons and for avoiding unnecessary additional tests, it has been suggested to delay late in the morning the sample collection for prolactin assays, i.e. around 11 am (75). The measure of PTH is a second-level examination, requested by physicians with other tests: e.g., calcium to elucidate a hyperparathyroidism hypothesis (76). It is recommended that blood samples for PTH measurement should be taken, ideally between 10:00 and 16:00, and plasma separated within 24 h of blood collection. Plasma samples should be stored at +4°C and analyzed within 72 h from collection (77). Therefore, considering that for several hormones the diurnal variations are critical for result interpretation, we suggest that blood withdrawal hours should be appropriately defined to guarantee suitable laboratory results and thus patient safety.

Variables due to sample matrix and evacuated tube

Different laboratory results, including hormones, were reported using different brands of serum- and plasma-evacuated tubes (13, 78, 79). The reasonable cause for these divergences was ascribed to interaction between blood and components in the evacuated tubes, e.g. surfactant(s), stopper(s), stopper-lubricant(s), separator gel(s), and additive(s) (80, 81). The commonly used tube surfactant – Silwet L-720 – has been reported to cause the desorption of antibodies from the solid phase (82). Moreover, samples

collected on SST tubes (BD Vacutainer) have been reported with values of total triiodothyronine, measured by the Immulite 2000, significantly higher than samples collected in glass or Vacuette tubes (Greiner Bio-One). Alterations in concentrations of triiodothyronine, thyroxine and cortisol in either quality control materials or in serum specimens have been reported after collecting or pouring in different blood-collection tube types (83). Discrepancies have been observed in C-reactive protein (CRP) assays measured in serum samples from SST tubes, in particular lower values in SST serum than in serum from plain tubes. It appears due to SST adsorption of some CRP macromolecules that form complexes with SST gel (84). Therefore, the need to verify the suitability of the evacuated tubes employed in sample collection for immunoassays is mandatory in order to avoid unwanted outcomes and to warrant the safety of the patients' results (8, 85, 86).

Plasma vs. serum samples reveal different hormone results due to interferences from lithium heparin, EDTA, sodium fluoride, or potassium oxalate (87). Parathormone (PTH) levels decrease after blood collection in either EDTA or serum samples, with divergent results. A greater stability has been reported for PTH collected in potassium EDTA (88).

The Clinical and Laboratory Standards Institute recommend to follow the instructions from evacuated tubes manufacturer regarding: sample mixing, resting time before centrifugation, centrifugation time, g-force (89). As far as we know, Vacumed ® (FL Medical, Torreglia) lacks a formal specification regarding sample processing and handling. Thus, after having checked other brands information (90-92), we decided to verify this evacuated tubes (Table 1). A sometimes-overlooked fact is that the accuracy of blood drawing with evacuated tube systems is influenced by altitude, because of variations of the atmospheric pressure. It is well known that reduced blood volumes are collected into the tubes in altitude settings (90), thus, altering the blood/additive ratio (91) (Table 2).

Table 1 Verification of Vacumed ®

Evacuated tubes	Additives	Mix by gently inversion	Centrifugation	
			g-force	time (min)
42010; 42011; 42012 43016 44019	K_2EDTA	2 X	1200	15
42110; 42111; 42112 43116 44119	K_3EDTA	2 X	1200	15
42210; 42211; 42212 43216 44219	Potassium Fluoride + K_3EDTA	3 X	1000	15

42310; 42311; 42312 43316 44319	Lithium Heparin	2 X	1200	15
43350; 43351; 43352 43356 44359	Sodium Heparin	3 X	1200	15
42410; 42411; 42412 42508 42510 42512	Sodium Citrate 3.2%	2 X	1500 NA NA NA	15
42611; 42612 43616 44619	Clot activator	2 X	1200	15
42691 43696 44698	Clot activator + sferes separator	3 X	1000	15
42711 43716 44718	Clot activator + gel separator	2 X	2000	10
42811 43816 44818	gel separator + Lithium Heparin	2 X	1800	10
42851 43856 44858	K_2EDTA + gel separator	2 X	1600	10

42910; 42911; 42912 43916 44919	without additive	NA	2200 for whole blood	10

Note: Serum tubes should rest at least 45 min in upright position after blood collection and before centrifugation. However, should be centrifuged within 2 h after blood collection. All tubes including tubes with gel separator should be transported in upright position after centrifugation process. The results reported in this table is part of the project 2451/15 of University of Verona.

Table 2 Mean volume filling regarding different altitudes

Altitude (m)	Mean volume filling of evacuated tubes			
	4.5 mL	3.5 mL	3 mL	2 mL
0	4.50	3.50	3.00	2.00
250	4.37	3.40	2.91	1.94
500	4.24	3.30	2.83	1.89
1000	4.00	3.11	2.67	1.78
1250	3.89	3.02	2.59	1.73
1500	3.78	2.94	2.52	1.68
1750	3.67	2.86	2.45	1.63
2000	3.57	2.78	2.38	1.59

Note: Results are presented as mean volume from five evacuated tubes producers – BD Vacutainer®, Becton, Dickinson and Company; Vacuette®, Greiner Bio-One; Vacumed®, FL Medical; Vacutest®, Kima; and S-Monovette, Sarstedt using the vacuum technique – filled with a solution with density and viscosity like whole blood. Volumes (mL) reported by the evacuated tube manufacturer at sea level (0 m) are presented in bold. Altitudes are referred in meters above sea level. All tubes used were 13x75 mm.

CONCLUSIONS

In immunochemistry assays the results can be significantly and positively influenced by the appropriateness of the procedures adopted along the process starting from test prescription and ending with result interpretation. Particular attention should be addressed separately to variables that on the contrary could be viewed as a collective whole in the pre-analytical phase, therefore resulting in less influence on the outcomes and possibly misleading in the

interpretation. In this context it should be summarized that:

- results can be significantly influenced by the techniques adopted, that in turn can be affected by some preanalytical conditions;
- age and gender are important variables, particularly in newborn, during sexual maturation and childbearing;
- stress can influence GH, prolactin cortisol and the total/free ratio of thyroid hormone;
- diurnal variations, day of cycle, influence by estrogens on thyroid hormone are to be accounted for result variability;
- in adults/elders some results can be interfered by autoantibodies;
- some additives and anticoagulants of collection tubes can influence specific assays;
- hemolysis, lipemia and bilirubin cause interferences on specific techniques/tests;
- paraproteins mainly cause interferences;
- specific issues should be considered as regards thyroid, pituitary and sex hormones;
- nicotine and cocaine addictions interfere with some hormones.

Therefore, in providing the results to the patients, laboratory professionals have the responsibility of ascertaining that all the above have been checked and duly evaluated and the appropriate actions have been accomplished to avoid any relevant interferences.

REFERENCES

1. Koivunen ME, Krosgrud RL. Principles of Immunochemical Techniques Used in Clinical Laboratories. Lab Med 2006;37:490-497.

2. Keffer JH. Preanalytical considerations in testing thyroid function. Clin Chem 1996;42:125-134.

3. Sam S, Frohman LA. Normal physiology of hypothalamic pituitary regulation. Endocrinol Metab Clin North Am 2008;37:1-22.

4. Veldhuis JD. Changes in pituitary function with ageing and implications for patient care. Nat Rev Endocrinol 2013;9:205-215.

5. Narayanan S, Guder WG. Preanalytical Variables and Their Influence on the Quality of Laboratory Results. Ejifcc 2001;13:9-12.

6. Bajaña W, Aranda E, Arredondo ME, Brennan-Bourdon LM, Campelo MD, Espinoza E, Flores S, Ochoa P, Vega V, Varela B, Lima-Oliveira G. Impact of an Andean breakfast on biochemistry and immunochemistry laboratory tests: an evaluation on behalf COLABIOCLI WG-PRE-LATAM. Biochem Med 2019;19:020702.

7. Arredondo ME, Aranda E, Astorga R, Brennan-Bourdon LM, Campelo MD, Flores S,Manriquez I, Ochoa P, Varela B, Salinas CV, Lima-Oliveira G. Breakfast can Affect Routine Hematology and Coagulation Laboratory Testing: An Evaluation on Behalf of COLABIOCLI WG-PRE-LATAM. TH open 2019;3:e367-e376.

