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# Call for manuscript submissions for a thematic eJIFCC issue on “Flow cytometry”

Guest Editor for the “Flow cytometry” issue: Katherina Psarra

Multiparameter Flow Cytometry (FCM) is a recent technology of very high performance developed for a vast variety of diagnostic applications.

Is there really a limit of future cytometry? Will the evolution and transformations arrive at a limit or will they go on and on? Will the effort be the one that really matters? We hope to offer you some insight into this bright future with an eJIFCC issue dedicated to cytometry.

We invite you to submit a paper on “**Flow cytometry**” to be published in this thematic issue.

That way the message will be carried on to fellow laboratorians about the importance, the magic and the charm of this evolving technology.

Submitted papers will be reviewed according to the regular procedure of the eJIFCC.

## **Important deadlines**

- Deadline for submission of the tentative title (to the Guest Editor): **April 1st, 2019**
- Deadline for submission of the manuscript: **May 31st, 2019**

## **Type of articles**

- Original Papers
- Critical Reviews
- Case studies

## **Manuscripts to be submitted by e-mail to:**

- the Editor-in-Chief: [ejifcc@ifcc.org](mailto:ejifcc@ifcc.org)
- with a copy to the Guest Editor: [kpsarra@outlook.com](mailto:kpsarra@outlook.com)

## **Guest Editor**

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# Call for manuscript submissions for a thematic eJIFCC issue on “Improving the preanalytical phase in laboratory medicine”

Guest Editor for the “Pre-analytical phase” issue: Gabriel Lima-Oliveira

The pre-analytical phase encompasses all the procedures before the start of laboratory testing. This phase of the testing process is responsible for a great deal, possibly the majority of the laboratory errors.

Diagnosis, management, treatment of patients and ultimately patient safety itself can be compromised by:

- patient preparation;
- patient posture;
- phlebotomy quality;
- kind/type of evacuated tube used to draw blood samples;
- sample centrifugation;
- sample transportation;
- sample contamination;
- time to analyze;
- sample storage;
- and more...

We aim to prepare a special thematic issue of the eJIFCC, entitled “**Improving the preanalytical phase in laboratory medicine**”, to be published in November 2019, to inform the laboratory

professionals and to seek to guarantee patient safety.

The electronic Journal of the IFCC (eJIFCC) is a platinum open-access journal, i.e. there is no charge to read, or to submit to this journal. Our numerous high-quality articles, debates, reviews, case studies and editorials are addressed to clinical laboratorians. We aim to assist the development of the field of clinical chemistry and laboratory medicine worldwide. Manuscripts are fully peer reviewed and immediately free to access and download from [www.ifcc.org](http://www.ifcc.org).

Submitted manuscripts shall be reviewed normally, according to the regular procedures of the eJIFCC.

As Guest Editor, I would like to invite researchers from a wide range of disciplines to contribute to papers on recent and innovative research on Pre analytical phase.

### **Important deadlines**

- Deadline for submission of the tentative title (to the Guest Editor): **May 1, 2019**
- Deadline for submission of the manuscript: **July 31, 2019**

***Types of articles***

- Original Papers
- Critical Reviews
- Case studies

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# The irreplaceable value of laboratory diagnostics: four recent tests that have revolutionized clinical practice

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## ABSTRACT

There is a common perception that laboratory medicine may be occasionally perceived as neglected discipline by clinicians, and that laboratory tests may be considered ordinary commodities. Although there is still debate on the real contribution of diagnostic testing in care pathways, many clinical diagnoses cannot be made without laboratory data. In support of evidence-based added value of laboratory diagnostics, this article aims to discuss the over-reaching contribution of some recent tests to the clinical decision making, and the unquestionable role they have played in revolutionizing clinical practice. These paradigmatic tests include highly-sensitive cardiac troponin immunoassays for diagnosing non-ST elevation myocardial infarction, hemoglobin A1c for diagnosis and therapeutic management of diabetes, procalcitonin for diagnosing severe bacterial infections and improving antibiotic stewardship, along with natriuretic peptides for early diagnosing and managing heart failure. It is advisable that altogether these paradigms will help reaffirming the vital role of laboratory medicine in modern healthcare.

## INTRODUCTION

Laboratory medicine is conventionally defined as a science devoted to generate clinically useful information by analyzing the concentration, composition and/or structure of analytes in biological fluids [1]. Throughout the relatively long history of this discipline as we currently know it, and which probably commenced around the 19th century [2], laboratory diagnostics is now providing an almost invaluable contribution to the clinical decision making. Although there is still an open debate on the real influence of diagnostic testing in care pathways, as mirrored by fierce controversies on the reiterated assumption that clinical laboratory intervenes in 70% of clinical decisions [3], it is now incontestable that many clinical diagnoses cannot be made without laboratory data. In support of the evidence-based added value of laboratory diagnostics, this article aims to discuss the over-reaching contribution of some recent tests to the clinical decision making, and the unquestionable role they have played in profoundly revolutionizing clinical practice.

## CARDIAC TROPONINS

Cardiac troponins are essential components of the muscle contractile apparatus, including myocardial tissue. In myocardial cells, two unique and exclusive isoforms of cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are present, so that their immunochemical measurement allows to accurately establishing whether or not the heart tissue has been injured, even in the absence of open signs and symptoms of heart damage [4]. Unlike former methods, the recent development of fourth-generation highly-sensitive immunoassays has enabled measuring physiological concentrations of both cTnI and cTnT, and to more accurately redefine the diagnostic thresholds for identifying myocardial injury, thus including acute myocardial

infarction. According to recent guidelines and recommendations, when the clinical presentation is suggestive for myocardial ischemia, a dynamic elevation of cardiac troponins in the absence of any other objective finding (e.g., normal electrocardiogram) is regarded as diagnostic of non-ST elevation myocardial infarction (NSTEMI) [5,6]. The first breakthrough occurred after introducing high-sensitivity immunoassays in routine clinical practice has been a substantial decrease in the number of diagnoses of unstable angina, in favor of an increment of those of NSTEMI, thus leading the way to hypothesize that a requiem should be prepared for unstable angina [7]. On the other hand, the improved accuracy of these last generation, high-sensitivity cardiac troponin immunoassays has contributed to amplify the rate of patients diagnosed with NSTEMI (i.e., by 20-30%) [5,6], who would have been earlier discharged with inaccurate diagnosis and without appropriate medical or pharmacological treatment. Is there any doubt left that high-sensitivity cardiac troponin immunoassays have revolutionized the diagnostics of myocardial infarction and improved the managed care of this condition? Certainly not.

## HEMOGLOBIN A1c

Hemoglobin A1c, also known as glycated hemoglobin, results from the nonenzymatic binding of hexose to the N-terminal amino acid of the hemoglobin molecule A1, which is contained into the erythrocytes. Its concentration is hence directly proportional to the average blood glucose level over the preceding 8-12 weeks [8]. Owing to this important biological information, hemoglobin A1c has been for long used for monitoring glucose control in diabetic patients. A major breakthrough has however occurred, when the American Diabetes Association (AHA) has published updated recommendations for classification and diagnosis of diabetes [9], according to which diabetes can now be diagnosed also

in the presence of a hemoglobin A1c value >48 mmol/mol (i.e., >6.5%) measured with an assay certified by the National Glycohemoglobin Standardization Program (NGSP) and standardized to the Diabetes Control and Complications Trial (DCCT) assay. The routine assessment of hemoglobin A1c has hence enabled overcoming many of the well-known drawbacks of plasma glucose measurement (either fasting, random or during an oral glucose tolerance test), which essentially include the relative instability of glucose concentration in uncentrifuged blood samples, the high intra-individual variation of blood glucose, as well as biological (i.e., acute stress, drugs) and analytical interference [10]. Moreover, the measurement of hemoglobin A1c will now enable garnering a dual clinical information, since it not only allows to diagnosing diabetes, but will contextually provide important clinical information on medium-term glycaemic control. Recent evidence supports the conclusion that the measurement of fasting plasma glucose may underestimate the real burden of diabetes compared to hemoglobin A1c assessment, leaving this condition undiagnosed (and hence untreated) in up to one-third of pre-diabetic or diabetic patients [11,12]. Due to the clinical, social and economic burden caused by a delayed diagnosis of diabetes, it seems reasonable to conclude that routine assessment of hemoglobin A1c has the potential to generate a highly favorable impact on both diagnosis and management of diabetes.

## PROCALCITONIN

The greatest drawback in sepsis diagnostics is that the current scoring systems based on integration of clinical and laboratory data, namely the host systemic inflammatory response syndrome criteria (SIRS), the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) and the quick SOFA (qSOFA) scores, have limited diagnostic efficiency, because they have been

mostly validated for predicting prognosis and death [13]. Therefore, their use for identifying sepsis would not permit an early diagnosis, and could even leave some patients underdiagnosed/untreated.

Procalcitonin is the 141 amino acids precursor of calcitonin, the leading hormone involved in calcium homeostasis [14]. In physiological conditions, procalcitonin is produced by thyroid C cells and then converted in the mature form calcitonin into the circulation. In patients with severe infections, the synthesis of procalcitonin occurs also in many extra-thyroid tissues (i.e., liver, kidneys, lungs, pancreas), thus boosting an increase of its circulating concentration over the physiological reference range (i.e., <0.05 ng/mL) [15]. This peculiar biological behavior is now exploited for diagnosing severe infections, especially sepsis.

The number of studies and meta-analyses which have analyzed the diagnostic performance of procalcitonin for both diagnosing and managing sepsis has exponentially increased over the past decade. According to a recent meta-analysis published by Tan et al [16], procalcitonin displays 85% diagnostic accuracy (with 0.80 sensitivity and 0.77 specificity) for diagnosing sepsis, which appears sensibly higher than that of C reactive protein (e.g., 73%, with 0.80 sensitivity and 0.61 specificity). Even more importantly, in another recent meta-analysis published by Meier et al [17], procalcitonin-guided antibiotic management was found to be effective to significantly shorten the duration of antibiotic therapy (mean variation, -2.86 days), thus representing a valuable step forward toward reducing the worldwide burden of antibiotic resistance [18]. It is also worthwhile mentioning here that procalcitonin-guided antibiotic management seems also associated with substantial economic savings, as recently highlighted by Schuetz et al [19]. It is hence reasonable to conclude that the use of this simple and rapid test holds great

promise to consistently improve clinical outcomes (i.e., earlier diagnosis), reduce the risk of antibiotic resistance (i.e., tailored therapy, shortened administration), but may also generate favorable revenues on healthcare budgets.

### NATRIURETIC PEPTIDES

Natriuretic peptides are a family of protein hormones exerting a vast array of metabolic functions, including natriuresis, diuresis, vasodilation and improved insulin sensitivity [20]. Among the four members of this family, b-type natriuretic peptide (BNP) and the N-terminal fragment of pro-BNP (NT-proBNP) are produced in the left ventricular myocardium in response to myocyte distension due to pressure overload or volume expansion [21]. This important biological property has catalyzed the measurement of both BNP and NT-proBNP in the diagnostics of heart failure. In the 2016 guidelines of the European Society of Cardiology (ESC) [22], the measurement of BNP or NT-proBNP has been included among the essential diagnostic tests in heart failure, alone or in combination with echocardiography. The reason underlying this assumption is that increased values of these peptides will help accelerating the diagnosis, identifying patients needing additional cardiac testing or accurately and safely ruling out heart failure in those with non-diagnostic values. Notably, in the meta-analysis of Roberts et al [23], both BNP and NT-proBNP displayed excellent performance for diagnosing acute heart failure (0.95-0.99 sensitivity and 0.94-0.98 negative predictive value, respectively). In another recent meta-analysis published by Pufulete et al [24], BNP-guided therapy was found to be effective in reducing by nearly 20% the number of further readmissions for heart failure. Even more importantly, in heart failure patients aged <75 years, BNP-guided therapy was also associated with 24% higher median survival and 13% quality-adjusted life-years gain [25].

Finally, stronger evidence was also found that BNP-guided care may be a cost-effective option to clinically-guided care in patients with heart failure and impaired ejection fraction [26]. It is hence undeniable that BNP-driven care has the great potential to improve the diagnosis of heart failure, lower the risk of developing left ventricular systolic dysfunction and ameliorate the quality of life of heart failure patients.

### CONCLUSIONS

There is a common perception that laboratory medicine may be occasionally perceived as a neglected discipline by clinicians [27]. The validity of this assumption is reflected by the many publications in which a deep knowledge of the real significance of laboratory tests is lacking, so that diagnostic testing is finally considered an ordinary commodity.

For example, in a recent article published in *JAMA Internal Medicine*, Morgan et al concluded that high-sensitivity troponin testing often yields a high number of false-positive results in patients with suspected myocardial infarction [28]. In another recent article published in the *British Medical Journal*, O'Sullivan et al hypothesized that many of the vitamin D tests ordered in the UK are unnecessary screening, and this conclusion was supported by the evidence that test prescriptions have increased by over 50% between 2000/1 and 2015/16 in that country [29].

These assumptions symbolize a limited appreciation of the actual significance and implication of laboratory tests. Regarding cardiac troponins, an increased value is indeed an essential criterion for diagnosing myocardial injury, although a concentration above the diagnostic threshold does not disclose the type of underlying cardiac damage. Therefore, cardiac troponin testing can be ordered for many important reasons other than for diagnosing myocardial infarction, such as in patients with myocarditis, cardiac contusion,

**Table 1** Paradigmatic examples of recent laboratory tests which have revolutionized clinical practice

Test	Disease
Highly-sensitive cardiac troponin immunoassays	Diagnosis of non-ST elevation myocardial infarction (NSTEMI)
Hemoglobin A1c	Diagnosis and therapeutic management of diabetes
Procalcitonin	Diagnosis of severe infections (sepsis) and antibiotic stewardship
Natriuretic peptides	Early diagnosis and therapeutic management of heart failure

cardiotoxicity. It is disappointing to infer that the clinical use of cardiac troponins remains still uncertain nearly 20 years after the publication of the first universal definition of myocardial infarction [6].

As regards vitamin D, the increased number of requests shall be interpreted according to the temporal trend of vitamin D deficiency. A recent study, analyzing the trends in diagnosing vitamin D deficiency in the UK, has concluded that this condition has increased by over 15-fold between 2008-2014, as consequence of many environmental factors [30]. Since vitamin D deficiency not only is a major contributor of skeletal health (by lowering the risk of osteoporosis and fractures), but also seems to play an essential role in decreasing the risk of many human pathologies (e.g., cancer, cardiovascular disease, auto-immune and infectious diseases, and so forth) [31], an increasing number of prescriptions is not certainly unexpected or unreasonable, and cannot be straightforwardly associated with inappropriateness.

This paradigmatic examples underscore the fact that clinical reasoning is unavoidable for accurate interpretation of laboratory test results, and that a deep knowledge of the real significance of

each laboratory test is essential for preventing a deplorable underestimation of the added value of in vitro diagnostic testing.

In conclusion, although it is predictable that the extent to which laboratory testing informs the clinical decision making will remain controversial [3], it cannot be denied that the contribution of laboratory medicine in modern healthcare remains pivotal, since it helps predicting susceptibility to disease, making accurate diagnoses, prognosticating and monitoring diseases [32], and will become even more important in the future for the ongoing diffusion of disruptive technologies (i.e., genomics, proteomics, theranostics) and personalized (precision) medicine [33].

The four paradigmatic cases described in this article (Table 1) represent just some arbitrary examples of how irreplaceable is the value of laboratory diagnostics, and in which way some diagnostic tests have recently revolutionized clinical practice. Indeed, many other examples could be brought here, even more straightforward than those discussed in this article. It is advisable that altogether these paradigms will help reaffirming the vital role of laboratory medicine in modern healthcare [34].

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# Factors affecting turnaround time in the clinical laboratory of the Kathmandu University Hospital, Nepal

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## ABSTRACT

### **Background**

The turnaround time (TAT) as defined by most of the laboratories is the time interval between the specimens received in the laboratory to the time of reports dispatched with verification. Nearly 80% of hospital-attached clinical laboratories receive complaints about delayed TAT. Reporting in time is a crucial indicator of quality services along with accurate, precise and reliable reports, thus each clinical laboratory should identify affecting factors to eliminate them for the enhancement of quality services.