8. Simundic AM, Bolenius K, Cadamuro J, Church S, Cornes MP, van Dongen-Lases EC, Eker P, Erdeljanovic T, Grankvist K, Guimaraes JT, Hoke R, Ibarz M, Ivanov H, Kovalevskaya S, Kristensen GBB, Lima-Oliveira G, Lippi G, von Meyer A, Nybo M, De la Salle B, Seipelt C, Sumarac Z, Vermeersch P ; Working Group for Preanalytical Phase (WG-PRE), of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin American Working Group for Preanalytical Phase (WG-PRE-LATAM) of the Latin America Confederation of Clinical Biochemistry (COLABIOCLI). Joint EFLM-COLABIOCLI Recommendation for venous blood sampling. Clin Chem Lab Med 2018;56: 2015-2038.

9. Lima-Oliveira G, Guidi GC, Salvagno GL, Danese E, Montagnana M, Lippi G. Patient posture for blood collection by venipuncture: recall for standardization after 28 years. Rev Bras Hematol Hemot 2017;39:127-132.

10. Ide BN, Souza-Junior TP, McAnulty SR, de Faria MAC, Costa KA, Nunes LAS. Immunological Responses to a Brazilian Jiu-Jitsu High-Intensity Interval Training Session. J Hum Kinet 2019;70:115-124.

11. Yao H, Rayburn ER, Shi Q, Gao L, Hu W, Li H. FDA-approved drugs that interfere with laboratory tests: A systematic search of US drug labels. Crit Rev Clin Lab Sci 2017;54: 1-17.

12. Lima-Oliveira G, Lippi G, Salvagno GL, Gaino S, Poli G, Gelati M, Picheth G, Guidi GC. Venous stasis and whole blood platelet aggregometry: a question of data reliability and patient safety. Blood Coagul Fibrinolysis 2015;26: 665-668.

13. Lima-Oliveira G, Salvagno GL, Lippi G, Brocco G, Voi M, Montagnana M, Picheth G, Guidi GC. Quality management of preanalytical phase: impact of lithium heparin

vacuum tubes changes on clinical chemistry tests. Accred Qual Assur 2013;18:429-434.

14. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Poli G, Solero GP, Picheth G, Guidi GC. K(3)EDTA Vacuum Tubes Validation for Routine Hematological Testing. ISRN hematolog 2012;2012:875357.

15. Grankvist K, Gomez R, Nybo M, Lima-Oliveira G, von Meyer A. Preanalytical aspects on short- and long-term storage of serum and plasma. Diagnosis 2019;6:51-56.

16. Lippi G, Lima-Oliveira G, Nazer SC, Moreira ML, Souza RF, Salvagno GL, Montagnana M, Scartezini M, Picheth G, Guidi GC. Suitability of a transport box for blood sample shipment over a long period. Clin Biochem 2011;44: 1028-1029.

17. Lima-Oliveira G, Lippi G, Salvagno GL, Gelati M, Bassi A, Contro A, Pizzolo F, Guidi GC. Abnormal gel flotation caused by contrast media during adrenal vein sampling. Biochem Med 2016;26:444-450.

18. Lippi G, Salvagno GL, Danese E, Lima-Oliveira G, Brocco G, Guidi GC. Inversion of lithium heparin gel tubes after centrifugation is a significant source of bias in clinical chemistry testing. Clin Chim Acta 2014;436:183-187.

19. Lima-Oliveira G, Cesare Guidi G, Guimaraes AVP, Abol Correa J, Lippi G. Preanalytical Nonconformity Management Regarding Primary Tube Mixing in Brazil. J Med Biochem 2017;36:39-43.

20. Hasanato R, Brearton S, Alshebani M, Bailey L, Aldugashim S, Alothaim A, Tamimi W. Effects of serum indices interference on hormonal results from the Abbott Architect i2000 immunoassay analyser. Br J Biomed Sci 2015;72:151-155.

21. Mendoza-Benitez PS, Aguilar-Ruiz SR, Romero-Tlalolini MD, Torres-Aguilar H. Evaluation of the Interference of Lipemia and Hemolysis in the Detection Limit of Anti-HIV-1 Antibodies. Clin Lab 2019;65.

22. Zhang YV, Fan SL, Kellogg MD. Effect of hemolysis, icterus, and lipemia on three acetaminophen assays: Potential medical consequences of false positive results. Clin Chim Acta 2018;487:287-292.

23. Saracevic A, Dukic L, Simundic AM. Haemolysis and lipemia interfere with resistin and myeloperoxidase Bio-Vendor ELISA assays. Biochem Med 2019;29:020703.

24. Consortium on Thyroid and Pregnancy-Study Group on Preterm Birth, Korevaar TIM, Derakhshan A, Taylor PN, Meima M, Chen L, Bliddal S, Carty DM, Meems M, Vaidya B, Shields B, Ghafoor F, Popova PV, Mosso L, Oken E, Suvanto E, Hisada A, Yoshinaga J, Brown SJ, Bassols J, Auvinen J, Brames WM, Lopez-Bermejo A, Dayan C, Boucai L, Vafeiadi M,Grineva EN, Tkachuck AS, Pop VJM, Vrijkotte TG, Guxens M, Chatzi L, Sunyer J, Jimenez-Zabala A, Riano I, Murcia M, Lu X, Mukhtar S, Delles C, Feldt-Rasmussen U, Nelson SM, Alexander EK, Chaker L, Mannisto T, Walsh JP, Pearce EN, Steegers EAP, Peeters RP. Association of Thyroid Function Test Abnormalities and Thyroid Autoimmunity With Preterm Birth: A Systematic Review and Meta-analysis. Jama 2019;322:632-641.

25. Wagner CL, Baatz JE, Newton D, Hollis BW. Analytical considerations and general diagnostic and therapeutic ramifications of milk hormones during lactation. Best Pract Res Clin Endocrinol Metab 2018;32:5-16.

26. Yu X, Rong SS, Sun X, Ding G, Wan W, Zou L, Wu S, Li M, Wang D. Associations of breast milk adiponectin, leptin, insulin and ghrelin with maternal characteristics and early infant growth: a longitudinal study. Brit J Nutr 2018;120: 1380-1387.

27. Soldin OP, Hoffman EG, Waring MA, Soldin SJ. Pediatric reference intervals for FSH, LH, estradiol, T3, free T3, cortisol, and growth hormone on the DPC IMMULITE 1000. Clin Chim Acta 2005;355:205-210.

28. Gunapalasingham G, Frithioff-Bojsoe C, Lund MAV, Hedley PL, Fonvig CE, Dahl M, Pedersen O, Christiansen M, Hansen T, Lausten-Thomsen U, Holm JC. Reference values for fasting serum concentrations of thyroid-stimulating hormone and thyroid hormones in healthy Danish/North-European white children and adolescents. Scand J Clin Lab Invest 2019;79:129-135.

29. Lobo RA, Pickar JH, Stevenson JC, Mack WJ, Hodis HN. Back to the future: Hormone replacement therapy as part of a prevention strategy for women at the onset of menopause. Atherosclerosis 2016;254:282-290.

30. Huhtaniemi IT. Andropause--lessons from the European Male Ageing Study. Ann Endocrinol 2014;75:128-131.

31. Duntas LH. Thyroid Function in Aging: A Discerning Approach. Rejuvenation Res 2018;21:22-28.

32. Messinis IE, Messini CI, Dafopoulos K. Novel aspects of the endocrinology of the menstrual cycle. Reprod Biomed Online 2014;28:714-722.

33. El Khoudary SR, Thurston RC. Cardiovascular Implications of the Menopause Transition: Endogenous Sex Hormones and Vasomotor Symptoms. Obst Gynecol Clin North Am 2018;45:641-661.

34. Herati AS, Cengiz C, Lamb DJ. Assays of Serum Testosterone. Urol Clin North Am 2016;43:177-184.

35. Chianese R, Cobellis G, Chioccarelli T, Ciaramella V, Migliaccio M, Fasano S, Pierantoni R, Meccariello R. Kisspeptins, Estrogens and Male Fertility. Curr Med Chem 2016;23:4070-4091.

36. Esposito S, Leonardi A, Lanciotti L, Cofini M, Muzi G, Penta L. Vitamin D and growth hormone in children: a review of the current scientific knowledge. J Transl Med 2019;17:87.

37. Murray PG, Dattani MT, Clayton PE. Controversies in the diagnosis and management of growth hormone deficiency in childhood and adolescence. Arch Dis Child 2016;101: 96-100.

38. Phillip M, Chalew SA, Kowarski AA, Stene MA. Plasma IGFBP-3 and its relationship with quantitative growth hormone secretion in short children. Clin Endocrinol 1993;39: 427-432.

39. Nunez SB, Municchi G, Barnes KM, Rose SR. Insulin-like growth factor I (IGF-I) and IGF-binding protein-3 concentrations compared to stimulated and night growth hormone in the evaluation of short children--a clinical research center study. J Clin Endocrinol Metab 1996;81: 1927-1932.

40. McTiernan A, Wu L, Chen C, Chlebowski R, Mossavar-Rahmani Y, Modugno F, Perri MG, Stanczyk FZ, Van Horn L, Wang CY; Women's Health Initiative Investigators. Relation of BMI and physical activity to sex hormones in postmenopausal women. Obesity 2006;14:1662-1677.

41. Valyasevi RW, Harteneck DA, Dutton CM, Bahn RS. Stimulation of adipogenesis, peroxisome proliferator-activated receptor-gamma (PPARgamma), and thyrotropin receptor by PPARgamma agonist in human orbital preadipocyte fibroblasts. J Clin Endocrinol Metab 2002;87:2352-2358.

42. Feldt-Rasmussen U. Thyroid and leptin. Thyroid 2007; 17:413-419.

43. Hankinson SE, Willett WC, Manson JE, Hunter DJ, Colditz GA, Stampfer MJ, Longcope C, Speizer FE. Alcohol, height, and adiposity in relation to estrogen and prolactin levels in postmenopausal women. J Natl Cancer Inst 1995;87: 1297-1302.

44. Ozarda Y, Sikaris K, Streichert T, Macri J; IFCC Committee on Reference intervals and Decision Limits (C-RIDL). Distinguishing reference intervals and clinical decision limits-A review by the IFCC Committee on Reference Intervals and Decision Limits. Crit Rev Clin Lab Sci 2018;55: 420-431.

45. LeGatt DF, Higgins TN. Paraprotein interference in immunoassays. Ther Drug Monit 2015;37:417.

46. Hirata K, Saruwatari J, Enoki Y, Iwata K, Urata Y, Aizawa K, Ueda K, Shirouzono T,Imamura M, Moriuchi H, Ishima Y, Kadowaki D, Watanabe H, Hirata S, Maruyama T, Fukunaga E. Possible false-negative results on therapeutic drug monitoring of phenytoin using a particle enhanced turbidimetric inhibition immunoassay in a patient with a high level of IgM. Ther Drug Monit 2014;36:553-555.

47. Cooper AA, Cowart K, Clayton A, Paul J. Undetectable Vancomycin Concentrations Utilizing a Particle Enhanced Turbidimetric Inhibition Immunoassay in a Patient with an Elevated IgM Level. Clin Lab 2017;63:1527-1532.

48. Ong MW, Salota R, Reeman T, Lapsley M, Jones L. Artefactual 25-OH vitamin D concentration in multiple myeloma. Ann Clin Biochem 2017;54:716-720.

49. LeGatt DF, Blakney GB, Higgins TN, Schnabl KL, Shalapay CE, Dias VC, Wesenberg JC. The effect of paraproteins and rheumatoid factor on four commercial immunoassays for vancomycin: implications for laboratorians and other health care professionals. Ther Drug Monit 2012;34: 306-311.

50. Serei VD, Marshall I, Carayannopoulos MO. Heterophile antibody interference affecting multiple Roche immunoassays: A case study. Clin Chim Acta 2019;497: 125-129.

51. Favresse J, Lardinois B, Nassogne MC, Preumont V, Maiter D, Gruson D. Anti-streptavidin antibodies mimicking heterophilic antibodies in thyroid function tests. Clin Chem Lab Med 2018;56:e160-e163.

52. Lam L, Bagg W, Smith G, Chiu WW, Middleditch MJ, Lim JC, Kyle CV. Apparent Hyperthyroidism Caused by Biotin-Like Interference from IgM Anti-Streptavidin Antibodies. Thyroid 2018;28:1063-1067.

53. Bowen RA, Chan Y, Cohen J, Rehak NN, Hortin GL, Csako G, Remaley AT. Effect of blood collection tubes on total triiodothyronine and other laboratory assays. Clin Chem 2005;51:424-433.

54. Samarasinghe S, Meah F, Singh V, Basit A, Emanuele N, Emanuele MA, Mazhari A, Holmes EW. Biotin Interference with Routine Clinical Immunoassays: Understand the Causes and Mitigate the Risks. Endocr Pract 2017;23: 989-998.

55. Bowen R, Benavides R, Colon-Franco JM, Katzman BM, Muthukumar A, Sadrzadeh H, Straseski J, Klause U, Tran N. Best practices in mitigating the risk of biotin interference with laboratory testing. Clin Biochem 2019;74:1-11.

56. Basolo A, Begaye B, Hollstein T, Vinales KL, Walter M, Santini F, Krakoff J, Piaggi P. Effects of Short-Term Fasting and Different Overfeeding Diets on Thyroid Hormones in Healthy Humans. Thyroid 2019;29:1209-1219.

57. Ho KY, Veldhuis JD, Johnson ML, Furlanetto R, Evans WS, Alberti KG, Thorner MO. Fasting enhances growth hormone secretion and amplifies the complex rhythms of growth hormone secretion in man. J Clin Invest 1988;81: 968-975.

58. Pradhan G, Samson SL, Sun Y. Ghrelin: much more than a hunger hormone. Curr Opin Clin Nutr Metab Care 2013;16: 619-624.

59. Kanakis GA, Tsametis CP, Goulis DG. Measuring testosterone in women and men. Maturitas 2019;125:41-44.

60. Guidi GC, Simundic AM, Salvagno GL, Aquino JL, Lima-Oliveira G. To avoid fasting time, more risk than benefits. Clin Chem Lab Med 2015;53:e261-e264.

61. Lima-Oliveira G, Valentim CD, Guidi GC. Lipid profile, changes in laboratory prescriptions are necessary. J Clin Lipidol 2017;11:768-769.

62. MacKenzie T, Comi R, Sluss P, Keisari R, Manwar S, Kim J, Larson R, Baron JA. Metabolic and hormonal effects of caffeine: randomized, double-blind, placebo-controlled crossover trial. Metabolism 2007;56:1694-1698.

63. Schubert MM, Irwin C, Seay RF, Clarke HE, Allegro D, Desbrow B. Caffeine, coffee, and appetite control: a review. Int J Food Sci Nutr 2017;68:901-912.

64. Kuang A, Erlund I, Herder C, Westerhuis JA, Tuomilehto J, Cornelis MC. Lipidomic Response to Coffee Consumption. Nutrients 2018;10:E1851.

65. Cornelis MC, Erlund I, Michelotti GA, Herder C, Westerhuis JA, Tuomilehto J. Metabolomic response to coffee consumption: application to a three-stage clinical trial. J Intern Med 2018;283:544-557.

66. Matta SG, Fu Y, Valentine JD, Sharp BM. Response of the hypothalamo-pituitary-adrenal axis to nicotine. Psychoneuroendocrinology 1998;23:103-113.

67. Mello NK. Hormones, nicotine, and cocaine: clinical studies. Horm Behav 2010;58:57-71.

68. Andersen AN, Ronn B, Tjonneland A, Djursing H, Schioler V. Low maternal but normal fetal prolactin levels in cigarette smoking pregnant women. Acta Obstet Gynecol Scand 1984;63:237-239.

69. Andersen AN, Lund-Andersen C, Larsen JF, Christensen NJ, Legros JJ, Louis F, Angelo H, Molin J. Suppressed prolactin but normal neurophysin levels in cigarette smoking breast-feeding women. Clin Endocrinol 1982;17:363-368.

70. Bastin P, Maiter D, Gruson D. Salivary cortisol testing: preanalytic and analytic aspects. Ann de Biol Clin 2018;76: 393-405.