### **Methodology**

Dhulikhel Hospital-Kathmandu University Hospital is a tertiary care hospital, where this observational descriptive study was conducted in 2017. Requested tests received on database in the Department of Clinical Biochemistry Laboratory along with test requisition form (TRF) were carefully screened for any possible error. When analysis of individual patient's tests was completed, results of individual parameters were entered in the database manually. TAT was

calculated as a time period between specimens received to analysis completed. Once test analysis has completed it was immediately followed by verification.

### Results

A total of 36,108 patients' reports generated from the Department of Clinical Biochemistry Laboratory during study period were analyzed. Nearly 36% of reports exceeded the predefined TAT in case of stat tests, while around 7% of reports were out of predefined TAT in case of routine tests. Among prolonged TAT, around 75% of reports were delayed due to various extra analytical reasons and approximately 48% of total delayed reports were found only due to error by cash unit.

### Conclusion

The major reasons of delayed laboratory reports were due to time consumed to fix the pre-analytical errors created by other departments rather than laboratory itself. Cash unit alone has the highest degree of error in total testing process and it is the most significant factor for prolonged TAT. However reasons for prolonged TAT may vary with hospital to hospital depending upon different factors.



## INTRODUCTION

Most of the medical decisions are made on the basis of laboratory findings<sup>1</sup>. So, clinical laboratory findings must be accurate and well-timed. Waiting for laboratory reports for long time is often disappointing for patients and clinicians too. Hence, it is better for each laboratory to have its own turnaround time (TAT)<sup>2-5</sup>. Accuracy, reproducibility and punctuality have their own grounds in the field of clinical laboratory science<sup>6,7</sup>. However, in general clinical

laboratories focus on accuracy and reliability of the test reports and pay less attention to the prompt release of laboratory reports<sup>8</sup>. Early diagnosis and appropriate treatment of the patients is an outcome of the calculated accuracy and well-timed execution of the work<sup>9,10</sup>. Analysis of the test report in time can also be an important factor for the patients to cut out their expenses by shortening the time of their hospital stay<sup>9,10</sup>.

The total/therapeutic TAT is the time "from vein to brain", it is the interval between the test request and the therapeutic decision, while the laboratory TAT starts when the sample arrives at the laboratory and ends when the report is released after the validation of the results<sup>11,12</sup>. However, many laboratories confine their definition of TAT to intra-laboratory due to limitations in control of extra laboratory factors<sup>13</sup>.

TAT varies from laboratory to laboratory and also depends on the varying explanation of the laboratories and the clinicians<sup>11,14</sup>. Furthermore, TAT can also be categorized on the basis of test types and patient's priority (outpatients, inpatients, emergency)<sup>11</sup>. Total Testing Process (TTP) in clinical laboratory, as described by Lundberg, has nine steps namely, ordering, collection, identification, transportation, preparation, analysis, reporting, interpretation and action<sup>15</sup>.

A study done by the College of American Pathologists, CAP Q-Probes, in 1998, found that, 41% of the laboratories defined emergency TAT as the interval between sample arrival and result reporting, 27% defined it as the time from test ordering to reporting of the results and 18% defined it as the interval between sample collection and result reporting<sup>16</sup>.

TAT is a measure of punctuality and is often regarded as one of the determinants of laboratory efficiency<sup>9</sup>. Humans, being impatient by nature, need everything to be done rapidly including the clinical laboratory reports. Thus, they prefer

the hospitals that guarantee fast service and do not make them wait for long hours for their test reports and proper diagnosis, treatment and management of their problems. These facts thus prove TAT crucial for both, medical as well as commercial point of view <sup>6</sup>.

Total laboratory testing process is divided into three phases, namely; pre-analytical, analytical and post-analytical, and TAT depends on these three phases <sup>17</sup>. The pre-analytical phase refers to the time period between requisition of test to the sample being reached to the hands of professionals and prepared for analysis. The analytical phase is the period of measurement; this is the interval between the beginning of the measurement (actual testing) and the confirmation of the test results. The post-analytical period indicates the time from result verification or printing to the time when the physician actually observes the results <sup>18, 19, 20</sup>. Among these three phases, pre-analytical and post-analytical phases contribute to nearly 96% of the TAT <sup>9</sup> and factors may vary depending upon the infrastructures of the institution, degree of automation, and experience and attribution of the employee <sup>21, 22</sup>. Dhulikhel Hospital-Kathmandu University Hospital (DH-KUH) has predefined TAT of one hour for tests requested from emergency department and intensive care unit (ICU) while two and half hour for tests requested from outpatient departments and indoor departments.

## METHODOLOGY

### Study design

This is a cross-sectional, descriptive and observational study based on the data obtained from the Department of Clinical Biochemistry Laboratory of Dhulikhel Hospital-Kathmandu University Hospital. Obtained data were closely analyzed to observe current TAT and factors affecting prolonged TAT. All the samples along with

their Test Requisition Form (TRF) available at Department of Clinical Biochemistry Laboratory of Dhulikhel Hospital in six months' time from March to August 2017 were analyzed.

### Selection criteria

#### Inclusion criteria

All the criteria matched specimens and TRF received at the Department of Clinical Biochemistry Laboratory of Dhulikhel Hospital-Kathmandu University Hospital were analyzed.

#### Exclusion criteria

- Specimens for fasting and postprandial blood glucose measurement. (As there is no system to record time of postprandial sample reception)
- Sudden addition or cancellation of the tests via telephone by clinician.
- Test for Sex Hormone Binding Globulin, Vitamin B12, Folic acid and Anti Cyclic Citrullinated Peptide. (These tests are performed twice a week only)
- Test for hemoglobin or serum protein Electrophoresis. (Performed weekly only)

### Data collection and TAT calculation

The time of sample arrival and time of analysis completed were recorded in Excel tables and the differences were calculated. Simultaneously, if any obstacle was found in TTP, it was mentioned in TRF and was noted in the spread sheet.

### Procedure

In the specimen collection unit, collection time was mentioned in TRF and collected samples and TRFs were transported to the Department of Clinical Biochemistry Laboratory by trained staff. The TRFs and samples were then received by technician in the Department of Clinical Biochemistry Laboratory and verified according

to pre-structured standard operating procedure (SOP). The sample receiving time and parameters were immediately entered in Midas Version 3.2. If any possible error was noticed during sample receiving process, that was mentioned over TRF of respective patients. The sample receiving time entered in Midas is automatically printed on the report as sample received time. After completion of the individual patient's

tests, the observed values for all the requested parameters were manually entered in reporting database software Midas. If any problem happened during analysis e.g. test was repeated or sudden machine breakdown, they were mentioned on the TRF without any delay by concerned staffs. The time when observed value is entered in Midas, appears as test analysis completed time in the patient's report.

**Figure 1** Flow chart of the Total Testing Process (TTP)

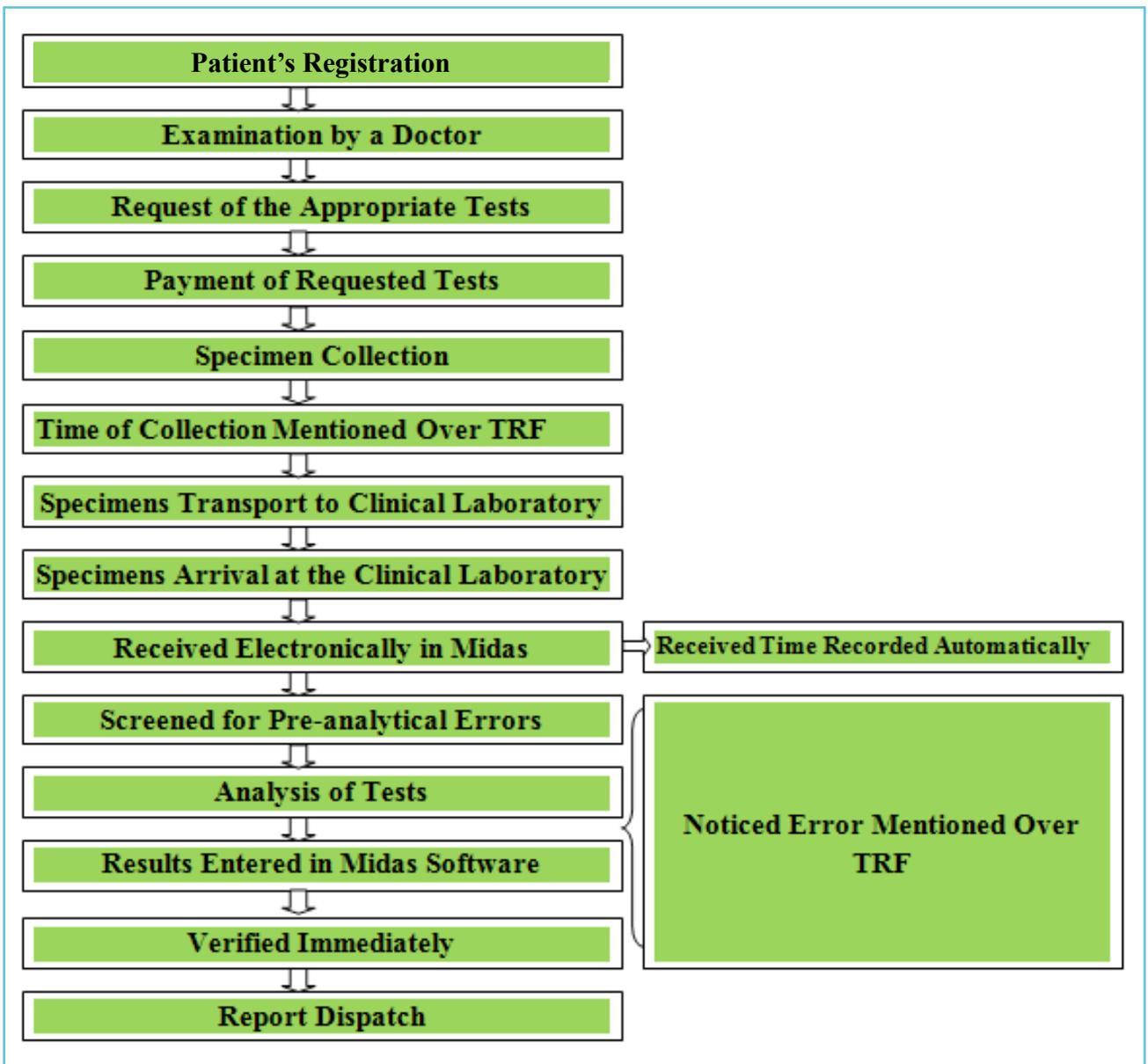


Figure 1 presents the flow chart of the total testing process in Dhulikhel Hospital-Kathmandu University Hospital.

Any factors that were encountered during the whole process, from receiving the samples to releasing the reports, might be responsible for prolonged TAT. They were mentioned on the TRF by concerned staff and were closely monitored by the author and research assistant. Every day all the criteria matching reports generated from the Department of Clinical Biochemistry Laboratory were analyzed for current TAT, together with the reasons of prolonged TAT mentioned on the TRFs. TTP was performed, as shown in Figure 1.

### **Ethical considerations**

The research proposal was submitted to the Institutional Review Committee (IRC) of the Kathmandu University School of Medical Sciences, and data collection was started after getting ethical clearance.

### **RESULTS**

This study was conducted in the year 2017 by analyzing a total of 36108 samples along with their TRF. Out of those, only 24644 samples were fit for the actual study. 11464 (31.74%) samples with TRF were not suitable because those requested tests had less than 20 minutes TAT which is practically not possible. This had happened because technical staff of clinical laboratory did not follow the standard operating procedure of TTP. Especially, in the late evening and night, technical staffs had done analysis of tests without receiving sample electronically in Midas, instead the samples were electronically entered only after completion of analysis or in between of analysis. So, the observed TAT was very short which is not possible in real situation (Table 1).

When 24644 patients' reports were analyzed for TAT and affecting factors, 2434 (9.8%) of them had prolonged TAT in comparison to predefined TAT. But only 2010 patients out of 24644 were

found to have reasons documented on TRF for stretched TAT. When specific reasons for prolonged TAT were analyzed in 2010 patient's reports, 973 (48.4%) patients' reports were delayed due to problem created in cash unit either incomplete payment or payment not according to test requested in TRF.

Nearly half of the total affected TAT was observed due to problem in cash unit and unfortunately patients from all the departments had to pay before test proceeds. Department of Clinical Biochemistry Laboratory reporting database is designed in such way that report cannot be generated unless a proper payment is made therefore the cash unit is the most important factor for prolonged TAT in Dhulikhel Hospital.

Repetition of tests is another leading factor of prolonged TAT in the Department of Clinical Biochemistry Laboratory of DH-KUH which is 494 (24.5%) out of 2010 patients. 313 (15.5%) patients did not get their reports on predefined time due to sample related factors. Visually detected hemolyzed specimens were absolutely rejected which resulted in delayed reports of 210 patients. It contributed delay of 10.44% patients' report. Furthermore, lingering of laboratory reports were due to poor inventory, failure of analyzers either due to irregular maintenance or lack of properly functioning analyzers were noticeable factors responsible for delay in 230 (11.4%) patients' reports.

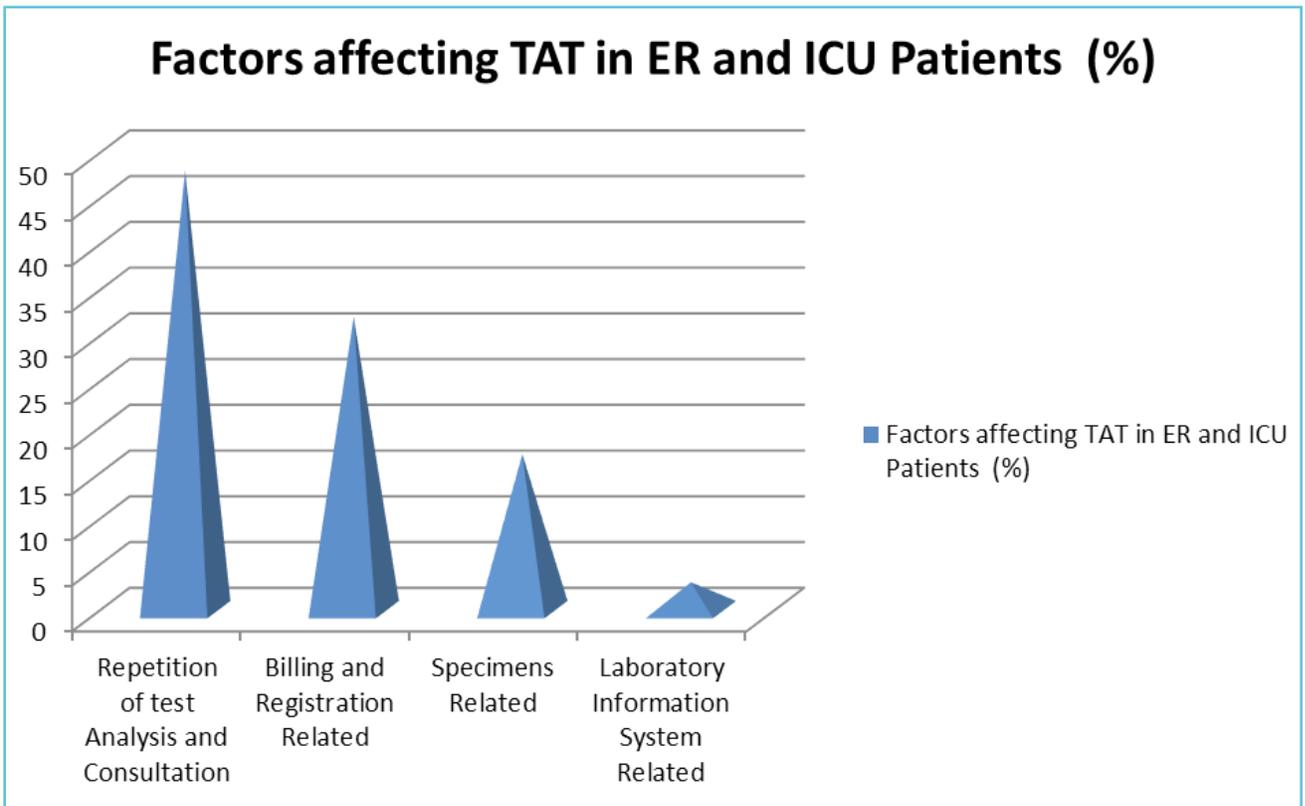
Table 2 clearly illustrates the number of samples from different departments in a particular time frame and their mean TAT. The frequency of long TAT is quite different among samples from ER/ICU and OPD/Indoors. Frequency of abnormal TAT for samples received from ER/ICU is five times higher than samples received from OPD and other indoor departments.