71. Restituto P, Galofre JC, Gil MJ, Mugueta C, Santos S, Monreal JI, Varo N. Advantage of salivary cortisol measurements in the diagnosis of glucocorticoid related disorders. Clin Biochem 2008;41:688-692.

72. Raff H, Singh RJ. Measurement of late-night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. Clin Chem 2012;58:947-948.

73. Lewy AJ, Sack RL, Blood ML, Bauer VK, Cutler NL, Thomas KH. Melatonin marks circadian phase position and resets the endogenous circadian pacemaker in humans. Ciba Found Symp 1995;183:303-321.

74. Samperi I, Lithgow K, Karavitaki N. Hyperprolactinaemia. J Clin Med 2019;8:2203.

75. Lewandowski KC, Skowronska-Jozwiak E, Szosland K, Lewinski A. Effect of timing of prolactin sampling on the incidence of spurious hyperprolactinaemia. Endocr Abstracts 2005;9:223.

76. Lima-Oliveira G, Lippi G, Salvagno GL, Brocco G, Guidi GC. In vitro diagnostic company recalls and medical laboratory practices: an Italian case. Biochem Med 2015;25:273-278.

77. Hanon EA, Sturgeon CM, Lamb EJ. Sampling and storage conditions influencing the measurement of parathyroid hormone in blood samples: a systematic review. Clin Chem Lab Med 2013;51:1925-1941.

78. Lima-Oliveira G, Lippi G, Salvagno GL, Montagnana M, Picheth G, Guidi GC. Preanalytical management: serum vacuum tubes validation for routine clinical chemistry. Biochem Med 2012;22:180-186.

79. Zungun C, Yilmaz FM, Boru EG, Topcuoglu C. Comparison of Improvacuter tubes with BD Vacutainer tubes for various hormones in the aspects of stability and influence of gel separators. Clin Chem Lab Med 2015;53:231-238.

80. Bowen RA, Remaley AT. Interferences from blood collection tube components on clinical chemistry assays. Biochem Med 2014;24:31-44.

81. Bowen RA, Adcock DM. Blood collection tubes as medical devices: The potential to affect assays and proposed verification and validation processes for the clinical laboratory. Clin Biochem 2016;49:1321-1330.

82. Bowen RA, Chan Y, Ruddel ME, Hortin GL, Csako G, Demosky SJ Jr, Remaley AT. Immunoassay interference by a commonly used blood collection tube additive, the organosilicone surfactant silwet L-720. Clin Chem 2005;51: 1874-1882.

83. Bowen RA, Sattayapiwat A, Gounden V, Remaley AT. Blood collection tube-related alterations in analyte concentrations in quality control material and serum specimens. Clin Biochem 2014;47:150-157.

84. Chang CY, Lu JY, Chien TI, Kao JT, Lin MC, Shih PC, Yan SN. Interference caused by the contents of serum separator tubes in the Vitros CRP assay. Ann Clin Biochem 2003;40: 249-251.

85. Giavarina D, Banfi G, Daves M, Dolci A, Farci Santarcangeli D, Lima-Oliveira G, Miconi D, Milanesi B, Montagnana M, Morandini M, Piva E, Salvano GL, Troiano T, Lippi G. Blood collection systems in clinical laboratories: local adaptation of the EFLM guidelines. Biochim Clin 2016;40:347-352.

86. International Organization for Standardization. Medical laboratories-Requirements for quality and competence ISO document 15189. Geneva, Switzerland: International Organization for Standardization; 2012.

87. Evans MJ, Livesey JH, Ellis MJ, Yandle TG. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. Clin Biochem 2001;34: 107-112.

88. English E, McFarlane I, Taylor KP, Halsall DJ. The effect of potassium EDTA on the stability of parathyroid hormone in whole blood. Ann Clin Biochem 2007;44:297-299.

89. Clinical Laboratory Standards Institute. Procedures for the handling and processing of blood specimens for common laboratory tests. CLSI H18-A4 document. 4th ed. Wayne, PA: Clinical Laboratory Standards Institute; 2010.

90. BD Diagnostics - Preanalytical Systems. Product Catalogue https://www.bd.com/resource.aspx?IDX=10155 [Accessed June 10th 2019].

91. Greiner Bio-One Product Catalog. Vacuette blood collection tubes. https://www.gbo.com/fileadmin/user_upload/1729004RN_Joint_Catalog_2017_Web.pdf [Accessed June 10th 2019].

92. Sarstedt full product catalogue. https://www.sarstedt.com/fileadmin/user_upload/Katalog/2019/45_200_catalogue_en_eu_eu_2019_2020_hp.pdf [Accessed June 10th 2019].

93. MacNutt MJ, Sheel AW. Performance of evacuated blood collection tubes at high altitude. High Alt Med Biol 2008;9(3):235-7.

94. Chuang J, Sadler MA, Witt DM. Impact of evacuated collection tube fill volume and mixing on routine coagulation testing using 2.5-ml (pediatric) tubes. Chest. 2004;126(4): 1262-6.

In this issue: Improving the preanalytical phase in laboratory medicine

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Methods for hemolysis interference study in laboratory medicine – a critical review

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ABSTRACT

Hemolysis represents an important source of error associated with the pre-analytical phase. Improving the protocols for detection, measurement, management of the parameters affected by the interference, and differentiation between hemolysis *in vivo* and *in vitro*, would favor a personalized management of hemolysis by increasing patient safety.

For this, it is essential to agree on the definition of "hemolysis". From this definition, a critical point is to establish cut-offs of hemolysis management for each analyte studied in the clinical laboratory.

Thus, in this review, the main methods described in the literature developed for obtaining a hemolysate are grouped, that simulate in controlled laboratory protocols what happens with a hemolyzed sample of a patient.

These methods are grouped into 3 categories according to their basis of lysing cells: freezing-thawing, osmotic shock and shear stress. In addition to development and improvement of methods for the study of

hemolysis, it is necessary to carry out comparative studies to determine which one offers the best capabilities. Harmonization of the methods will allow to include them in working guidelines. All these strategies will allow to move from managing hemolysis on whole-sample basis to customize it analyte by analyte.

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INTRODUCTION

Most of the errors associated with the total testing process occur in the pre-analytical phase (70-80%) (1,2). This phase includes sample collection, handling and transport, whose inadequate realization entails, inter alia, the possible appearance of analytical interferences, the main one being hemolysis (3). The presence of this interference in the samples affects the analytical determination of the biochemical tests, having as a consequence the need to recollect samples, delays in patient diagnosis and follow-up (crucial in the emergency laboratories), decreased patient safety, increased costs, …, etc. (4-6).

The hemolysis process is characterized by accelerated breakdown of the erythrocyte (RBC) membranes, releasing intra-erythrocyte content to the extracellular compartment (7). In this process not only hemoglobin is released, other components of the erythrocyte cytoplasm such as potassium, lactate dehydrogenase (LDH), or neuro-specific enolase (NSE) among other components.

All these elements can interfere with the measurements of the biochemical tests, and this interference will be more or less important depending on the magnitude assayed and the degree of hemolysis (8).

Due to the importance of knowing the degree of interference of the different levels of hemolysis over biochemical determinations, it is crucial to develop and harmonize appropriate methods to establish as closely as possible cut-off points for the proper hemolysis management (9).

This step is very important for the subsequent development of rules that allow harmonized handling of hemolyzed samples. Thus, this review aims to provide insight into the methods available for studying hemolysis interference in clinical laboratories.

CAUSES OF HEMOLYSIS

The knowledge of the hemolysis degree of a sample is very important. This enables a different handling of the sample, appropriate to the type of hemolysis, and to perform corrective actions of the interferences.

Hemolysis can be due either to pathophysiological reasons or to causes exogenous to the patient. Thus, hemolysis can be classified into two large blocks:

- ***Hemolysis* in vivo**

  It is characterized by the breakdown of RBCs due to endogenous causes. Different degrees of anemia can be observed in the affected patient, able to increase the activity of the bone marrow as a compensatory mechanism (6). This premature rupture of RBCs may be due to antigen-antibody reactions, chemical reactions, hemolytic anemias, toxins or physical agents such as artificial heart valves, particularly mechanical ones (5). Less than 2% of the hemolysed samples detected in the clinical laboratory are due to *in vivo* processes (10). The handling of this type of samples will be aimed at knowing the patient's clinical situation and communicating the interferences to the clinician, in order to consider any possible treatments of the patient relevant to his/her pathophysiological situation.