Bar graph in Figure 2 reflects that repetition of test analysis for reconfirmation and consultation

**Table 1** Specific reasons and their frequencies for prolonged TAT

Factors	Specific reason	Number	Frequency (%)
Payment for Tests in Cash Unit	Payment Missing (Incomplete payment)	542	26.96
	Wrong payment (Not according to tests prescribed)	297	14.77
	Wrong registration (Paid in another patients account)	127	6.31
	Excess payment (Mostly double payment)	07	0.34
Tests Repetition	Critical value reconfirmation and consultation	494	24.57
Specimen related	Hemolyzed	210	10.44
	Insufficient	40	1.99
	Not Received	17	0.84
	Unlabelled	15	0.74
	Unsuitable Specimen/test	10	0.49
	Marked Lipemic	08	0.39
	Mislabeled	07	0.34
	Clotted	06	0.29
Reagents related	Out of Stock/Not Provided/Supplied	101	5.02
	Expired	20	0.99
Machine Breakdown	Random Breakdown	66	3.28
	Preventive Maintenance Schedule	23	1.14
Reporting Software Breakdown	Reporting System (Midas) Down	20	0.99
<b>Total</b>	-	<b>2010</b>	-

**Figure 2** Factors affecting TAT in ER and ICU patients (%)



In the case of the laboratory reports of ER and ICU patients, the leading cause of TAT prolongation is the repetition of test analysis and consultation of the results.

**Table 2** Number of samples from different departments and their average TAT

Departments	ER and ICU	OPD and Indoors
Total Number of Samples	2403	22241
Prolonged TAT Samples	881 (36.66%)	1553 (6.98%)
Average TAT of Total Samples	1 hour 3 minutes	1 hour 21 minutes
Average TAT of prolonged TAT samples	1 hour 45 minutes	4 hours 30 minutes

of observed value of samples received from ER/ICU is the most prominent factor covering around 45% for prolonged TAT. Factors related to billing and registrations are the second most important factors for delaying laboratory reports which is followed by specimen and laboratory information system related factors.

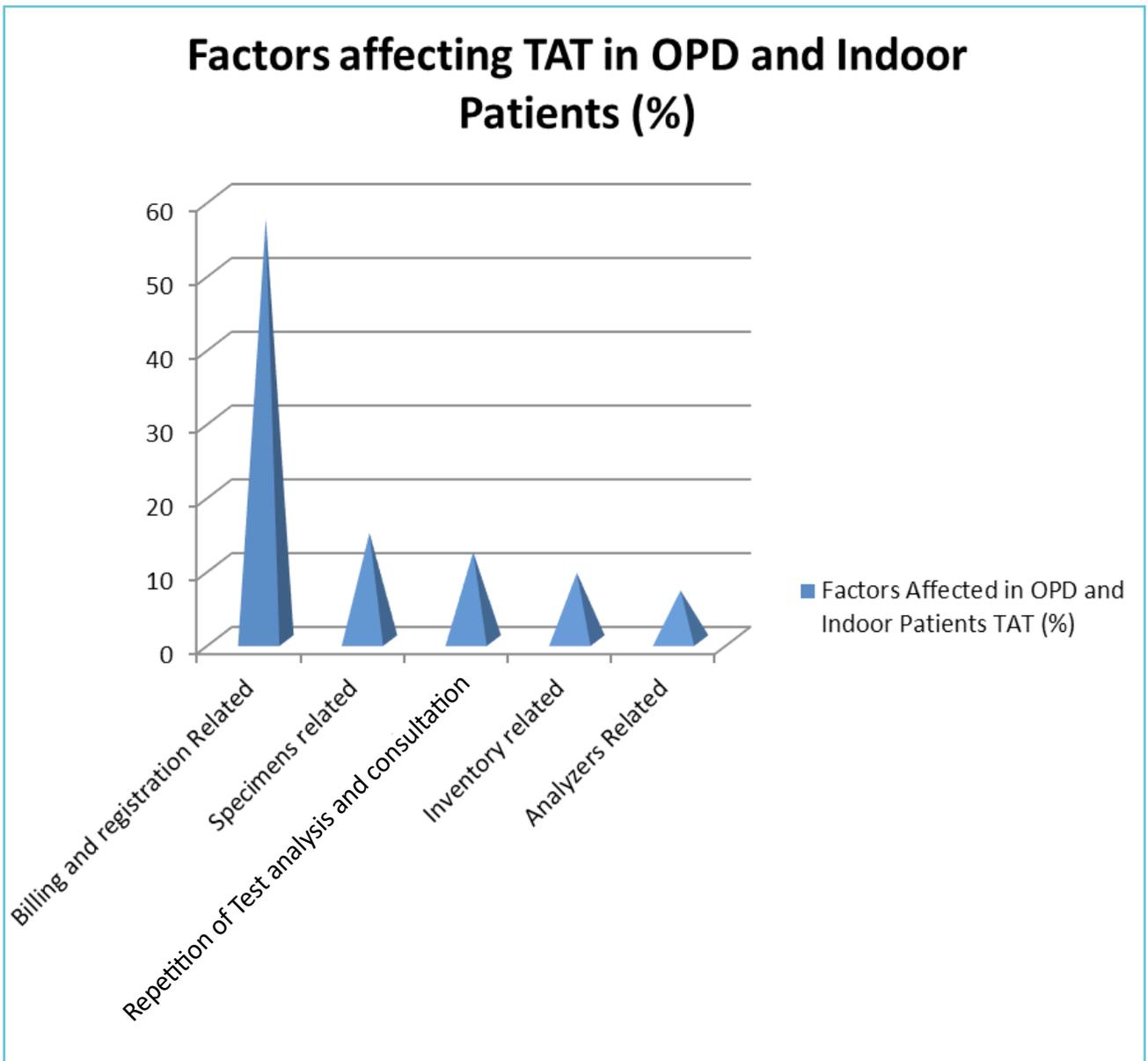
As shown in Figure 3, reports of tests requested from OPD and other wards had prolonged TAT due to different factors. Out of all five major factors for delayed reports, billing in cash unit is the highest, covering around 57%. Specimens related factors were the second highest, affecting nearly 15% of the delayed TAT.

When affecting factors for prolonged TAT were analyzed for individual departments, they varied according to types of services they provide. Such as the nature of service provided in emergency room (ER) and intensive care unit (ICU), where prompt action is needed and of course the laboratory reports must be delivered in time and must have less TAT than other departments

like general wards and outpatients departments (OPD).

As shown in bar graph, 48% of reports requested from ER or ICU were delayed due to time wasted in reconfirmation of obtained critical values of test. Mostly reconfirmation was done by repeating the tests or informing laboratory consultant before releasing such reports. Payment related

**Figure 3** Factors affecting TAT in case of OPD and indoor patients



In case of the laboratory reports of OPD and indoor patients, the TAT prolongation is mainly billing and registration related.

issues were found to be the second most significant factor (32%) for long TAT for tests ordered from ER and ICU followed by sample related issues like hemolysis, low volume, wrong sample etc. playing role in delaying reports of 17% patients.

In addition to the above-mentioned factors, transportation of the sample is also one of the factors affecting total TAT. Transportation time can be varied, which can be from specimen collection center to the laboratory or from other wards. It is not actually affecting laboratory TAT but it may affect the total TAT. Hence, it may lead to patients' prolonged stay in hospital and dissatisfaction, ultimately affecting hospital service. In general, average transportation time of collected specimens from the collection unit to the Department of Clinical Biochemistry Laboratory was found 24 minutes.

Analyzing factors responsible for prolonged TAT in ER and ICU, this study shows critical value reconfirmation and consultation time playing a major role for delay in predetermined TAT covering 48% of the total whereas billing and registration related issues were the next significant reason (32%) for the same. Sample related issues contributed around 17% to the total delay in TAT.

## DISCUSSION

How punctually a hospital provides service to the patient is an important determinant of quality of that hospital. In this study samples along with their TRFs were analyzed to generate relevant information about the TAT. Statistical analysis was applied for samples taken from ER and ICU departments and OPD and indoor departments. The average TAT for the samples received from all departments was 1 hour and 19 minutes.

Specific analysis for individual departments showed average TAT for ER and ICU departments and OPD and indoor departments as 1 hour and

3 minutes and 1 hour and 21 minutes respectively. The study carried out by *F. Bilwani et al.* showed that only 2.03% of the stat samples had longer TAT than acceptable range <sup>6</sup> while our study suggested that for ER and ICU departments a total of 36.66% of all samples investigated showed prolonged TAT.

This variation in TAT was mainly due to the delayed entry of the time at which the report was generation. The laboratory technicians generally inform the ER and ICU department immediately via phone call and enter the data later when they are relatively free.

Tests requested from OPD and indoor departments showed nearly four times less prolonged TAT in comparison to emergency and ICU, and the ratio of samples showing TAT prolongation stood at 6.98%.

A study performed by *K. P. Chauhan et al.* suggested that percentage of specimens exceeding TAT in 2011 was 6.4% which decreased to 4.6% by year 2012 <sup>14</sup>. The slightly higher prolonged TAT in our case was due to the registration and billing issues, analyzer errors, inventory of reagent related issues and sample related issues along with reconfirmation and consultation time. In this study, among all factors involved for excessive TAT, preanalytical factors were responsible for nearly 75% of the delay whereas around 24% of the delay was due to analytical factors.

Similarly, a study done by *KN. Desai et al.* suggests that 74.2% of the samples were delayed due to preanalytical phase <sup>7</sup>.

In contrast to this result, a study performed by *F. Bilwani et al.* emphasizes that most of the delays were due to analyzer error constituting 40% of the total specimens <sup>6</sup>.

In our study analyzer error was responsible for only 6.83% of the TAT prolongation observed exclusively for OPD and indoor departments.

## CONCLUSION

Achievement of quality service is not simply possible in hospital attached laboratories without finding the factors for prolonged TAT and immediate improvement of that area by hospital management. However, factors affecting TAT in clinical laboratories may vary from institute to institute depending upon institutional infrastructure, their own setup, policy, system and attributes of employees working in different departments of the hospital. In case of the Department of Clinical Biochemistry Laboratory of Dhulikhel Hospital-Kathmandu University Hospital, the major reasons for delay in laboratory reports were due to the time burnt out to fix the preanalytical errors created by other departments and cash unit alone was the major factor with highest degree of error in total testing process.



### Acknowledgements

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# Hemolysis interference studies: the particular case of sodium ion

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All authors declare no conflicts of interest.

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## ABSTRACT

### **Background**

Despite many studies assessing hemolysis interference in almost every clinically relevant magnitude, sodium has poorly been assessed. Our aim was to evaluate hemolysis interference on plasma sodium, using different strategies of hemolysis preparation, at different baseline sodium ion concentrations and bias specifications.

### **Methods**

Two different strategies were used for the preparation of hemolysis from lithium heparin blood samples. Repeatability was calculated at two levels for each strategy and interferograms were outlined for both approaches at sodium concentrations between 130-145 mmol/L. Results were interpreted according to different specifications: reference change value, RiLiBÄK, Westgard's database, RCPA-QAP and CLIA.

### **Results**

The coefficients of variation of the hemolyzed samples using the first strategy were lower than for the second strategy (0.23-0.78% vs 0.57-48.6%, for 0.2 g/dL

free Hb and 0.28-0.44% vs 0.40-135.1%, for 0.9 g/dL free Hb). Statistically significant differences were seen when comparing the slopes of the pairs of interferograms at each sodium concentration obtained by both strategies ( $p < 0.001$  for 130 mmol/L;  $p = 0.068$  for 135 mmol/L;  $p = 0.002$  for 140 mmol/L and  $p = 0.001$  for 145 mmol/L). Hemolysis cut-off values were generally independent of the sodium concentration.

### Conclusions

Reproducibility of hemolysate preparation is procedure-dependent. A greater standardization is needed for the preparation of a true hemolysate to better quantify the degree of interference of clinically relevant analytes, especially those with higher complexity such as sodium. We found a concentration-independent cut-off value for the hemolysis index that allows the establishment of a single and robust value in every laboratory, according to their quality specifications.



## INTRODUCTION

In the preanalytical phase, the *in vitro* lysis of red blood cells (hemolysis), which implies a release of hemoglobin and other intraerythrocyte elements, is the main cause of interference and rejection in the biochemical analytical methods worldwide (1–3). *In vitro* hemolysis depends mainly on blood sample drawing techniques and subsequent treatment (agitation, transportation, storage), whereas *in vivo* hemolysis may have at least 50 causes (4,5), including Gram-positive bacteria, parasites, toxins or autoimmune disorders. Genetic disorders such as sickle-cell disease or glucose-6-phosphate dehydrogenase deficiency may also lead to hemolytic crises with high free hemoglobin levels in blood. There are two central mechanisms of interference by hemolysis in clinically relevant tests:

spectral (especially in spectroscopic methods, due to an overlapping of absorption spectra) and chemical (due to a release of components from red blood cells which alter the *in vivo* concentration of the analyte) (6). Other hemolysis-derived interfering mechanisms may be due to other causes (e.g. magnesium in the measurement of total calcium concentration, or adenylate kinase in the measurement of creatine kinase activity).

A great number of studies have assessed the effect of hemolysis on almost every clinically relevant analyte. Towards a minimization of variability and a higher reproducibility, guidelines have been published both for the performance of such studies and for the *in vitro* simulation of hemolysis and the handling and processing of such blood specimens (7–10). The quantification of the degree of hemolysis is also fundamental for the proper management of samples and test results (11,12).

In spite of their almost universal applicability for biomarkers in laboratory medicine, there are a few exceptions still needing a thorough examination, and the paradigm of such exceptions is sodium ion. As previously reported, *in vitro* hemolysis is known to negatively interfere with sodium due to a diluting effect (13,14), as the intracellular concentration of sodium is significantly lower than the concentration in serum or plasma. The degree of hemolysis in a sample is frequently assessed by measuring the free hemoglobin in serum or plasma.

The preparation of a true hemolysate is crucial for the performance of studies assessing hemolysis interference. This term refers to the absence of unhemolyzed red blood cells or other intact cells after the preparation of hemolysate. One of the procedures most commonly was first described by Meites (15), and includes a water-dilution step before freezing and thawing an anticoagulated blood sample. Other useful

strategies in literature include microwave radiation, ultrasounds or mechanic lysis (16).

Nevertheless, the preparation of valid hemolysate for the study of sodium in serum or plasma should not have a water-addition step, as it would decrease the concentration of the ion in the solution, hence altering the (direct) relationship with the hemolysis index. According to literature, intraerythrocyte sodium concentration is 10-15 mmol/L (17,18), whereas sodium concentration in distilled water is negligible. As a result, the water-dilution step in the preparation of a hemolysate would alter the relationship between hemoglobin (hemolysis index) and sodium, as well as with any other intraerythrocyte biomolecule. If including this step, the greater water volume added for the preparation of the hemolysate, the greater interference observed (negatively). The equilibration with distilled water would impede the detection of the strictly negative effect of hemolysis.

Our aim was to comprehensively assess the magnitude of hemolysis interference of plasma sodium, using different strategies of hemolysate preparation, different baseline sodium ion concentrations and different bias specifications in their interpretation.

## **METHODS**

Two different study procedures were suggested in this study to parallelly assess and quantify the magnitude of interference of hemolysis on plasma sodium (Figure 1).

A total of 40 volunteers were recruited for the performance of this study: 20 for the first approach, and 20 for the second.