- ***Hemolysis* in vitro:**

  In this case, the rupture of the RBCs is due to improper blood collection or sample handling (11). This kind of hemolysis may be due to phlebotomst using too thin needle size, inappropriate tubes mixing, or a too prolonged tourniquet time (12), as well as due to causes related to the transport or storage of the samples (13,14). In addition, this type of hemolysis is the one on which the clinical laboratory plays a more important role trying to reduce its incidence. To do this, it must first be estimated and managed properly by including in the report the degree of interference, e.g. through comments, as well as by managing indicators to know their impact on the blood collection centers, strengthening the training where sample hemolysis rates are higher than the quality objectives set.

The knowledge of the type of hemolysis is important in order to perform the more appropriate management of the interfered parameters (Table 1).

Table 1 List of different causes of hemolysis occurring *in vivo* and *in vitro* *

Causes of hemolysis *in vivo*	
Inherited	**Acquired**
Defects in hemoglobin	Immune-mediated causes
Defects of RBC membrane	Hypersplenism
Defective red cell metabolism	Burns
Glucose-6-phosphate dehydrogenase deficiency	Infections
Pyruvate kinase deficiency	Mechanical damage in circulation
Other RBC enzyme deficiencies	Inmunophenotyping errors in blood transfusion
	Drugs and toxins
Causes of hemolysis *in vitro*	
Operator-dependent	Handling of the specimen
	Transport of the specimen
Device-dependent	Sample processing
	Storage of the specimen

**Table adapted from Lippi et al (7).*

HEMOLYSIS MECHANISMS

Biochemical tests are interfered by RBCs breakdown as a result of the release of their cellular content. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) defines interference as "the systematic error of measurement caused by a sample component, which does not, by itself, produces a signal in the measuring system" (15). Interference (bias) in hemolysis processes will occur through four types of mechanisms described below:

- ***Increase in the concentration of the constituent by intracellular release***

  Inside the red blood cell there are several analytes with higher concentration than in the surrounding fluid. Thus, their release as a result of hemolysis generates a positive interference on the relevant assays, causing overestimation of the real values. Potassium, LDH, AST, magnesium or phosphorus are the parameters that have the greatest difference between the intracellular medium of the RBC and the extracellular medium showing significant interference already at low hemolysis levels (7).

- ***Chemical interference***

  In this mechanism, the interfering substance acts on one of the phases of the chemical reaction of the assay. In general, the interaction or competition of some substances released from the RBC, such as proteins or lipids, with reagents or reaction products will occur, being altered the concentration of the analyzed parameter (16). As examples of this interaction, the effect that hemoglobin exerts on the measurement of bilirubin, or the overestimation of creatin kinase by interference with adenylate kinase released by RBC.

- ***Spectrophotometric interference***

  The wavelength at which the parameters that are measured spectrophotometrically are determined can overlap with the hemoglobin absorption spectrum. Thus, the assay of the analyte will undergo interference depending on the hemolysis degree. Both oxyhemoglobin and deoxyhemoglobin have a maximum absorption at 415 nm with a detection range between 320 nm and 450 nm, and between 540 nm and 589 nm respectively. The assays whose detection wavelength is between the above intervals will be affected, e.g. lipase, albumin and g-glutamyl transferase (GGT) (17,18). These analytes will be overestimated, while others such as alkaline phosphatase (ALP) will be underestimated when hemoglobin is degraded in alkaline medium (17).

- ***Interference by dilutional effect***

  The analytes whose concentration is lower inside the RBC with respect to the extracellular fluids, will undergo further diminution when hemolysis occurs, due to dilutional effect. Thus, sodium, chloride and glucose are classic examples of this type of interference (7). Such an interference on sodium, which has a very narrow homeostatic regulation, can have important clinical relevance.

It is possible that, when hemolysis levels are very high, several described mechanisms happen simultaneously (17). The interference degree caused by hemolysis is not only important in spectrophotometric biochemical tests, but also in gasometric, hematocytometric, coagulometric and immunoassay tests.

MEASUREMENT OF HEMOLYSIS IN CLINICAL LABORATORY

Both types of hemolysis, *in vitro* and *in vivo*, are classically assessed by visual inspection comparing

the serum or plasma with a color scale after centrifuging the sample (19,20). This way of measuring hemolysis has important disadvantages (21), since it is a time-consuming method, therefore for laboratories with a high workload it is not very viable (5,22). Moreover, it is dependent on the observer thus on the degree of the observer training (23,19), and hardly standardizable to avoid intralaboratory biases (24). On the other hand, the assessment of hemolysis by visual inspection can be hindered by the presence of other interferers such as bilirubin, that adds color to the sample preventing a correct reading (25). Finally, this kind of hemolysis management could have consequences on patient safety (26).

To overcome the above difficulties in the assessment of hemolysis, the analyzers have progressively incorporated automated methods for the determination not only of hemolysis but also of icterus and lipemia (27). All these methods are based on performing a sample dilution and making multiple readings at different wavelengths using spectrophotometry (17). Farrell and Carter describe the characteristics of the automated HIL (hemolysis, icterus and lipemia) measurement methods of different analytical platforms currently available (17). With these methods, a qualitative or semi-quantitative determination is transformed to a quantitative determination allowing the development of control materials to implement both internal quality control programs (IQC) (28), as well as external quality control programs (EQA) for comparison among laboratories (29). But there are still challenges, making it difficult to implement this type of method in laboratories. Among the unresolved problems, Lippi lists several such as poor harmonization of detection techniques, non-standardization of the measurement units, improvement of the quality control systems available, and heterogeneity of the cut-offs between the different analytical platforms (30). Efforts are currently being made to try to solve these problems (28, 31), as well as demonstrating the low impact of any of them (32).

STUDIES FOR DETERMINATE HEMOLYSIS *IN VIVO*

Being able to differentiate hemolysis *in vivo* from hemolysis *in vitro* is presently a current challenge in the clinical laboratory, because of the impact more at the clinical level than at the analytical level. Currently there is no established consensus for the differentiation of hemolysis *in vivo* from hemolysis *in vitro*, but there are examples in the literature of strategies that try to improve differentiation (33). A described strategy is to compare different samples received in the laboratory of the same patient (34). When receiving multiple samples from a patient, the hemolysed sample is compared with the rest of the patient's samples; if all the samples show hemolysis levels above the set cut-off for hemolysis, a high probability exists of *in vivo* hemolysis. That should be confirmed with the medical history. On the other hand, if only one sample is hemolysed, an *in vitro* hemolysis can be suspected.

Lippi et al propose an algorithm for screening samples in which hemolysis is suspected *in vivo* (35). The hemolysis index value is transformed to g/L of hemoglobin. The samples with a free hemoglobin value greater than the upper reference limit (URL) and without suspected *in vitro* hemolysis will be tested further to confirm hemolysis *in vivo*. The development of mathematical models to allow screening of whole blood samples with hemolyzed *in vitro* has been proposed (36).

In addition to the algorithms, there are biomarkers that can help to determine hemolysis *in vivo.* These biomarkers have variable sensitivities and specificities for the study of hemolysis. Analytes such as potassium, LDH and aspartate aminotransferase (AST) increase markedly in hemolysis *in vitro* (34,37), however the concentration of potassium is not interfered always by hemolysis

in vivo (38). These samples can show increased potassium value for other physiopathological reasons, making screening difficult.

The decrease in haptoglobin concentration is considered the best marker to evaluate accelerated destruction of intravascular erythrocytes (39). In *in vitro* hemolysis this parameter is not affected since the resulting haptoglobin-hemoglobin complexes are rapidly eliminated from the circulation by monocytes and tissue macrophages CD163 receptor pathway (40). However, haptoglobin is decreased by hemolysis *in vivo*. But haptoglobin expression also decreases in liver function diseases which makes it difficult to use in the hemolysis assessment (41). Thus it should not be viewed as a gold standard (39).

Other typical *in vivo* hemolysis markers are indirect bilirubin and reticulocyte count increase, as well as decreased erythrocyte indices such as Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH). But none of the biomarkers described are going to be affected only by the presence of hemolysis, therefore it is necessary to develop specific biomarkers to help us differentiate *in vivo* hemolysis from *in vitro* hemolysis.