The first approach consisted in blood extraction into two simultaneous 3.5-mL lithium heparin tubes without gel from each volunteer (ref. 368884, BD Vacutainer). One of them was directly centrifuged (10min 1500g; Sample A)

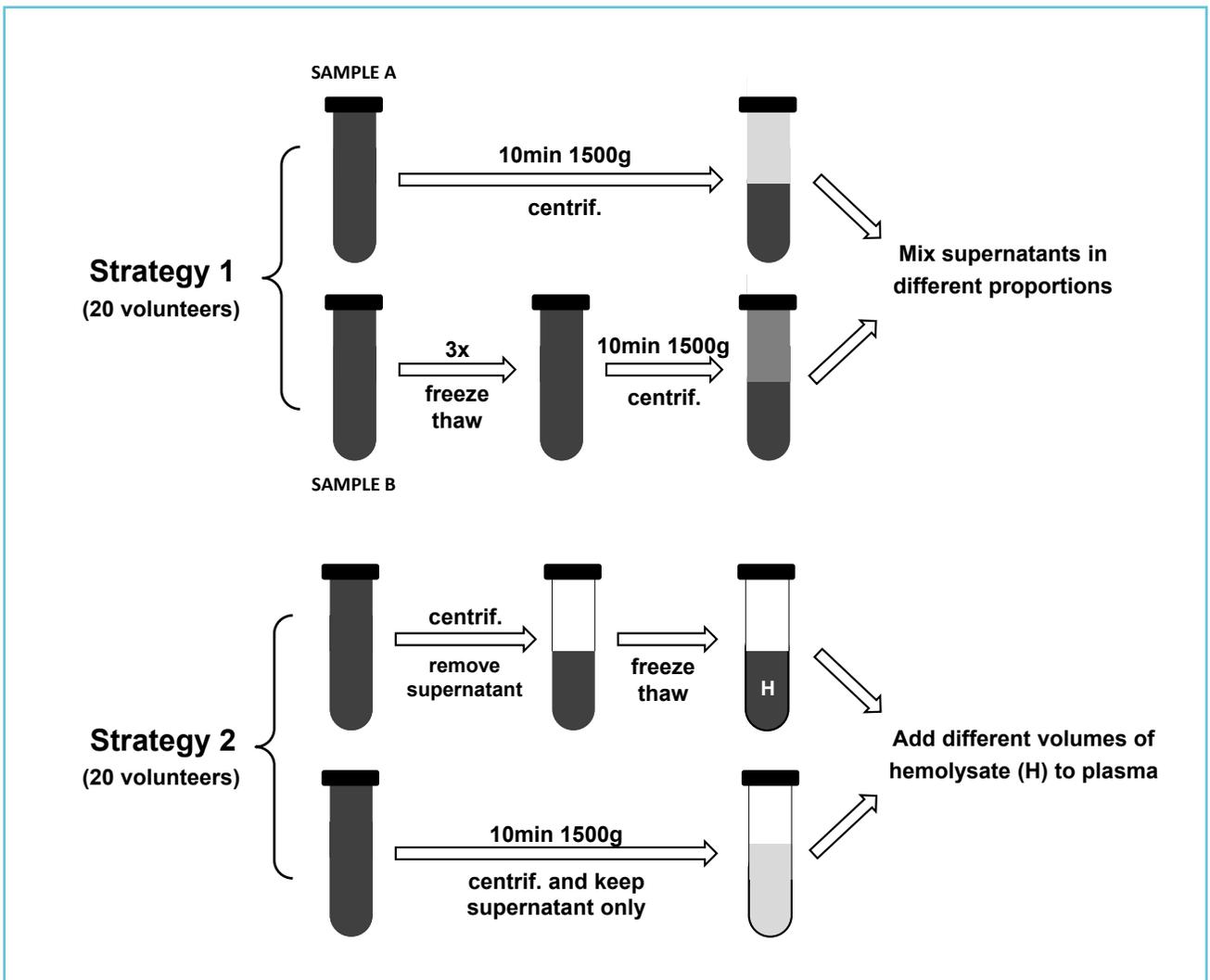
while the other was previously frozen-thawed 3 times to induce hemolysis and subsequently centrifuged (Sample B). Both plasma samples A+B were mixed in different proportions, starting from [1000  $\mu$ L A + 0  $\mu$ L B] to [1000  $\mu$ L A + 200  $\mu$ L B] (greater proportions of B yielded excessive hemolysis, not quantifiable by the analyzer). Sodium ion was measured by indirect potentiometry, whereas hemolysis index was analyzed by dichromatic spectrophotometry at different wavelengths and calculated using an algorithm (Architect c16000 platform, Abbott Diagnostics, USA). Sodium in sample A (pure) was taken as reference. The experiment was performed at four concentrations of sodium: approximately 130, 135, 140 and 145 mmol/L. None of the samples was seen to be hyperlipidemic.

The second approach consisted in the simulation of hemolysis by removing the supernatant and the buffy coat of a plasma heparin tube, freezing-thawing it 3 times and further centrifuging it (10min 1500g). No addition of distilled water or washing step was carried out. Pools of lithium heparin plasma were prepared at different sodium concentrations (approximately 130, 135, 140 and 145 mmol/L) using plasma from 5 different participants for each, and aliquoted into 1-mL tubes. Increasing volumes of the hemolyzed supernatant (5, 10, 20, 25, 30, 35 and 40  $\mu$ L) were added to each aliquot, which were further centrifuged to remove possible intact red blood cells.

Repeatability of both strategies was assessed by measuring 10 times the hemolysis index at 5-minute intervals in 10 samples, at two different hemolysis indices (approx. 0.2 and 0.9 g/dL) for each strategy in different days, and calculating the coefficients of variation.

Interferograms were outlined for both approaches and different initial sodium concentrations, according to guidelines (7).

**Figure 1** Strategies for the evaluation of hemolysis interference



The critical hemolysis index causing interference was evaluated in every interferogram as the difference from the baseline value, and established according to five different performance specifications:

- the reference change value in our laboratory (RCV), which integrates the within-subject biological variation (0,6%) and our analytical coefficient of variation (0,67%);
- the RiLiBÄK specification of 3%;
- the desirable quality specification of 0.73% for total error on the Westgard database

(available at: <https://www.westgard.com/biodatabase1.htm>);

- the allowable limit of performance of 2% by the Royal College of Pathologists of Australasia (RCPA-QAP) (available at: <https://www.westgard.com/rcpa-biochemistry.htm>); and
- the CLIA specification of  $\pm 4$  mmol/L (available at: <https://www.westgard.com/clia.htm>).

The Shapiro-Wilk's test was used to assess normality, and outliers were removed using the Reed/Dixon's test. The Fisher's F-test was carried

out to compare variances and the Student's t-test was used in order to compare the slopes and intercepts of both hemolysis-preparing strategies at each sodium concentration. Statistical significance was set at 5%. The software SPSS v.20 was used for all statistical analyses.

## RESULTS

The coefficients of variation for the repeated measures were 0.26-0.78% and 0.28-0.44% for hemolysis levels obtained by the first strategy (parallel extraction and mixing in different proportions), and 0.57-48.6% and 0.40-135.1% for the samples obtained using the second strategy (hemolysate generation and subsequent addition to a normal plasma) (Table 1).

Sodium concentration was seen to decrease with hemolysis in every concentration assessed, independent of the hemolysis strategy (Figure 2). When comparing the slopes of the pairs of interferograms at each sodium concentration obtained by both strategies, statistically significant differences were detected (Table 2).

As outlined in Table 3, when the approach based on hemolysis generation in whole blood, centrifugation and mixing in different proportions with a paired normal plasma was used (strategy 1), the mean hemolysis index exceeding the RCV specification for a sodium concentration of 130 mmol/L was 0.95 g/dL hemoglobin (CI 95%: 0.88-1.02), while this critical value is reduced when a more strict limit is used, such as the one suggested on the Westgard database

**Table 1** Repeatability (coefficients of variation) of hemolysis index obtained by different strategies

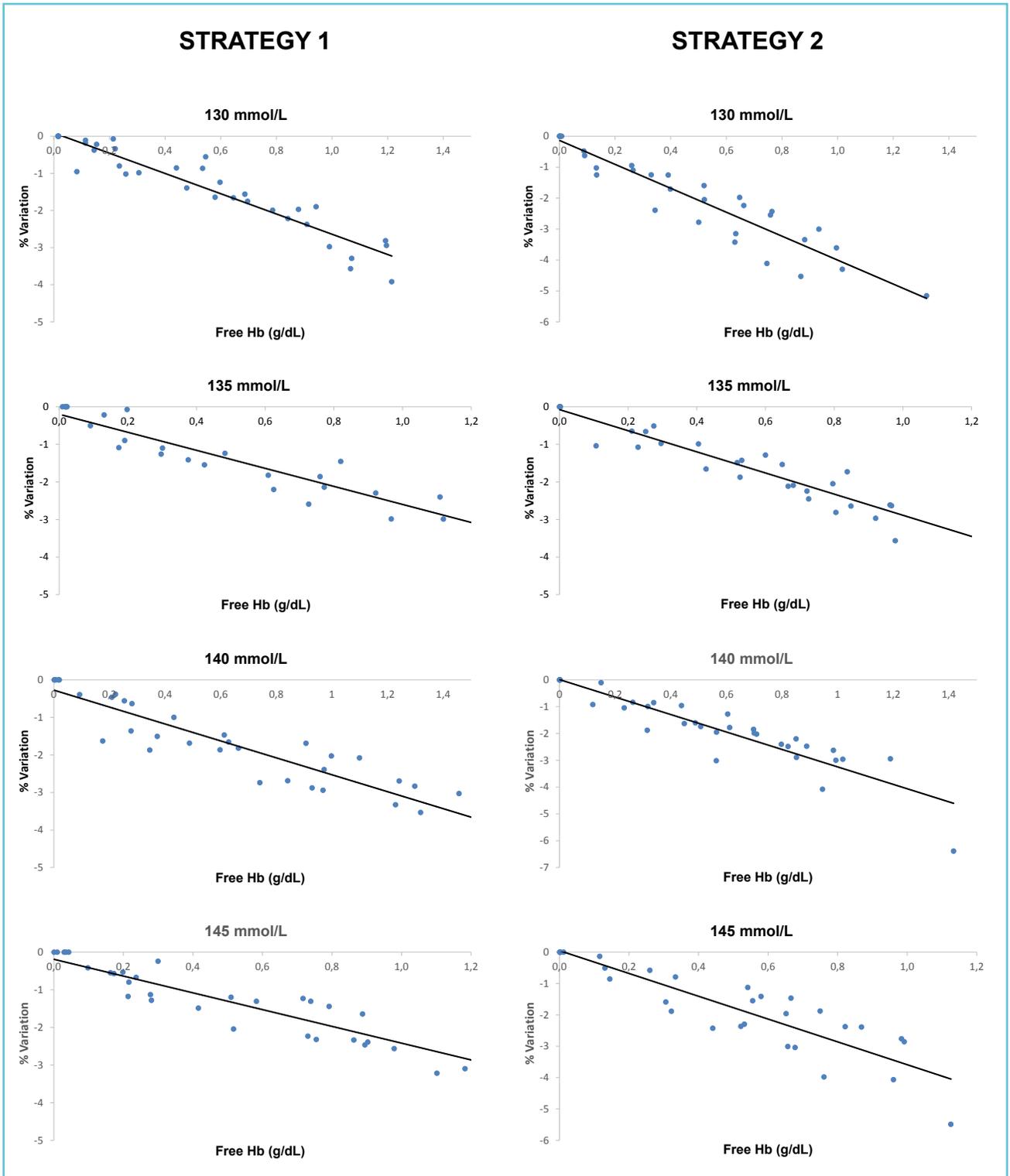
	Plasma [Na <sup>+</sup> ] (mmol/L)	Low Hemolysis Assay (0.2 g/dL Hb)	High Hemolysis Assay (0.9 g/dL Hb)
Strategy 1	144.1	0.26-0.78%	0.28-0.44%
Strategy 2	144.3	0.57-48.6%	0.40-135.1%

**Table 2** Comparison of slopes and intercepts of interferograms obtained at a specified sodium concentration (y: deviation from baseline as %; x: free hemoglobin in g/dL)

Plasma [Na <sup>+</sup> ] (mmol/L)	Regression equation strategy 1	Regression equation strategy 2	p-value (slope)
130	$y = -0.003x + 0.088$	$y = -0.004x - 0.136$	<0.001*
135	$y = -0.002x - 0.198$	$y = -0.003x - 0.076$	0.068
140	$y = -0.002x - 0.272$	$y = -0.003x + 0.013$	0.002*
145	$y = -0.002x - 0.190$	$y = -0.004x - 0.046$	0.001*

\* Significant at  $p < 0.05$

**Figure 2** Interferograms for different sodium concentrations using both hemolysis preparing strategies



Percentage deviation from sodium baseline value according to increasing hemolysis in the samples.

**Table 3** Hemolysis interference cut-off on plasma sodium for different bias specifications

		Free hemoglobin (g/dL)			
		[Na <sup>+</sup> ] 130mmol/L	[Na <sup>+</sup> ] 135mmol/L	[Na <sup>+</sup> ] 140mmol/L	[Na <sup>+</sup> ] 145mmol/L
CI for samples used in Strategy 1		(130.7-133.0)	(136.2-137.7)	(140.3-142.7)	(145.6-148.5)
CI for samples used in Strategy 2		(131.7-133.1)	(135.7-137.7)	(139.3-142.5)	(145.8-147.0)
RCV (2.5%)	Strategy 1	0.95 (0.88-1.02)	0.96 (0.88-1.04)	0.99 (0.90-1.08)	1.04 (0.95-1.13)
	Strategy 2	0.61 (0.56-0.67)	0.86 (0.80-0.92)	0.77 (0.70-0.84)	0.70 (0.62-0.78)
RiLiBÄK (3%)	Strategy 1	1.13 (1.05-1.22)	1.17 (1.07-1.26)	0.93 (0.84-1.01)	0.84 (0.74-0.94)
	Strategy 2	0.74 (0.68-0.80)	1.04 (0.97-1.12)	0.93 (0.84-1.01)	0.84 (0.74-0.94)
Westgard (0.73%)	Strategy 1	0.30 (0.38-0.49)	0.22 (0.30-0.45)	0.20 (0.27-0.46)	0.24 (0.33-0.48)
	Strategy 2	0.15 (0.18-0.32)	0.23 (0.31-0.42)	0.23 (0.26-0.42)	0.21 (0.23-0.40)
RCPA-QAP (2%)	Strategy 1	0.88 (0.81-0.94)	0.84 (0.77-0.90)	0.81 (0.74-0.88)	0.86 (0.78-0.93)
	Strategy 2	0.56 (0.51-0.61)	0.76 (0.70-0.81)	0.65 (0.59-0.71)	0.59 (0.52-0.66)
CLIA (±4mmol/L)	Strategy 1	1.17 (1.08-1.26)	1.17 (1.08-1.26)	1.17 (1.06-1.27)	1.17 (1.06-1.28)
	Strategy 2	1.17 (1.08-1.26)	1.17 (1.08-1.26)	1.17 (1.06-1.27)	1.17 (1.06-1.28)

Confidence intervals are shown in parentheses. Abbreviations: RCV: reference change values; RCPA-QAP: Royal College of Pathologists of Australasia Quality Assurance Program.

for desirable quality specifications for total error (0.30 mg/dL hemoglobin). When the approach based on the preparation of hemolysate and small-volume addition into normal samples was followed (strategy 2), lower hemolysis cut-off values were obtained at most concentrations. At a sodium concentration of 130 mmol/L, a hemolysis of 0.61 g/dL Hb was shown to interfere when the RCV specification was used, while small amounts as 0.15 g/dL Hb were seen to interfere when the Westgard's specification was followed.

There is no fixed tendency in the hemolysis cut-off at different sodium concentrations. Hemolysis cut-off values are generally independent of the baseline sodium concentration.

## **DISCUSSION**

There is currently an objective and evident improvement in patient safety thanks to the automatization in the measurement of hemolysis index in the clinical laboratories, which replaces behind the visual inspection for the decision upon their adequacy (19). The optimal hemolysis index cut-off value for each clinically relevant test is method- and instrument-dependent, and always subject to the previously defined quality specifications in every specific laboratory (5). In hemolysis interference studies, the procedure for the preparation of hemolysate is of utmost importance (20).

### ***Hemolysis-preparing strategies***

In our study, two different strategies were used to assess hemolysis interference at different plasma sodium concentrations. The first strategy, based on a parallel blood drawing, freeze-thawing one whole-blood sample, centrifuging and mixing in different proportions with non-hemolyzed plasma, showed a better repeatability than the second strategy, based on hemolysate generation and subsequent addition to a normal plasma.

This better repeatability yields a greater reproducibility and robustness of the first strategy.

### ***Hemolysis index cut-off establishment***

Many studies may be found in literature assessing hemolysis interference for chemistry analytes, although sodium ion is only assessed in very few of them.

Steen and colleagues (21) assayed two different sodium concentrations (127 and 140mmol/L), using an hemolysate prepared by osmotic disruption with distilled water, and could not detect any significant difference in the values, thus interpreting an absence of interference. As stated, the inclusion of a water-dilution step in the preparation of the hemolysate could most probably add a bias in the results.

Another approach by Lippi et al (22) followed a freeze-thaw procedure for hemolysis preparation and, with a critical difference set at  $\pm 0.3\%$ , found that even small amounts of hemolysis (0.016 g/dL of hemoglobin) could interfere in the measurement of sodium (seen at  $[\text{Na}^+] = 140.1 \pm 1.5 \text{ mmol/L}$ ).

Another study by Saldaña and collaborators (23) introduced a washing step with NaCl in the preparation of the hemolysate. After correcting for the dilution, an hemoglobin concentration of 0.21 g/dL was shown to induce interference.

A third study evaluated one single sodium concentration (146 mmol/L) and found a deviation of 2% from baseline when hemolysis was 0.66 g/dL. Greater concentrations of free hemoglobin were not assessed (24).

In our study, given the interval of sodium concentrations, we found a concentration-independent cut-off value for the hemolysis index, at different bias specifications. This allows the establishment of a single cut-off value for hemolysis in every laboratory, according to their quality specifications.

### Apparent hyponatremia

There are varied and important clinical implications derived from a falsely reduced plasma sodium result due to hemolyzed samples, which is the main cause of preanalytical rejection of samples (14,25). In addition, there is no consensus whether sample transportation to the laboratory may influence the degree of *in vitro* hemolysis, whether by pneumatic tube or not (26–28).

Hyponatremia, defined as a sodium concentration <135 mmol/L, is reported to have an incidence between 4-19% of outpatients, being also associated with a mortality increase (29). After the diagnosis of a *in vivo* hyponatremia, a comprehensive differential diagnostic study is essential to determine the exact etiology, including volemia, sodium clearance and serum osmolality, among other biochemical tests (30). The treatment of hyponatremia needs to be directed to correct the etiologic cause and restore blood sodium levels with a rate 6-12 mmol/L in the first 24 hours, as a greater rate would trigger an osmotic demyelination (31). Therefore, robust and reliable sodium results by the laboratory are crucial not only for the diagnosis of electrolyte imbalance disorders, but also for the decision of treatment strategies and follow-up.

The wide range seen in previous bibliography for the establishment of a hemolysis cut-off for sodium interference highlights the challenging aspect of this cation. Our study is the first to comprehensively assess the impact of hemolysis on plasma sodium, including two strategies for sample preparation, several sodium concentrations (130 to 145 mmol/L), and interpreting such results according to different deviation specifications. The inclusion of different specifications in the establishment of a cut-off value makes our approach of greater applicability for laboratories worldwide. The main limitations of our study relate to the use of a single analytical

platform, given that hemolysis index is measured by different spectroscopic methods and mathematical algorithms among clinical laboratories, as well as the low number of samples included, although in line with previous literature.

### CONCLUSIONS

Our study brings to light the importance of the proper preparation of hemolyzed samples for the interference quantification in such a particular case as sodium ion. The negative effect by dilution seen in this case makes the protocols including water-dilution steps unsuitable for sodium ion studies, and other more appropriate strategies need to be followed. The establishment of a valid and concentration-independent hemolysis cut-off value will lead to more reliable laboratory results for sodium ion results.

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# Prevalence of anemia and associated factors among hospitalized children attending the University of Gondar Hospital, Northwest Ethiopia

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## ABSTRACT

### **Background**

Anemia in children continues to be a major public health challenge in most developing countries, particularly in Africa.

In the early stages of life, it leads to severe negative consequences on the cognitive functions as well as growth and development of the children, which may persist even after treatment.

### **Objective**

The main aim of this study was to assess the prevalence and associated factors of anemia among hospitalized children attending at university of Gondar comprehensive and specialized referral hospital, Northwest Ethiopia.

## **Method**

A cross sectional study was conducted on 384 hospitalized children, between February and June, 2018. Data of socio demographic characteristics and clinical conditions of the study individuals were collected using questionnaire after taking appropriate written informed consent and assent. Then 3 mL of blood was collected for complete blood count analysis and also stool examination was done for intestinal parasites. Data were coded, cleared and entered into SPSS version 20 for analysis.

Bivariate and multivariate logistic regression models were used to identify associated factors of anemia. P-value  $\leq 0.05$  was considered as statistically significant.

## **Result**

The overall magnitude of anemia among hospitalized children was 58.6%; of them 56.4% were males. Of anemic children, 28% had mild, 51.1% moderate and 20.9% severe anemia. The magnitude of anemia among children aged 6-59 months, 5-11 years and 12-14 years were 54.1%, 58.9% and 67.5%, respectively.

In this study, anemia was positively associated with parasitic infection (AOR= 2.541; 95% CI: 1.363, 4.737), not eating meat and animal products (AOR = 1.615; 95% CI: 1.014, 2.574).

## **Conclusion**

Anemia among hospitalized children in this study was found to be a severe public health problem. It was strongly associated with intestinal parasitic infection and not eating meat and animal products.

Focused polices and strategies should be designed to reduce anemia among hospitalized children in Ethiopia.



## **BACKGROUND**

Anemia is one of the major public health concerns that cause significant morbidity and mortality in children worldwide (1). It is one of the most prevalent public health problems in the world, affecting both affluent and poor countries with major consequences of human health as well as social and economic developments (1,2). Even its prevalence has shown decrement across the regions; still it remains a serious public health problem around the globe (3).

Although anemia occurs at all stages of human life cycle, its prevalence is higher especially in younger preschool aged children due to increased demand of iron for fast growth (4).

It adversely affects the cognitive and physical development of children which in turn results in a significant impairment of work capacity and school educational performance (3, 5-7).

The magnitude of anemia in children substantially varies across the world's regions, whereby the global prevalence is estimated to be 42.6%, and its magnitude in Africa, South East Asia, America and European regions is 62.3%, 53.8%, 23.3% and 22.9%, respectively. Globally, on average, around 9.6 million children are severely anemic (1).

Anemia with prevalence of  $\geq 40\%$ , 20-39.9%, 5-19.9% and  $<5\%$  in the community is categorized as severe, moderate, mild and no public health problem, respectively (8).

The 2008 WHO report also has revealed that more than half (56.3%) of the world's preschool aged children reside in developing countries where anemia is a severe public health problem including sub-Saharan Africa with a prevalence of 40% and above (7, 9, 10).

Similarly, the 2015 WHO report, from the global anemia prevalence in 2011 showed that the highest figure (42.6%) was in children compared to other age groups around the globe

and its prevalence in Africa and Ethiopia was 62.3% and 50%, respectively (1).

Anemia is associated with socioeconomic, biological, environmental and nutritional factors. Nutritional deficiencies (iron deficiency, vitamin B12 and folate deficiency), lack of awareness among the mothers about the problem together with their low educational status, unhealthy food habits and parasitic infestations were considered the main factors associated with anemia among children (1, 3, 4, 7, 11-13).

The prevalence of anemia among hospitalized children in different parts of the world has been studied; 33.2% in Lebanon (14), 61.6% in Turkey (15), 56.3% in Uganda (16), 83.2% in Southern Tanzania (17), and 77.2 % in Mwanza, Tanzania (13). However, in our country Ethiopia, there is lack of information about the prevalence and risk factors of anemia among hospitalized children.

Most studies in Ethiopia focus on the prevalence of anemia under age five and for school aged children (18-20). Therefore, this study was aimed to determine the prevalence and associated factors of anemia among hospitalized children attending university of Gondar referral and comprehensive hospital, Gondar, Northwest Ethiopia. Identifying risk factors and determining the magnitude of anemia have paramount importance to reform the regional and national public policy for ensuring sustainable improvement.

## **METHODS**

### ***Study setting and population***

A cross sectional study was done among 384 hospitalized children aged 6 months to 14 years at university of Gondar comprehensive and specialized referral hospital, between February and June, 2018. Gondar is located at a distance of 737 km from the country's capital, Addis Ababa—in

the North Gondar zone of the Amhara regional state, in North-Western Ethiopia. The city's latitude and longitude coordinates are 12°03'61" N and 37°02'81" E, respectively; and it is located at elevation of 2,133 m above sea level. Children with chronic kidney disease, active bleeding, HIV/AIDS, those who have undergone surgery in the previous one month and who had chronic illness were excluded from the study.

### ***Data collection procedures***

Upon obtaining a signed consent form and additional assent from children aged 7 years and above, the children who fulfilled the inclusion criteria were enrolled into the study. A pre-tested and standardized questionnaire was used to collect social demographics and social economic history. The caretaker/ family's level of income was assessed by asking the caretaker how much they earned per month including donation or handout from other persons or organizations. Other clinical data of hospitalized children were also collected by nurses working in pediatric ward.

### ***Laboratory analysis***

#### ***Hematology analysis***

About 3mL of venous blood was collected from each subject for the analysis of hematological parameters; hemoglobin (Hgb), hematocrit (%), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red blood cell count (RBC) and red cell distribution width (RDW) were determined using Cell-Dyn1800 automated blood analyzer.

#### ***Stool examinations***

For intestinal parasite examination, stool samples were collected and both wet mount and concentration technique were employed by an experienced laboratory technologist.

### Anemia definition and severity classification

Since the study area altitude is 2133 meter above sea level, results of Hgb values were adjusted by subtracting 0.8g/dl to its respective sea level as it is recommended by WHO. Then Hgb cutoff value 11g/dl for children 6-59 months, 11.5g/dl for children 5-11 years and 12g/dl for children 12-15 years were considered to define anemia. Regarding to its severity of anemia, Hgb value of 10.0-10.9 g/dl, 7.0-9.9 g/dl and less than 7 g/dl were considered as mild, moderate and severe anemia, respectively for children aged 6-59 months and Hgb value of 11.0-11.4 g/dl for children 5-11 years and 11.0-11.9 g/dl for children aged 12-14 years was considered as mild type of anemia while Hgb value of 8.0-10.9 g/dl and below 8.0 g/dl were considered as moderate and mild type of anemia for both age groups; children aged 5-11 years and 12-14 years (8).

### Statistical analysis methods

Data were cleaned, edited, checked for completeness and entered into SPSS version 20 statistical software for analysis. Descriptive statistics were used to summarize the characteristics of the study population. To determine factors

associated with anemia binary logistic regression analysis was done; and odds ratio with its 95% confidence level was used to determine the strength of association between the predictors and dependent variables. A p-value of less than 0.05 were considered as statistically significant.

## RESULTS

### Socio-demographic and clinical characteristics of study participants

In this study, a total of 384 hospitalized children aged between 6 months and 15 years were included, of which 212 (55.2%) are males in gender and 55.7% were from rural setting. The median age of the children was 5 years with interquartile range (IQR) of 2-10 years. Nearly half (45%) of the hospitalized children were in the age group between 6 months to 5 years. From the total hospitalized children, 18% were infected with intestinal parasites, 20% were hospitalized for five or more days and 21.6% (83/384) were malnourished. From malnourished hospitalized children, 45.8% (38/83) were severely malnourished. Regarding educational status, 58.1% of mothers and 51.6% fathers of hospitalized children were unable to read and write.

**Table 1** Socio-demographic and clinical characteristics of hospitalized children attending University of Gondar comprehensive and specialized referral hospital, Northwest Ethiopia

Characteristics		Frequency	Percentage
Sex	Male	212	55.2
	Female	172	48.8
Age in years	0.5- 5	172	45.0
	5-11	129	33.1
	12-14	83	21.9

Residence	Rural	214	55.7
	Urban	170	44.3
Nutritional status	Severe malnutrition	38	9.9
	Moderate malnutrition	45	11.7
	Normal	301	78.4
Intestinal parasite infection	Yes	69	18.0
	No	315	82.0
Length of hospitalization	Up to 2 days	112	29.2
	3-5 days	195	50.8
	Above 5 days	77	20.0
Maternal educational status	Unable read and write	223	58.1
	Primary education	44	11.5
	Secondary education	47	12.2
	College/ University	70	18.2
Paternal educational status	Unable read and write	198	51.6
	Primary education	42	10.9
	Secondary education	57	14.8
	College/ University	87	22.7
Monthly household income in ETB	<1000	66	17.2
	1001-2000	181	47.1
	Above 2000	137	35.1

ETB: Ethiopian Birr; 28ETB = 1 \$USD.

For nearly two thirds (64.3%) of the hospitalized children, their family/care giver monthly household income was below 2,000 Ethiopian Birr (70 US dollars) (Table 1).

### Prevalence of anemia

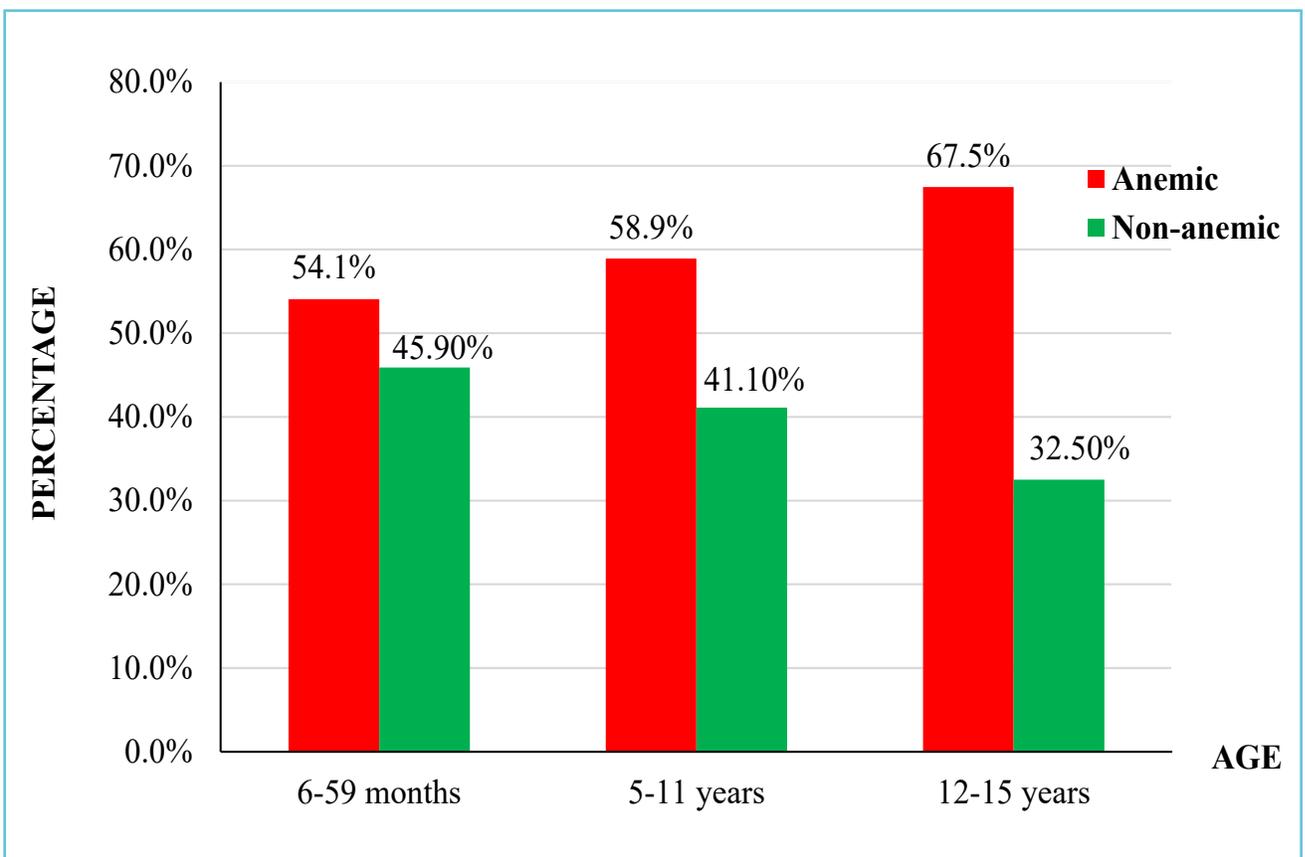
The mean ( $\pm$  SD) value of altitude adjusted Hgb level among hospitalized children was  $10.5 \pm 2.6$  g/dL, with values ranging from 2.3 g/dL to 19.3 g/dL. The overall prevalence of anemia in this study was 58.6% (225/384), with 65.9% (141/214) and 49.4% (84/170) magnitude among rural and urban hospitalized children, respectively.