Currently there is no established consensus to distinguish hemolysis *in vivo* and *in vitro* (7). Thus it is necessary to continue working on the development of algorithms, that allow us to differentiate the origin of the hemolysis, due to its clinical significance.

ASSESSMENT OF *IN VITRO* HEMOLYSIS

Due to the high impact that *in vitro* hemolysis has on the assays in the clinical laboratory, homogeneous procedures are needed that allow us to determine cut-off points for the proper handling of hemolyzed samples. There is currently a high degree of heterogeneity in these studies (42,18). Thus we can decide whether or not to report the result obtained (43,44), or which is the most appropriate handling procedure depending on the parameter. In 2018, the Working Group for Preanalytical Phase (WG-PRE) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) (45), published a document of recommendations for the harmonized management of the results of hemolyzed samples, in order to know the cut-offs for each analyte in each analytical platform, since the values are dependent on method and instrument (46). Thus, an individualized management of each parameter can be achieved, all not being influenced in the same way by the same degree of hemolysis (46).

All the methods described in the literature and used for *in vitro* hemolysis studies are based on three fundamental strategies:

- **Sample Freezing:** i.e. Freezing of the sample or freeze-thaw cycles to breakdown the cells.
- **Osmotic shock:** i.e. Rupture of the cells when resuspended in hypotonic medium, usually distilled water.
- **Shear stress:** i.e. Mechanical rupture of the cells by passing them through a needle.

In this section, different strategies for estimating the degree of interference by hemolysis, of the analytes to be measured in the clinical laboratory will be described.

CLASSICAL METHODS FOR HEMOLYSIS INTERFERENCE STUDY

The key point is to obtain a hemolysate that adequately represents the working conditions. Different strategies for obtaining hemolysate have been described. Having a hemolysate that mimics most closely hemolyzed patient samples will allow the most appropriate handling of the samples. Lovelock describes a method for obtaining hemolyses based on the freezing of the sample (47), e.g. starting from the whole blood sample and freezing it or freezing washed red

blood cells. Freezing is performed over a period of 30 min, which is insufficient to break the platelets and leukocytes if whole blood is used, requiring longer periods of freezing (48).

Twenty years later, Meites published a modified method based on the Lovelock method (49). The Meites method is the classic method used to obtain hemolysis recommended by different guides (50). In this method a hemolysate is generated which will be used to add it to serum or plasma samples of patients where interference is studied. Anticoagulated whole blood is centrifuged to obtain the sedimented RBCs and washed 3 times with isotonic saline. The cells are frozen overnight in water and thawed at room temperature next day. It is centrifuged to remove cell debris and the hemoglobin concentration in the hemolysate is determined. Finally, hemolyzate is added to serum samples or plasma at different concentrations. Freezing-thawing of these methods breaks the blood cells and centrifugation removes cell debris, similar to the handling of the samples after venipuncture. To try to improve this method, studies have been done modifying the conditions of freezing temperature and freezing time (51).

It is important to consider that in these methods, starting from centrifuged and washed RBCs, the contribution that the breakdown of leukocytes and platelets have on the interference caused by hemolysis is lost.

Another alternative is the method described by Glick et al (52). This method starts with washed erythrocytes that are only going to be lysed using distilled water (osmotic shock). The hemolysate is cleaned by filtering on the glass wool and subsequent centrifugation. Like the Meites method, it has the limitation that only the breakdown of RBCs contributes to hemolyzing. Other method, less used, is proposed by Frank et al (53). This method also starts with washed erythrocytes to which distilled water and detergent are added to favor its rupture.

NEW APPROACH FOR HEMOLYSIS INTERFERENCE STUDY

Based on the work of Meites and Glick, different strategies have been developed with the aim of achieving better hemolysates. Some of them are new strategies and others are modifications of those already available. The classical methods were based on freezing strategies (Lovelok and Meites) or osmotic shock (Glick) using distilled water. In 2004, Dimeski described a new method based on passing the anticoagulated whole blood sample through a needle several times to cause the breakdown of cellular components (54). The number of times the sample is passed through the needle determines the degree of hemolysis achieved.

Lippi et al (55) made a modification of Dimeski method, by standardizing the number of times the sample was passed through the needle (1 to 4 times), the type of syringe used (insulin type, 0.5 mL), as well as the thickness of the needle (30 gauge). The sample was centrifuged to remove the debris before determining the amount of free hemoglobin. As in the freeze-thaw models, with this method the erythrocytes were not selectively broken as in the osmotic shock models, since all blood cells in the sample (erythrocytes, leukocytes and platelets) are broken (9). In addition, an advantage of Dimeski's method is that by passing the sample through a needle simulates what is happening when collecting a patient's sample (56). The Lippi modified Dimeski method has been used for studies of hemolysis interference in parameters such as gasometry (56), coagulation (57), and hematimetry (58). Using this method, Lippi et al obtained the following conclusions (58):

- The hematology equipment used does not discriminate erythrocyte hemoglobin to free hemoglobin.
- A decrease in the RBC and hematocrit count due to mechanical rupture is

observed, confirmed by a parallel increase in LDH and hemolytic index.

- A significant decrease in the number of total leukocytes is not detected
- Problems were observed in leukocyte formula assessment due to interference with RBC and platelet clumps.

In this type of samples, the main interference is not associated with the presence of free hemoglobin, but with the rupture of the different blood cell lines, leading to significant errors in the cell count. In hematological samples it is not appropriate to apply the methods of osmotic shock and freezing because all the cells in the sample would be broken and could not be assessed in the hematological counter.

In methods employing whole blood (gasometry and hematocytometry), it has been proposed to perform a previous screening of the samples by collecting an aliquot, centrifuging and determining visually the degree of hemolysis (59). This approach, especially for a laboratory with high workloads, would increase response times and increase workloads on the whole. These disadvantages would be more critical in emergency laboratories where the number of samples of gasometry and hematocytometry is high.

Recently Delgado et al, based on freezing-thawing methods for hemolysis studies, proposed two different strategies (60):

- ***Strategy 1***

  Collection of two anticoagulated whole blood tubes. One was centrifuged and the supernatant (plasma) was collected. The other tube was frozen-thawed 3 times, centrifuged and the supernatant was collected. The two supernatants (plasma) obtained in different proportions were mixed.

- ***Strategy 2***

  Collection of two anticoagulated whole blood tubes. One of them was centrifuged, the supernatant (plasma) was removed and the pellet was frozen-thawed (without using distilled water). The other tube was centrifuged and the supernatant (plasma) was collected. Different volumes of hemolysate to the plasma of the second tube were added.

In both strategies a negative interference (decrease in concentration) was observed in the determination of Na^+, but strategy 1 presented better repeatability. The interference on Na^+ was due to dilution, therefore not including dilution with water in these strategies allowed a better assessment of Na^+ interference compared to previous studies that did include the dilution step (61).

In order to evaluate the suitability of the methods in the study of hemolysis interference, and to verify which one has better performance, it is necessary to carry out comparison studies between them. Studies have been carried out to compare the methods of obtaining hemolysate by osmotic shock and shear stress. In both cases they have been studied to determine the effect of hemolysis on the determination of ammonium (54,62), showing a better detection of interference using the osmotic shock method.

These studies did not include the freezing-thawing method, so Gidske et al (63) first compared the three main methods, namely: freezing, osmotic shock and shear stress. They studied 10 biochemical parameters on two different analytical platforms based on liquid chemistry or alternatively on dry chemistry assays. LDH was the parameter that provided more information, the interference observed was greater in the freezing-thawing and shear stress methods than the osmotic shock methods. The shear stress method produced the breakdown of erythrocytes,

platelets and leukocytes but it was a laborious method, with a high variability in the release of hemoglobin. On the other hand, the freezing method is simpler, with less variability and the three cell lines are also broken. In this study, freezing-thawing method was recommended as the most appropriate for obtaining hemolysates in hemolysis interference studies.

OTHER METHODS

In addition to the methods described above, other methods for the breakdown of blood cells based on different physical principles have been published. Yücel et al described the mechanical rupture of cells by stirring whole blood with a metallic bar (64). In another paper, Larga et al proposed hemolysis using tissue homogenization equipment (65). For the realization of these methods, equipment is needed that is not normally available in clinical laboratories, limiting its use in hemolysis interference studies.

Table 2 summarizes the characteristics of the methods for performing *in vitro* hemolysis studies in the clinical laboratory.

Table 2 Main methods reported for obtaining in *vitro* hemolysates and their characteristics

Method	Basis	Sample	Use water	Cell type break
Lovelock (47)	Freezing-thawing	Whole blood/ Washed Erythrocytes	No	Erythrocytes-Leukocytes-Platelets/ Erythrocytes
Meites (49)	Osmotic shock/ Freezing-thawing	Washed Erythrocytes	Yes	Erythrocytes
Glick et al. (52)	Osmotic shock	Washed Erythrocytes	Yes	Erythrocytes
Frank et al. (53)	Osmotic shock	Washed Erythrocytes	Yes (+detergent)	Erythrocytes
Dimeski (54)	Shear stress	Whole blood	No	Erythrocytes-Leukocytes-Platelets
Dimeski adapted by Lippi et al. (58)	Shear stress	Whole blood	No	Erythrocytes-Leukocytes-Platelets
Delgado et al. (60)	Freezing-thawing	Whole blood	No	Erythrocytes-Leukocytes-Platelets/ Erythrocytes

CONCLUSIONS

The harmonization/standardization of the processes performed in the clinical laboratory is an important objective to reduce variability and generate high quality results. The proper handling of hemolysis interference, in addition to correct analytical measurements, is particularly influenced by the determination of precise cut-offs for each analyte studied.

At first, it should be defined "hemolysis", i.e. whether it is the rupture of only the erythrocytes or of all blood cells (erythrocytes, leukocytes and platelets). Following a classical approach, e.g. Meites et al and Glick et al, only washed erythrocytes are employed. On the other hand, more recent studies, such as Lippi et al, take into account the contribution of the three cell lines, and Delgado et al and Gidske et al test different strategies, concluding that methods based on whole blood samples are more suitable for interference studies by hemolysis. Once the term "hemolysis" is defined, it is necessary to develop consensus protocols for the study of interference by hemolysis, by exchanging results between laboratories. In this review we have described the three groups of procedures currently used: freezing-thawing, osmotic shock and shear stress. Among these, a lot of heterogeneity in the method used to obtain hemolysate is reported in literature. To solve the problem it is necessary to carry out comparative studies of the available methods, such as that carried out by Gidske et al, in order to select the most appropriate protocol.

Another possible approach, as done by Delgado et al., is based on a method already described that introduces modifications allowing a better assessment of the degree of interference in the samples. All these studies should be extended to all the currently available analytical methods as well as to all analytical platforms. The availability of consensual method(s), could allow their introduction into international guides, such as CLSI, harmonizing the protocols for carrying out hemolysis studies. The proper definition of the methods to be used in each situation would allow to obtain precise cut-offs independent of the method used to determine the degree of hemolysis interference. Thus, the management of hemolysis through interpretative comments proposed by the WG-PRE of the EFLM, would allow better assessment of each parameter that is being interfered and to obtain information useful for either the management of the clinical laboratory or the interpretation of the result at the clinical level.

Finally, a need is apparent to continuously progress in developing algorithms to differentiate hemolysis *in vivo* from *in vitro,* wieved as a challenge for the future in the clinical laboratory. In summary, with the harmonization/standardization of all these strategies, the integral management of hemolysis as a whole (clinical-analytical) will be favored with a notable increase in patient safety.

REFERENCES

1. Plebani M. The detection and prevention of errors in laboratory medicine. Ann Clin Biochem. 2010; 47: 101–110.

2. Plebani M, Lippi G. Closing the brain-to-brain loop in laboratory testing. Clin Chem Lab Med. 2011;49:1131–3.

3. Chawla R, Goswami B, Tayal D, Mallika V. Identification of the types of preanalytical errors in the clinical chemistry laboratory: 1-year study at G.B. Pant Hospital. Lab Med. 2010; 41(2):89–92.

4. Lima-Oliveira G, Volanski W, Lippi G, Picheth G, Guidi GC. Pre-analytical phase management: a review of the procedures from patient preparation to laboratory analysis. Scand J Clin Lab Invest. 2017;77(3):153-163.

5. Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, Vassault AJ, Plebani M. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med. 2008;46(6):764-72.

6. Lippi G, Plebani M, Di Somma S, Cervellin G. Hemolyzed specimens: a major challenge for emergency departments

and clinical laboratories. Crit Rev Clin Lab Sci. 2011;48(3): 143-53.

7. Lippi G, Cervellin G, Favaloro EJ, Plebani M. In vitro and in vivo hemolysis. An unresolved dispute in laboratory medicine. Berlin, Germany: Walter de Gruyter GmbH, 2012.

8. Heireman L, Van Geel P, Musger L, Heylen E, Uyttenbroeck W, Mahieu B. Causes, consequences and management of sample hemolysis in the clinical laboratory. Clin Biochem. 2017;50(8):1317–1322.

9. Lippi G. Interference studies: focus on blood cell lysates preparation and testing. Clin Lab. 2012;58(3-4):351–5.

10. Carraro P, Servidio G, Plebani M. Hemolyzed specimens: a reason for rejection or a clinical challenge? Clin Chem. 2000; 46(2):306-7.

11. Bush V, Mangan L. The hemolyzed specimen: causes, effects, and reduction. BD Vacutainer Syst Preanalytical Solut. 2003;2003:1-8.

12. Lippi G, Salvagno GL, Solero GP, Guidi GC. The influence of the tourniquet time on hematological testing for antidoping purposes. Int J Sports Med. 2006;27(5):359–362.

13. Simundic AM, Topic E, Nikolac N, Lippi G. Hemolysis detection and management of hemolyzed specimens. Biochem Med (Zagreb). 2010;20(2):154-159.

14. Lippi G, Avanzini P, Pavesi F, Bardi M, Ippolito L, Aloe R, Favaloro EJ. Studies on in vitro hemolysis and utility of corrective formulas for reporting results on hemolyzed specimens. Biochem Med (Zagreb). 2011;21(3):297-305.

15. Thomas L. Haemolysis as influence and interference factor. EJIFCC. 2002;13(4):95-98.

16. Gonzalez-Porras JR, Graciani IF, Alvarez M, Pinto J, Conde MP, Nieto MJ, Corral M. Tubes for pretransfusion testing should be collected by blood bank staff and labeled until the implementation of new technology for improved sample labelling. Results of a prospective study. Vox Sang. 2008;95:52–6.

17. Farrell CJL, Carter AC. Serum indices: managing assay interference. Ann Clin Biochem. 2016;53:527-38.

18. Lippi G, Salvagno G, Montagnana M, Brocco G, Guidi GC. Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med. 2006; 44(3): 311–6.

19. Hawkins R. Discrepancy between visual and spectrophotometric assessment of sample haemolysis. Ann Clin Biochem. 2002;39:521-2.

20. Simundic AM, Nikolac N, Ivankovic V, Ferenec-Ruzic D, Magdic B, Kvaternik M, Topic E. Comparison of visual vs. automated detection of lipemic, icteric and hemolyzed specimens: can we rely on a human eye? Clin Chem Lab Med. 2009;47(11):1361-5.

21. Lippi G, Cadamuro J. Visual assessment of sample quality: quo usque tandem?. Clin Chem Lab Med. 2018; 56(4):513-515.

22. Howanitz PJ, Lehman CM, Jones BA, Meier FA, Horowitz GL. Practices for identifying and rejecting hemolyzed specimens are highly variable in clinical laboratories. Arch Pathol Lab Med. 2015;139(8):1014-9.

23. Glick MR, Ryder KW, Glick SJ, Woods JR. Unreliable visual estimation of the incidence and amount of turbidity, hemolysis, and icterus in serum from hospitalized patients. Clin Chem. 1989;35(5):837-9.

24. Kato GJ, McGowan V, Machado RF, Little JA, Taylor J VI, Morris CR, Nichols JS, Wang X, Poljakovic M, Morris SM Jr, Gladwin MT. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. Blood. 2006;107(6): 2279-85.