Of the 225 anemic cases, 127 (56.4%) were males. Of the total anemic hospitalized children, 28% (63/225), 51.1% (115/225) and 20.9% (47/225) had mild, moderate, and severe anaemia,

respectively. Ninety-three (41.3 %) anemic cases were in the age group between 6 months to 5 years, and the rest 76 (33.8%) and 56 (24.9%) are in the age group 5 to 12 years and 12 to 14 years, respectively.

The most common type of anaemia was the microcytic hypochromic anemia observed in 62.2% (140/225) of children, followed by 36.9% (83/225) with normocytic normochromic anaemia. Two children (0.9%) had macrocytic normochromic anaemia. There was no significant difference in the prevalence of anemia between females and males which was 57.0% and 59.9%, respectively. The prevalence of anemia was higher among older children (65.7%) in 12-15 years, and it had shown increased as the child's age increased (Figure 1).

**Figure 1** Distribution of anemia among different age groups of hospitalized children



### Factors associated with anemia

In order to determine child related factors that were associated with anemia, bivariate logistic regression analysis followed by multivariate logistic analysis was done.

As indicated in Table 2, in the bivariate logistic regression analysis, children related factors such being 12-15 years old, infection with intestinal parasites, not eating meat and animal products, edema and fever were significantly associated

with anemia. Then those variables having p-value less than 0.25 were subjected to multivariate logistic regression.

In multivariate logistic regression analysis, being infected with parasitic infection (AOR = 2.541; 95% CI: 1.363, 4.737) and not eating meat and animal products (AOR = 1.615; 95% CI: 1.014, 2.574) were statistically associated with anemia. In this study, vomiting (AOR = 0.446; 95% CI: 0.208, 0.96) and diarrhea for more than

**Table 2** Association of child related factors with anemia among hospitalized children

Characteristics		Anemic	Non-anemic	COR (95% CI)	AOR (95% CI)
Sex	Male	127 (59.9)	85 (40.1)	1	-
	Female	98 (57)	74 (43)	0.886 (0.589,1.333)	-
Age in years	0.5-5	93 (54.1)	79 (45.9)	1	1
	5-11	76 (58.9)	53 (41.1)	1.218 (0.768,1.932)	1.02 (0.603,1.726)
	12-15	56 (67.5)	27 (32.5)	1.762 (1.018,3.049)	1.53 (0.829,2.822)
Nutritional status	Malnourished	49 (59)	34 (41)	1.024 (0.625,1.677)	-
	Normal	176 (58.5)	125 (41.5)	1	-
Intestinal parasites	Yes	50 (72.5)	19 (27.5)	2.105 (1.187,3.734)	2.541 (1.363,4.737)*
	No	175 (55.6)	140 (44.4)	1	1
Hospitalization length	Up to 2 days	69 (61.6)	43 (38.4)	1	1
	3-5 days	105 (53.8)	90 (46.2)	0.727 (0.453,1.167)	0.685 (0.414,1.132)
	Above 5 days	51 (66.2)	26 (33.8)	1.222 (0.666,2.242)	1.29 (0.681,2.442)

Do you eat meat	Yes	101 (51.8)	94 (48.2)	1	1
	No	124 (65.6)	65 (34.4)	1.775 (1.177,2.678)	1.615 (1.014,2.574)*
Edema	Yes	71 (70.3%)	30 (29.7)	1.982 (1.219,3.225)	1.56 (0.898,2.71)
	No	154 (54.4)	129 (45.6)	1	1
Fever	Yes	27 (45.8)	32 (54.2)	0.541 (0.31,0.946)	0.606 (0.324,1.136)
	No	198 (60.9)	127 (39.2)	1	1
Vomiting	Yes	18 (46.2)	21 (53.8)	0.571 (0.294,1.112)	0.446 (0.208,0.96)*
	No	207 (60)	138 (40)	1	1
Diarrhea >1 week	Yes	14 (43.8)	18 (56.3)	0.52 (0.25,1.079)	0.402 (0.178,0.907)*
	No	214 (58.8)	150 (41.2)	1	1

\* Indicates statistically significant association;

COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence interval

**Table 3** Association of family/caregiver related factors with anemia in among hospitalized children

Characteristics		Anemic	Non-anemic	COR (95% CI)	AOR (95% CI)
Residence	Urban	84 (49.4)	86 (50.6)	1	1
	Rural	141 (65.9)	73 (34.1)	1.977 (1.309,2.988)	1.638 (0.844,3.177)
Maternal educational status	Unable read & write	144 (64.6)	79 (35.4)	1.823 (1.059,3.137)	1.657 (0.299,9.182)
	Primary education	21 (47.7)	23 (52.3)	0.913 (0.429,1.942)	0.908 (0.191,4.305)
	Secondary education	25 (53.2)	22 (46.8)	1.136 (0.542,2.382)	1.113 (0.278,4.455)
	College / University	35 (50)	35 (50)	1	1

Paternal educational status	Unable read & write	125 (63.1)	73 (36.9)	1.673 (1.005,2.786)	0.318 (0.064– 1.588)
	Primary education	25 (59.5)	17 (40.5)	1.437 (0.682,3.03)	0.455 (0.103,2.003)
	Secondary education	31 (54.4)	26 (45.6)	1.165 (0.597,2.276)	0.558 (0.156,1.996)
	College / University	44 (50.6)	43 (49.4)	1	1
Mother occupation	Government employee	32 (51.6)	30 (48.4)	0.66 (0.38 -0 1.145)	1.112 (0.241,5.128)
	Private	12 (41.4)	17 (58.6)	0.437 (0.201,0.949)	0.467 (0.166,1.315)
	House wife	181 (61.8)	112 (38.2)	1	1
Father occupation	Government employee	40 (48.2)	43 (51.8)	1	1
	Farmer	134 (63.5)	77 (36.5)	1.871 (1.119,3.127)	1.553 (0.305,7.892)
	Private	51 (56.7)	39 (43.3)	1.406 (0.772,2.56)	1.955 (0.463,8.255)
Monthly income in ETB	<1000	43 (65.2)	23 (534.8)	1.842 (1.004,3.381)	1.528 (0.569,4.102)
	1001-2000	113 (62.4)	68 (37.6)	1.638 (1.044,2.569)	1.283 (0.563,2.926)
	Above 2000	69 (50.4)	68 (49.6)	1	1

ETB = Ethiopian Birr, 28.40 ETB = 1 \$USD;

COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence interval.

one week (AOR = 0.402; 95% CI: 0.178, 0.907) were also showed a statistically significant association with anemia. This is due to the fact that during vomiting and diarrhea, there will be a decrease in body fluid and mask the low level of Hgb value.

Family/care giver-related factor association with anemia was determined as indicated in Table 3.

In bivariate logistic analysis, being rural in residence, having no formal maternal education and a monthly income below 2,000.00 Ethiopian Birr (28 Ethiopian Birr = 1 \$USD) were statistically associated with anemia among hospitalized children. However, there is no family/care giver related factors associated with anemia in multivariate logistic regression analysis.

## DISCUSSION

The findings from this study indicate that the prevalence of anemia among hospitalized children aged 6 months to 15 is high. The prevalence was found to be 58.6 %. The overall prevalence is higher than reported from other studies in India (21), Lebanon (14), Nepal (22) and Nigeria (23), where the prevalence were between 32 and 50%. The variations in percentage in different regions might be due to heterogeneity of the studied population, dietary habits, different nutritional status and incidence of worm infestation in a defined geographical spots (21).

In our study, prevalence of anemia was elevated as age increase and it was quite high among older children aged 12-14 years (65.7%). This finding is contrary to that describe by others where the prevalence of anemia was highest among children under age of five (14). The highest prevalence in age group between 12 and 15 may be attributed to low intake of iron rich food or inappropriate dietary choices in children, poor iron absorption due to iron absorption inhibiting factors such as tannine in tea which reduces hemoglobin synthesis or inadequate iron absorption enhancers (vitamin C and hydrochloric acid) (24). Based on our finding, anemia prevalence in children under five which is 54.1% is lower than the result found in Ghana, Brazil, Bangalore, and Tanzania (13, 17, 25-27).

Different studies had different result on the association between anemia and gender. This study found insignificant difference in anemia prevalence between male and female, 57.0% and 59.9% respectively. While others have demonstrated that the prevalence of anemia can vary between male and female children (28). They argued that the high prevalence of anemia in boys may be due to the faster growth of preschool boys than girls that has high iron demand which cannot be met by diet alone.

Morphological classification of anemia showed microcytic hypochromic anemia was the most predominant type occurring in 62.2% of children. This finding is similar to the study done in Tanzania, and it is assumed that iron depletion is the main factor responsible for the high percentage of microcytic-hypochromic anemia (13). On the other hand, normocytic normochromic anemia was the second type of anemia in this study. This may be due to acute blood loss, drug therapy and chronic diseases (anemia of inflammation) (29, 30).

Child related factors like being infected with parasitic infection and not eating meat and animal products were statistically associated with anemia. The lower immune response in children compared to adults, poor hygiene and environmental conditions favor the susceptibility of children to parasitic infections (31). In our finding, children with parasitic infestation are two and half times more likely to have anemia than children who are free from parasitic infection, (AOR= 2.541; 95% CI: 1.363, 4.737). This finding is similar to study done in Bangladesh (32). The mechanism behind this relation can be due to the fact that the parasite directly induces iron deficiency through blood loss by mechanical rupture of host capillaries and arterioles followed by the release of a battery of pharmacologically active polypeptides including anticoagulants, antiplatelet agents, and antioxidants (31).

Not eating meat and animal products were also found to be related to a risk of acquiring anemia in our study and this is similar to the study done in Uganda (12). Iron can be found in two forms in foods heme and non-heme. Heme iron is only found in animal products, whereas non-heme iron is only found in plants (33). Iron deficiency and/or low bioavailability account for half of the anemia in developing countries (34) accessed 28 November, 2018.

In this study univariate analysis showed anemia that was significantly associated with maternal related variables like being rural in residence (COR= 1.977 (1.309, 2.988)), have no formal education (COR= 1.823 (1.059, 3.137)) and low monthly income (COR= 1.842 (1.004, 3.381)), but in multivariate analysis this factor was not significant statistically. Since mothers are mostly care givers for the child, maternal education has always been linked to many child health outcomes. It may also affect health decision making and thus influence the probability of a child meeting certain nutrition-related requirements.

In addition, anemia among children was also associated with household income. Children living in household with lower monthly income (<2000 Ethiopian Birr) were more likely to have anemia compared to those with higher income. Similar finding was from study conducted in Brazil and northern Ethiopia (9). The reason might be due to children from poor households are less likely to get iron-rich foods.

This study has some limitations in that we lack data on the prevalence of blood parasite like *plasmodium falciparum*, one of the risk factors considered to be the principal cause of severe anemia in malaria-endemic areas in Africa. The clinical diagnoses of the children were also not included in this study therefore clinical causes of anemia could not be identified.

## **CONCLUSIONS**

Prevalence of anemia in hospital admitted children aged between 6 months and 15 years is high and it was found to be a severe public health problems. Being more common in children may predispose this vulnerable population to future infections, hematological and developmental disorder. Microcytic, hypochromic and normocytic normochromic pattern were the common morphological types of anemia. Parasitic infection and not eating meat and animal products

significantly associate with anemia. Deworming and interventions like iron supplementation and nutritional education activities are important to decrease the prevalence of anemia.



### **Abbreviations**

**AOR:** Adjusted odds ratio

**COR:** Crude odds ratio

**EDTA:** Ethylene Die-amine Tetra-acetic Acid

**FA:** Fanconi Anemia

**HCT:** Hematocrit

**Hgb:** Hemoglobin

**IDA:** Iron Deficiency Anemia

**MCH:** Mean Cell Hemoglobin

**MCHC:** Mean Cell Hemoglobin Concentration

**MCV:** Mean Cell Volume

**RBC:** Red Blood Cell

**RDW:** Red Cell Distribution width

**WHO:** World Health Organization



### **Ethical approval and consent to participate**

Ethical clearance and approval was granted from University of Gondar, Collage of Medicine and Health Sciences, School of Biomedical and Laboratory Sciences Ethical Review Committee. Permission letter was also obtained from the hospital administrators. The purpose of the research was explained to the study subjects and written informed consent and assent was obtained from each hospitalized children and their care givers and then those who were willing to participate were included in the study. Participation was fully voluntarily, refusal at any time during data collection was permitted. Confidentiality was kept. When haemoglobin level of the children was below 11 g/dl, the result was communicated with the physicians.

### **Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

### **Competing interests**

The authors have declared that they have no existing competing interests.

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### **Authors' contributions**

- BE & AA: conceived and designed the experiments
- YW, ST, EM, MM: performed the experiments
- BE & MG: analyzed and interpreted results
- All authors contributed to the writing and editing of the manuscript and approved the final version submitted.

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# Waist circumference cutoff point determination for defining metabolic syndrome in type 2 diabetes mellitus in Ethiopia

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## ABSTRACT

### **Background**

Metabolic syndrome (MetS) is a complex disorder characterized by a cluster of interrelated cardiovascular risk factors. So far, cutoff point variability of waist circumference was documented to define MetS.

### **Objective**

To determine the classification power and cutoff point of waist circumference to define MetS among patients with type 2 diabetes.

### **Methods**

An institution-based cross sectional study was conducted from March to April 2017 at Ayder Comprehensive Specialized Hospital among patients with type 2 diabetes. Using systematic sampling technique, 520 participants were enrolled into the study. Data were collected by checklist, anthropometric measurements and biochemical analyses. Data were entered to Epi-info 3.5.1 and transferred to SPSS 20 for analysis. Participants having more than one abnormal MetS components were categorized as patients and the

others were considered as control. The classification power of waist circumference to distinguish patients from controls was determined by ROC curve analysis. Waist circumference cutoff points were determined by taking the point that had a maximum youden index.

### Results

Among the 520 participants, 308 (59.2%) were females. The mean age of the participants was  $56 \pm 10.8$  years for males and  $55 \pm 11.4$  years for females. The classification power of waist circumference was 0.67 (0.58-0.75) for male and 0.63 (0.52-0.73) for females. The optimal waist circumference cutoff point to distinguish patients from controls were 95.5 cm (sensitivity 39.8%, specificity 86.3%,  $p < 0.001$ ) for males, and 87.5 cm (sensitivity 73.1%, specificity 54.5%,  $p < 0.017$ ) for females.

### Conclusion

The positive predictive value of waist circumference was 93% for females and 90% for males in Northern Ethiopia using 87.5 and 95.5 cm points cut-off for females and males, respectively.



## INTRODUCTION

Metabolic syndrome (MetS) is a complex disorder characterized by a cluster of interrelated factors that increase the risk of cardiovascular disease (CVD). In type 2 diabetic patients (T2DM) and those with other non-communicable diseases (1), MetS increases the complication rate (2).

Various international organizations are using five parameters including elevation of waist circumference (WC), blood pressure (BP), triglycerides (TG), fasting blood glucose (FBG), and reduced HDL-C level to define MetS (3).

Elevated WC is characterized by the accumulation of fat in the visceral part of the abdomen and plays a central role in the pathogenesis of MetS (4).

To precisely assess the visceral fat volume, abdominal computed tomography (CT) has been considered as the most accurate and reproducible technique (5). However, CT scans are costly and time consuming (6). Because of this limitation, a variety of alternative methods are applied to assess abdominal obesity.