25. Jeffery J, Sharma A, Ayling RM. Detection of haemolysis and reporting of potassium results in samples from neonates. Ann Clin Biochem. 2009;46:222-5.

26. Luksic AH, Gabaj NN, Miler M, Dukic L, Bakliza A, Simundic AM. Visual assessment of hemolysis affects patient safety. Clin Chem Lab Med. 2018; 56(4): 574-581.

27. Lippi G, Plebani M. Continuous-Flow automation and hemolysis index: A crucial combination. J Lab Autom. 2013; 18(2):184-8.

28. Lippi G, Cadamuro J, Danese E, Gelati M, Montagnana M, von Meyer A, Salvagno GC, Simundic AM. Internal Quality Assurance of HIL indices on Roche Cobas c702. PlosOne. 2018;13(7):e0200088.

29. Programa de Garantía de la Calidad para Laboratorios Clínicos-Preanalitica. Sociedad Española de Medicina de Laboratorio (SEQCML): www.seqc.es/es/programas-garantia-calidad.

30. Lippi G. Systematic assessment of the hemolysis index: pros and cons. Adv Clin Chem. 2015;71:157-70.

31. Von Mayer A, Cadamuro J, Lippi G, Simundic AM. Call for more transparency in manufactures declarations on serum indices (HIL): On behalf of the working group for preanalitycal phase (WG-PRE), European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). Clin Chim Acta. 2018;484: 328-332.

32. Lippi G, Avanzini P, Campioli D, Da Rin G, Dipalo M, Aloe R, Giavarina D, Salvagno GL. Systematical assessment of serum indices does not impair efficiency of clinical chemistry testing: A multicenter study. Clin Biochem. 2013;46(13-14):1281-4.

33. Ismail A, Shingler W, Seneviratne J, Burrows G. In vitro and in vivo haemolysis and potassium measurement. BMJ. 2005;330(7497):949.

34. Abd Elrouf MB, Amanullah M, Zaman GS. Interference of hemolysis in the estimation of plasma aspartate aminotransferase, potassium and phosphate. Journal of Investigational Biochemistry. 2014;3(1):12-16.

35. Lippi G, Favaloro EJ, Franchini M. Haemolysis index for the screening of intravascular haemolysis: a novel diagnostics opportunity? Blood Transfus. 2018; 16(5): 433-437.

36. Lippi G, Pavesi F, Avanzini P, Chetta F, Aloe R, Pipitone S. Development of simple equations for effective screening of spurious hemolysis in whole-blood specimens. Int J Lab Hematol. 2015;37(2), 253-8.

37. Withold W. Monitoring of bone turnover biological, preanalytical and technical criteria in the assessment of biochemical markers. Eur J Clin Chem Clin Biochem. 1996; 34(10):785-99.

38. Blank DW, Kroll MH, Ruddel ME, Elin RJ. Hemoglobin interference from in vivo hemolysis. Clin Chem. 1985;31(9): 1566-9.

39. Shih AW, McFarlane A, Verhovsek M. Haptoglobin testing in hemolysis: Measurement and interpretation. Am J Hematol. 2014;89(4):443-7.

40. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. Nature. 2001;409(6817): 198-201.

41. Körmöczi GF, Säemann MD, Buchta C, Peck-Radosavljevic M, Mayr WR, Schwartz DW, Dunkler D, Spitzauer S, Panzer S. Influence of clinical factors on the haemolysis marker haptoglobin. Eur J Clin Invest. 2006;36(3):202-9.

42. Zemlin AE, Essack Y, Rensburg M, Keller T, Brinkmann T. Stability of red blood cell folate in whole blood and haemolysate. Clin Lab. 2010;56(9-10):391-6.

43. Cadamuro J, Mrazek C, Haschke-Becher E, Sandberg S. To report or not to report: a proposal on how to deal with altered test results in hemolytic samples. Clin Chem Lab Med. 2017;55(8):1109-1111.

44. Lippi G, Cervellin G, Plebani M. Reporting altered test results in hemolyzed samples: is the cure worse than the disease? Clin Chem Lab Med. 2017;55(8):1112-1114.

45. Lippi G, Cadamuro J, von Meyer A, Simundic AM. European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group of Preanalitycal Phase (WG-PRE). Practical recommendations for managing hemolyzed samples in clinical chemistry testing. Clin Chem Lab Med. 2018;56(5):718-727.

46. Lippi G, Salvagno GL, Favaloro EJ, Guidi GC. Survey on the prevalence of hemolytic specimens in an academic hospital according to collection facility: opportunities for quality improvement. Clin Chem Lab Med. 2009;47(5): 616-8.

47. Lovelock JE. The haemolysis of human red blood-cells by freezing and thawing. Biochim Biophys Acta. 1953;10(3): 414–26.

48. Lippi G, Salvagno GL, Montagnana M, Brocco G, Cesare Guidi G. Influence of the needle bore size used for collecting venous blood samples on routine clinical chemistry testing. Clin Chem Lab Med. 2006;44(8):1009-14.

49. Meites S. Reproducibly simulating hemolysis, for evaluating its interference with chemical methods. Clin Chem. 1973;19(11):1319.

50. Clinical and Laboratory Standards Institute. Interference testing in clinical chemistry; approved guideline, 2nd ed. CLSI document EP07-A2, 2005.

51. Lippi G, Musa R, Aloe R, Mercadanti M, Pipitone S. Influence of temperature and period of freezing on the generation of hemolysate and blood cell lysate. Clin Biochem. 2011; 44:1267-9.

52. Glick M, Ryder K, Jackson S. Graphical comparisons of interferences in clinical chemistry instrumentation. Clin Chem. 1986;32(3):470-5.

53. Frank JJ, Bermes EW, Bickel MJ, Watkins BF. Effect of in vitro hemolysis on chemical values for serum. Clin Chem. 1978; 24(11):1966-70.

54. Dimeski G. Effects of hemolysis on the Roche ammonia method for Hitachi analyzers. Clin Chem. 2004;50(5): 976-7.

55. Lippi G, Avanzini P, Dipalo M, Aloe R, Cervellin G. Influence of hemolysis on troponin testing: studies on Beckman Coulter UniCel Dxl 800 Accu-TnI and overview of the literature. Clin Chem Lab Med. 2011;49(12):2097-100.

56. Lippi G, Fontana R, Avanzini P, Sandei F, Ippolito L. Influence of spurious hemolysis on blood gas analysis. Clin Chem Lab Med. 2013;51(8):1651–4.

57. Lippi G, Plebani M, Favaloro EJ. Interference in Coagulation Testing: Focus on Spurious Hemolysis, Icterus, and Lipemia. Semin Thromb Hemost. 2013;39(3):258–66.

58. Lippi G, Musa R, Avanzini P, Aloe R, Pipitone S, Sandei F. Influence of in vitro hemolysis on hematological testing on Advia 2120. Int. J Lab Hematol. 2012;34(2):179–84.

59. Lippi G, Ippolito L, Fontana R. Prevalence of hemolytic specimens referred for arterial blood gas analysis. Clin Chem Lab Med. 2011;49(5):931–2.

60. Delgado JA, Morell-Garcia D, Bauça JM. Hemolysis interference studies: the particular cases of sodium ion. EJIFCC. 2019;30(1):25-34.

61. Steen G, Vermeer HJ, Naus AJ, Goevaerts B, Agricola PT, Schoenmakers CH. Multicenter evaluation of the interference of hemoglobin, bilirubin and lipids on Synchron LX-20 assays. Clin Chem Lab Med. 2006;44(4):413–9.

62. El-Khoury JM, Bunch DR, Wang S. Is the effect of hemolysis on plasma ammonia measurement overrated? Arch Pathol Lab Med. 2012;136(5):471–2.

63. Gidske G, Solvik UO, Sandberg S, Kristensen GBB. Hemolysis interference studies: freeze method should be used in the preparation of hemolyzed samples. Clin Chem Lab Med. 2018;56(9):e220–e222.

64. Yücel D, Dalva K. Effect of in vitro hemolysis on 25 common biochemical tests. Clin Chem. 1992;38(4):575-7.

65. Laga AC, Cheves TA, Sweeney JD. The effect of specimen hemolysis on coagulation test results. Am J Clin Pathol. 2006;126(5):748-55.

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