Waist to hip ratio and WC are correlated with abdominal imaging. Among them, WC is considered to be simple and inexpensive measure with excellent correlation with the CT results (7). In addition, it is used to define MetS based on various international criteria and different scholars indicted that WC appears to be the best central obesity indicator than BMI and waist to hip ratio (8).

Many organizations and expert groups recommend WC as one of the criteria to define MetS. International Diabetes Federation (IDF), particularly, needs elevation of WC as an obligatory criterion with a different cutoff point in accordance with ethnicity and gender. Recent publications report that WC cutoff point is quite variable among different ethnic groups and between genders. However, based on ethnicity and gender, until now, the IDF established WC cutoff point for the general populations for two regions only to define MetS, for the European and Asian populations.

In this regard, the African region has no cutoff point for WC to define MetS yet (9). A number of studies support the necessity of locality based WC cutoff points. Consequently, this study aimed to determine WC cut points among T2DM patients in Ayder Comprehensive Specialized Hospital (ACSH), Northern Ethiopia.

## MATERIALS AND METHODS

### *Samples and design*

An institution based cross-sectional study was conducted among T2DM patients from March to April 2017 in ACSH. Five hundred twenty participants were selected based on systematic random sampling techniques.

### *Data collection*

Data were collected on the basis of self-administered standardized questionnaires and the measurement of anthropometric, blood pressure and biochemical parameters. A self-administered standardized questionnaire was customized on the basis of the WHO STEP wise approach to non-communicable disease risk factor surveillance (10).

### *Measurements*

Blood pressure was measured after the participants had rested for at least 5 minutes. The measurements were taken using left arm at the heart level, using BP monitor MB 300-D digital instrument. In each case, the mean of two results was used for analysis (11).

BMI was calculated as weight (kg) divided by height squared (m<sup>2</sup>). WC was taken by positioning the non-elastic measuring tape midway between the lower rib margin and the iliac crest. It was measured to the nearest 0.5 cm (12). Fasting (8 to 12 hour) 5 mL venous blood was also collected in clot activated test tubes. Serum was separated within 30 minutes (2000 rpm, 5 minutes). FBG, TG and HDL-C were determined using a Pentra C400 clinical chemistry analyzer.

### *Data analysis and interpretation*

The data were entered into EpiData 3.1 and then transferred to SPSS version 20. Data cleaning was done before analysis, descriptive and summary statistics were also performed. Participants who had one or more abnormal MetS components

(excluding WC and FBS), were coded as case and the others as control (9, 13). The classification potential (AUC) of WC to distinguish cases from control was determined using ROC curve for both sexes separately. Sensitivity and specificity of WC to distinguish cases from controls was calculated at several WC points. For each WC point Youden index (YI) was calculated. The WC points, having the maximum YI was taken as the optimum cutoff point of WC to classify case from controls. In all conditions, *P*-value of < 0.05 was considered as statistically significant.

## RESULTS

Among the 520 participants, 308 (59.2%) were females. The mean ( $\pm$  SD) ages of the participants were  $56 \pm 10.8$  and  $55 \pm 11.4$  years for males and females, respectively. A majority of the participants, 457/520 (87.9%), were from the urban area (Table 1).

The mean duration of diabetes co-morbidity after diagnoses was  $4.7 \pm 2.9$  years for males and  $4.6 \pm 2.5$  years for females. All of the study participants are taking anti-diabetic drugs. Seventy two (35%) male and 144 (46.8%) female participants were overweight and obese (Table 2).

Four hundred thirty six patients were investigated for MetS (275 females and 161 males). Reduced HDL-C was the most typical abnormal component of MetS in both genders, it was present in 221 (42.5%) patients. High blood pressure was detected in 157 patients (30.2%) (Table 3).

The classification power of WC to define MetS was characterised by AUC. The value of AUC was 0.67 (95% CI, 0.58-0.75) for males and 0.63 (95% CI, 0.52-0.73) for females, respectively (Figure 1).

The classification power of WC to define the reduced HDL-C was lower as compared to the other MetS components in both sexes (0.60 in males and 0.53 in females) (Table 4).

Among men, WC at a cut-off value of 95.5 cms yielded the highest YI (0.261) with a corresponding sensitivity of 39.8% and specificity of 86.3%.

For women, the WC at a cut-off value of 87.5 cms yielded the highest YI (0.276) with a sensitivity of 73.1% and specificity of 54.5 % (Table 5).

**Table 1** Socio-demographic characteristics of patients with diabetes mellitus at Ayder Comprehensive Specialized Hospital, Northern Ethiopia, 2017 (N=520)

Variables		Male n (%)	Female n (%)	Total N (%)
Age ( years)	30-39	11 (5.2)	18 (5.8)	29 (5.6)
	40-49	45 (21.2)	81 (26.3)	26 (24.2)
	50-59	8 (36.8)	95 (30.8)	173 (33.3)
	60-69	55 (26.0)	76 (24.7)	131 (25.2)
	≥ 70	23 (10.8)	38 (12.4)	61 (11.7)
Occupation	Governmental	80 (37.7)	68 (22.1)	148 (28.5)
	Non-governmental	19 (9.0)	13 (4.2)	32 (6.1)
	Self employee	68 (32.0)	56 (18.2)	124 (23.8)
	House wife	0 (0.0)	128 (41.5)	128 (24.6)
	Farmer	22 (10.4)	11 (3.6)	33 (6.35)
	No	23 (10.9)	32 (10.4)	55 (10.6)
Marital status	Single	17 (8.0)	9 (6.2)	36 (6.9)
	Married	181 (85.4)	212 (68.8)	393 (75.6)
	Divorced	8 (3.8)	19 (6.2)	27 (5.2)
	Widowed	6 (2.8)	58 (18.8)	64 (12.3)
Ethnicity	Tigris	208 (98.1)	305 (99.0)	513 (98.6)
	Other	4 (1.9)	3 (1.0)	7 (1.4)

Educational level	No formal schooling	29 (13.7)	79 (25.7)	108 (20.7)
	Less than Primary school	14 (6.6)	43 (13.9)	57 (10.8)
	Primary school completed	47 (22.2)	60 (19.5)	107 (20.6)
	Secondary school completed	38 (17.9)	53 (17.2)	91 (19.0)
	College/University completed	84 (39.6)	73 (23.7)	157 (28.9)
Residence	Rural	29 (13.7)	34 (11.1)	63 (12.2)
	Urban	183 (86.3)	274 (88.9)	457 (87.8)
Religion	Orthodox	191 (90.1)	288 (93.5)	479 (92.1)
	Muslim	18 (8.5)	18 (5.8)	36 (6.9)
	Other	3 (1.4)	2 (0.7)	5 (1.0)

**Table 2** Frequency of clinical, behavioral and anthropometric measurements of T2DM patients at Ayder Comprehensive Specialized Hospital, Northern Ethiopia, 2017 (N=520)

Variables		Male n (%)	Female n(%)	Total N (%)
Medication status	Anti-DM Only	123 (58.0)	170 (55.2)	293 (56.3)
	Anti-DM +Anti-HT	34 (16.0)	27 (8.8)	61 (11.7)
	Anti-DM +Anti-Dyslipidemia	25 (11.8)	58 (18.8)	83 (16.0)
	Anti-DM + HT + Anti-Dyslipidemia	30 (14.2)	53 (17.2)	83 (16.0)
Duration of diabetes comorbidity (Yrs)	Less than one year	20 (9.4)	25 (8.1)	45 (8.7)
	1-5 years	101 (47.6)	154 (50.0)	255 (49.0)
	6-10 years	39 (18.4)	75 (24.4)	114 (21.9)
	More than10 years	52 (24.5)	54 (17.5)	106 (20.4)

Alcohol consumption	Yes	46 (21.7)	14 (4.5)	60 (11.5)
	No	166 (78.3)	294 (95.5)	460 (88.5)
Cigarette Smoking	Yes	5 (2.4)	0 (0.0)	5 (1.0)
	No	207 (97.6)	308 (100)	515 (99.0)
BMI	Under weight	11 (5.0)	10 (3.2)	21 (4.0)
	Normal weight	129 (60.0)	154 (50.0)	283 (54.5)
	Over weight	65 (31.7)	116 (37.7)	181 (34.8)
	Obese	7 (3.3)	28 (9.1)	35 (6.7)

DM: diabetic mellitus; HT: hypertension.

**Table 3** Frequency of individual components of MetS among T2DM patients in Ayder Comprehensive Specialized Hospital, Northern Ethiopia, 2017 (n=520)

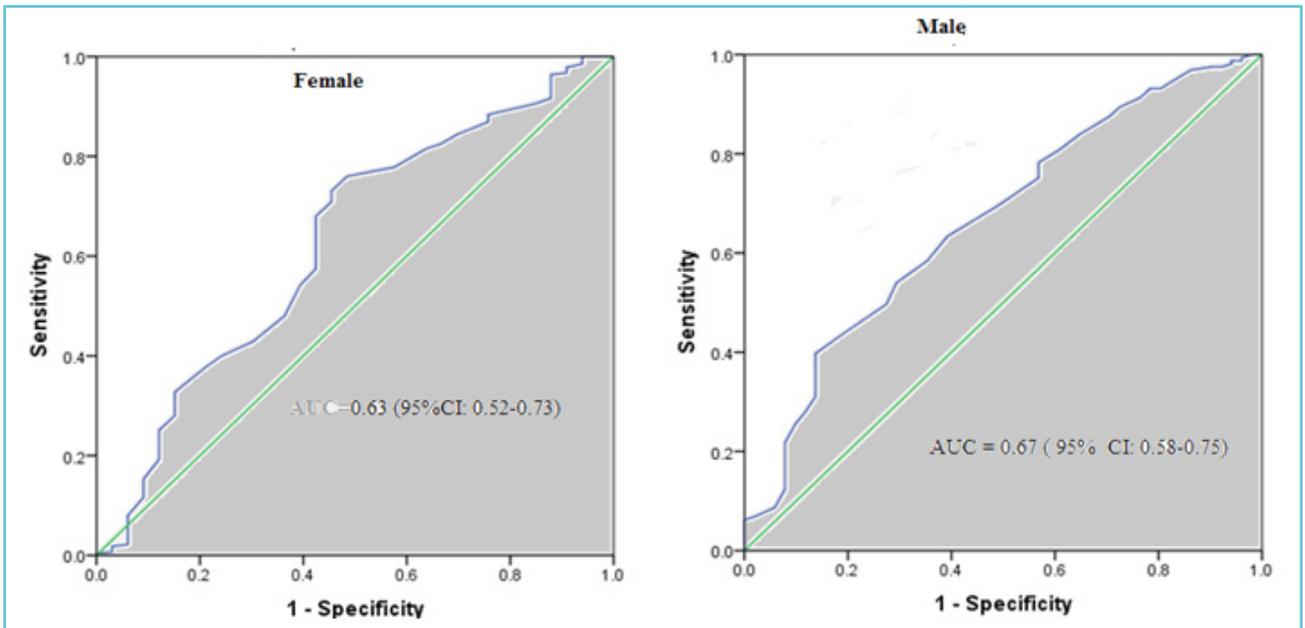
Variables	Male		Female		Total (N%)
	n (%)	95 % CI	n (%)	95 % CI	
High TG	79 (37.3)	30.7-44.0	134 (56.5)	37.1-49.0	213 (40.9)
high BP	75 (35.4)	28.9-42.3	82 (26.6)	21.2-31.3	157 (30.2)
low HDL-C	116 (54.7)	47.6-61.7	105 (34.1)	20.6-47.6	221 (42.5)

#### Clusters of abnormal components

At least 1 component	161 (75.9)	70.2-81.6	275 (89.3)	85.4-92.4	436 (83.8)
At least 2 components	78 (36.8)	30.7-43.5	131 (42.5)	36.7-47.7	209 (40.2)
At least 3 components	57 (26.9)	20.6-32.9	99 (32.1)	27.3-37.2	156 (30.0)
All components	26 (12.3)	7.9-16.7	45 (14.6)	11.0-19.2	71 (13.6)

BP: Blood Pressure; HDL-C: High density lipoprotein cholesterol; TG: Triglyceride.

**Figure 1** ROC curves of WC cutoff value to discriminate cases from controls among T2DM patients for both sexes in Ayder Comprehensive Specialized Hospital, Northern Ethiopia



**Table 4** Characteristics of AUC value of the ROC curves WC to define cases and MetS components among T2DM patients in Ayder Comprehensive Specialized Hospital, Northern Ethiopia, 2017 (N=520)

Sex	MetS components	AUC (95% CI)	Sensitivity	Specificity	P value
Male	Hypertension	0.67 (.60 -.75)	0.51	0.76	≤0.001
	Hypertriglyceridemia	0.67 (.60 -.74)	0.49	0.76	≤0.001
	Low HDL-C	0.60 (.52 -.67)	0.39	0.73	0.013
	Cases	0.67 (.58 -.75)	0.40	0.86	≤ 0.001
Female	Hypertension	0.63 (.56 -.70)	0.79	0.33	0.001
	Hypertriglyceridemia	0.57 (.52 -.65)	0.75	0.34	0.010
	Low HDL-c	0.53 (.44 -.61)	0.72	0.59	0.017
	Cases	0.63 (.52-.73)	0.73	0.45	0.01

**Table 5** Performance of different WC cutoff-points for discriminating patients from controls among T2DM patients at Ayder Comprehensive Specialized Hospital, Northern Ethiopia, 2017 (N=520)

Sex	WC cutoff points (cm)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Youden index
Male (n=212)	90	63.4	60.8	83.6	34.4	0.242
	92	54.4	70.6	85.3	32.7	0.250
	94	44.1	80.4	87.7	31.3	0.245
	95.5	39.8	86.3	90.1	31.2	0.261
	98	27.9	88.2	88.2	27.9	0.161
	100	21.7	92.2	89.7	27.2	0.139
Female (308)	80	88.4	24.2	87.3	20.0	0.126
	82	84.4	30.3	91.0	18.9	0.147
	85	81.4	36.4	91.4	19.0	0.178
	87.5	73.1	54.5	93.0	19.6	0.276
	88	70.9	54.5	92.8	18.4	0.254
	90	61.8	57.6	92.4	15.3	0.194

CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value.

## DISCUSSION AND CONCLUSIONS

Central obesity is the major component of metabolic risk factors. WC is considered to be simple and inexpensive measure with excellent correlation with the results of CT. As a good indicator of visceral fat, WC is widely used to predict the outcome of MetS. WC cutoff values are age, gender and ethnicity specific for MetS.

Therefore, in the current study, WC had classified patients from controls with statistically

significant power for both sexes (P-value <0.001 and <0.017 for males and females, respectively), but it had a poor classification power to categorize patients from controls in both sexes. The optimal WC cutoff value was 87.5 cm (sensitivity 73.1% and specificity 54.5%) for females and 95.5 cm (sensitivity 39.8% and specificity 86.3 %) for males for identifying patients from controls.

Even though the classification power of WC was statistically significant, it had poor classification

power for males (AUC=0.67; 95% CI: 0.58-0.75), and for females (AUC =0.63; 95% CI 0.52-0.73) to discriminate cases from controls (14). Similar to our study, the classification power of WC to classify patient from control was poor in a study conducted in Benin, where, the AUC was 0.67 for males and 0.68 for females (15). Moreover, in Egypt it was also 0.69 for males and 0.63 for females (16).

On the contrary, in several studies such as in Angola (0.85 in males and 0.79 in females) (17), and in Congolese adults (0.899 in males and 0.844 in females) (18), WC had a good classification power to classify patient from control in both sexes.

In our study, the optimal WC cutoff point that best predicts cases was 95.5 cm for the T2DM males, which is relatively higher than the value for females. The finding from the present study is slightly higher than the IDF recommended value for males, which is 94 cm (9).

In contrast to our study, WC cutoff point in urban African teachers was 90 cm in men (19) and the same to that in black South African T2DM male patients were also 90 cm (20). These values were somewhat different to that found in T2DM male patients in our study area.

The WC cutoff value for T2DM females determined in this study is 87.5 cm which is similar to the study in University employees in Angola (87.5 cms) (21).

The result from this study was higher than the recommended cutoff points to define cases for African females, which is  $\geq 80$  cms. Whereas our finding is lower than the study conducted with African females, which was 98 cm (19) and a study from Egyptian adults (96.25 cms) (16).

The present study suggested that the WC cutoff point for females is lower than males, which is in agreement with the recommended value.

Some studies show that the WC cutoff point for African populations is higher in rural South African females (92 cms) than males (86 cms) (22), in Cape Town 94 cm for females and 83.9 cm for males (23).

Similarly, in Benin, the cutoff is 94 cm for females and 80 cm for males, which is the exact reverse of the recommended value (24). Furthermore, a study in Congolese community also shows higher WC value for females (99 cms) compared to the males (95 cms) (18).

In general, several studies in Africa that determined WC cutoff points showed a higher WC value in females as compared to males. On the contrary, our finding is higher in males than females, which is in agreement with the IDF.

On the other hand, a study from Tunisian adults showed equal value of WC for both males and females which was 85 cm (25). Although the WC value in adult Egyptian females (96.2 cms) wasn't higher than males (100.5 cms), it is higher than the IDF recommended value for African females (16).

Further study in prospective cohort is better to determine the optimal WC cutoff point among T2DM patients to define MetS. In addition, studies which can consider the confounders such as lipid lowering medications, age of the participants and duration of diabetes are advisable in order to increase the classification power of WC.



#### **List of abbreviations**

**AUC:** Area Under the Curve

**BMI:** Body Mass Index

**BP:** Blood Pressure

**CT:** Computed Tomography

**CV:** Cardiovascular

**CVD:** Cardiovascular Disease

**FBG:** Fasting Blood Glucose

**HDL-C:** High Density Lipoprotein cholesterol

**HT:** Hypertensive

**IDF:** International Diabetes Federation

**IR:** Insulin Resistance

**LDL-C:** Low Density Lipoprotein cholesterol

**MetS:** Metabolic syndrome

**NCD:** Non Communicable Disease

**NCEP/ATPIII:** National Cholesterol Education Program Adult Treatment Panel III

**NT:** Normotensive

**ROC:** Receiver Operating Characteristics

**T<sub>2</sub>DM:** Type two Diabetes Mellitus

**TG:** Triglyceride

**VAT:** Visceral Adipose Tissue

**WC:** Waist Circumference

**WHO:** World Health Organization

**YI:** Youden Index



### **Ethical issue**

Ethical clearance was obtained from the Institutional Review Board of the University of Gondar. The purpose and importance of the study were explained to the participants. The data were collected after a full written consent was received.

### **Author's contribution**

SH conceived the idea, carried out the proposal writing, participated in the data collection, data analysis and drafted the manuscript. TM and MA participated in data analysis and interpretation of the findings. TM participated on final write-up of the paper. All authors reviewed the manuscript and approved for publication.

### **Conflict of interests**

The authors declared no conflict of interests.

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# Critical issues and new trends on stat tests in clinical laboratory

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## ABSTRACT

### ***Meeting Report on the IX European Symposium on Clinical Laboratory and In Vitro Diagnostics Industry (Barcelona)***

The IX European Symposium of the Clinical Laboratory and In Vitro Diagnostics Industry, entitled “Stat Tests in Clinical laboratory”, took place in Barcelona, Catalonia (Spain), between May 17–18, 2017.

The scientific program was structured in several roundtables that dealt with the following topics: emergency laboratory models, accreditation of stat tests by ISO 15189, critical issues of stat tests and the new proposals of the in vitro diagnostics industry for emergency laboratories. The aim of the Symposium was the discussion of the transformation that stat tests have generated on clinical laboratories in terms of organization, turnaround time, accreditation, and probable evolution of these laboratories coming years.

## INTRODUCTION

The IX European Symposium of the Clinical Laboratory and In Vitro Diagnostics Industry, entitled “Stat Tests in Clinical laboratory”, was held in a most welcoming yet modern environment at the old gothic Hospital de la Santa Creu. The event was co-organized between the Catalan Association of Clinical Laboratory Sciences (ACCLC) and the Catalan Society of Biology (SCB). The symposium was sponsored by the International Union of Pure and Applied Chemistry (IUPAC) and under the auspices of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

The topic of stat testing in clinical laboratories was chosen as an opportunity to discuss the current state of emergency laboratories and also analyse the probable evolution of these laboratories in the coming years. Stat (from the Latin *statim*, immediately, but also considered as an acronym for “short turnaround time”) identifies laboratory tests that should be made available within a defined, as short as possible time, according to clinical necessity (1,2).

There are several reasons that make stat testing an interesting topic for a Symposium. Mainly, organizational and economic aspects: collecting blood outside scheduled activity, the need of quick sample transportation to the laboratory in some cases with pneumatic tube systems, activation of specific paths for managing specimens with priority over routine samples, in many of laboratories entails exclusive staff, specific instrumentation and the need to maintaining back-up instrumentation (3).

## EMERGENCY LABORATORY MODELS

Currently, three laboratory models are adopted for the management and performance of stat tests. Each of these solutions is related to the

size and type of hospital or institution in which they are employed (2).

Generally, in small- and medium-size laboratories, ordinary and stat tests are integrated, and their analysis is performed in the same place using the same instrumentation. The situation in large laboratories is rather more heterogeneous, with the majority of organisations continuing to separate stat from ordinary tests, using different instrumentation, personnel and locations. An intermediate option also exists, in which stat test analyses are semi-integrated in an automated core chain with routine samples, all of which are processed at the same time. However, each approach requires specific workflow processes, leading to different timeliness to produce a validated result. Generally speaking, the stat testing process should be structured to fit the context of care in which the testing services are required.

There was a strong discussion regarding how to prioritise these stat analyses in order to provide an adequate turnaround time (TAT), such as for samples in a specific emergency chain and branches of the chain for the highest-priority samples. It was concluded that large laboratories tend to incorporate stat tests in central automation areas (core), prioritising and adapting circuits to obtain response times appropriate to the needs of each situation.

For decades, stat tests have been performed in dedicated laboratories, either at stand-alone satellite locations or aggregated with central laboratories. However, considering that today all first-line tests can be managed rapidly via total laboratory automation (TLA) (24-hour laboratories), the traditional model appears outmoded for several reasons.

Firstly, a dedicated emergency laboratory represents a source of duplication of analytical platforms that perform the same assay in both emergency and ordinary situations, as well as the

duplication of operating staff and, sometimes, the duplication of orders for the same test.

Secondly, from an analytical perspective, parallel processing of tests across more than one laboratory location within a healthcare setting requires that the analyser alignment be checked continuously to assure comparability of patient results. Although ensuring that the difference between results produced for the same test in stat and central laboratories does not exceed a clinically acceptable difference is often a challenge, it is mandatory for patient monitoring during hospitalisation. All this complexity represents a significant drain on laboratory resources and makes the dedicated emergency laboratory/section model outdated and more expensive than TLA, especially when the latter can manage modern testing processes effectively and with a short TAT (2).

Finally, the chairman enquired as to the role of point-of-care testing (POCT) in emergency laboratories. Bedside testing through implementation of point-of-care devices in the emergency department, intensive care unit or any other ward that more often would require urgent test results for patient management.

The experts explained that POCT at satellite locations may represent a new laboratory model. Stat tests may sometimes be performed near an intensive care department as part of critical analyses used in the evaluation of vital functions. POCT is typically evaluated positively by clinicians because it allows a reduction in TAT and a reduced length of stay in the emergency department (4).

All the experts agreed that POCT should depend on laboratory staff because it is a stat test activity. The involvement of laboratory staff in the management of POCT should therefore be total, from the choice of measurement system, staff training, and quality control assurance to the integration of the results into the clinical history of the patient.

## **ACCREDITATION OF STAT TESTS BY ISO 15189**

The second roundtable discussion addressed issues regarding the accreditation of stat tests by the International Standard ISO 15189. All experts work at emergency laboratories, accredited by UNE-EN ISO 15189, and which perform a high percentage of accredited stat tests. Accreditation is a procedure by which an authoritative body gives formal recognition that an organisation is competent to carry out specific tasks according to certain standards. The National Accreditation and Certification Authority (ENAC) is the agency appointed by the Spanish government to operate as the only national accreditation body. Accreditation of stat test laboratories according to ISO 15189 is becoming more and more a matter of course in Catalonia. However, there are currently only accredited stat tests laboratories in the province of Barcelona, with those in Girona, Lleida and Tarragona certified by ISO 9001:2008 or ISO 9001:2015. The essential difference between certification and accreditation is that the latter, as well as management requirements, also refers to technical competence (5).

To accredit or not to accredit, that is the question. All speakers agreed that the process of accreditation would improve the quality of lab services due to the better documentation of processes and the increased responsibilities or interest of management. The first step prior to accreditation is building an enthusiastic team that is educated regarding the development of quality management systems.

There was an interesting debate as to whether the accreditation of stat tests is more difficult than the accreditation of routine analyses. The former is certainly more laborious because emergency laboratories contain many members of staff and accreditation requires an increase in staff competence. Frustration of evaluated

staff must be avoided, and they must be shown that accreditation is a continuous process of improvement. Laboratory leaders must ensure that staff technical competence is highly praised, as this can provide a great boost to team spirit, as well as instil a sense of achievement and pride in their accreditation.

It was agreed that quality standards for stat tests should be just as rigorous as those for routine tests. Moreover, internal and external quality controls must be available for all parameter devices and must fulfil the same quality requirements specified for non-stat test analysis (5).

In addition, interchangeability studies must be carried out when emergency laboratories are separated from routine laboratories using different instrumentation. ISO 15189 requires that results obtained using different devices are interchangeable in order to assess non-different clinically relevant discrepancies (5).

It was deemed necessary to be proactive in risk management and to make good use of brands listed by ENAC in the final laboratory reports, although the experts outlined the potential difficulties of their use following the regulations.

During the roundtable, it was also emphasised that laboratories should evaluate the impact of work processes and potential failures on examination results, as they can affect patient safety. Laboratories should therefore modify these processes to reduce or eliminate the identified risks, as well as document decisions and actions taken.

It was noted that the presence of the ENAC mark on reports provides an assurance that the laboratory will be able to rely on this endorsement; only laboratories that are actually accredited can make use of the ENAC mark to indicate accredited status (5). However, the experts explained there can be difficulties in using the ENAC mark in final reports, with improper use potentially leading ENAC to bring legal action against the laboratory.

Finally, the speakers encouraged the attendees to consider and initiate actions that will lead them to accreditation, arguing that they themselves had perceived internal improvements in their processes and that they were very satisfied to have achieved this. Accreditation is a process that involves the whole laboratory and requires both good support from management and the positive assessment of clients (clinicians and patients).

Hence, higher quality laboratory testing associated with accreditation is expected to improve patient care by aiding the timeliness and accuracy of medical decision making.

Accreditation programs can help drive improvements in the management of individual laboratories and laboratory networks and may also have positive spill over effects on performance in other sectors of the health care system. In summary, laboratory accreditation can be achieved through leadership, vision and hard work.

## CRITICAL ISSUES OF STAT TESTS

The third debate was attended by a clinician who shared their vision of the laboratory from an outside perspective. Critical issues discussed included the non-conformities that laboratories detect in samples and the impact that they may have on result interpretation. All speakers agreed that a strict stance should be taken when dealing with stat test non-conformities such as haemolytic, poorly spotted or insufficient samples. Greater awareness should be promoted among clinicians, as should the continued training of nursing staff involved in sample extraction. It became clear during the debate that in special situations, such as with newborns, it would be necessary to consider a differentiated treatment.

Currently, serum index measurement (haemolysis, icterus and lipaemia) is automated and determination of the interfering substances is

objective (6). This approach provides benefits compared with subjective visual interpretation, such as increasing the traceability, efficiency and effectiveness of the work process.

In any case, internal and external serum index quality assurances are available for the vast majority of laboratories.

In summary, non-conformities must be dealt with and minimised systematically, as they can influence the reliability of test results, affect patient safety, delay result acquisition and, ultimately, are a drain on resources.

There was an interesting debate regarding the definition of turnaround time. Laboratories most commonly define turnaround time as the time taken from specimen receipt in the laboratory to the moment that test results are reported. However, clinicians may also define turnaround

time as the period from test ordering to reporting (7).

Speakers and assistants agreed that laboratory staff must be involved from the moment of sample arrival in the laboratory with the aim of optimising hospital circuits. Laboratory staff can help to achieve optimum turnaround time in a variety of ways, for example by introducing a pneumatic tube system. Many studies have proven the efficiency of this mechanism in reducing delays arising from human couriers (8).

The causes of poor satisfaction in laboratory users include stat test turnaround times; this parameter was considered by the majority as the most important indicator of laboratory functioning. Hospital computerisation, including the recording of time from test request, sample collection, report generation and report receipt by the clinician would help in generating

**Table 1** Turnaround times in stat laboratories

Parameters	TAT (min)				TAT goals (min)
Literature	S. Angeletti (Ref. 9)	A. Dolci (Ref. 10)	AH. Lou (Ref. 11)	TL. Ellison (Ref. 12)	Wu, Nichols, Hawkins (Refs. 13-15)
Blood gas	-	-	-	-	<15
Blood count cell	21	11-20	22	-	<20
P – Potassium	41	28-49	40	-	<30
P – Troponin	45	29-34	-	52	<60
Prothrombin time	42	36-44	34	-	<45
P – Creatinine or P – Urea	41	36-58	39	-	<45
P – glucose	40	-	-	55	<45
Urinalysis	-	-	-	-	<30

turnaround time data. Analysis of any outliers in turnaround times in a lab would then provide an insight into potential causes of delays and the areas that need improvement.

Table 1 shows summarizing data from previous publications on turnaround times for stat test results. Differences among laboratories have been shown. TAT goals for stat samples were established by different recommendations from clinical practice guidelines.

There are situations that these TAT have to be improved, for example: sepsis code, stroke code and myocardial infarction code. Emergency departments, primary care physicians, divisions of cardiology, hospital administrations, and laboratory staff should work collectively to develop an accelerated protocol for the use of biochemical markers in the evaluation of these patients.

Another topic discussed was the catalogue of a stat test laboratory. It was argued that several aspects should be considered before a stat test is included in a catalogue, with the following underlined: cost, knowledge of the property, technological availability, human resources, and characteristics of the test in terms of positive and negative predictive value, sensitivity and specificity, and above all clinical utility.

The catalogue should be adapted to the laboratory needs of each hospital and it is essential to reach agreement with clinicians. The control of demand must be based on scientific evidence and not guided by economic criteria alone.

In many laboratories, it is obvious that some tests are ordered as stat not for clinical needs but rather for other organisational reasons, such as quick retesting due to previously unsuitable samples, requests for previously forgotten tests, or a desire to receive routine results more rapidly (16).

Sometimes, users may simply be unaware of the difference between stat and ordinary tests.

However, logistical issues can never be considered a good reason for ordering stat tests, as inappropriately ordered tests may degrade the ability of the laboratory to deliver clinically urgent information in a timely fashion. Clinicians not receiving results in the expected timeframe typically order more urgent tests, further reducing laboratory effectiveness and increasing costs (17).

Finally, a discussion took place regarding critical results, i.e. those results that must be immediately sent, either by phone or electronically, to the patient care provider by laboratory staff. The question arose as to who should define a result as being of critical value; responses included the requirement for consensus with clinicians, published recommendations and laboratories' professional experience.

An expert outlined a project involving the Catalan Institute of Health (ICS) and other centres. As part of the project, a descriptive cross-sectional study was undertaken by laboratory professionals and clinicians from hospitals and primary care centres, with biological parameters reviewed whose values - are likely to be considered as possibly critical - and therefore must be reported. In addition, the critical limits considered and actions carried out after obtaining a critical result were also reviewed. From this approach an agreement between the participating laboratories was reached that subsequently formed the basis for the construction of an initial model, based on consensus with clinical practitioners.

To achieve consensus with clinicians, a Delphi model was applied in real time according to the "Health Consensus" methodology.

The model used data from a questionnaire that was sent to clinicians to value and identify, according to their criteria, when a result should be considered critical. The document prepared will be available soon.

## PROPOSALS OF THE IN VITRO DIAGNOSTICS INDUSTRY FOR EMERGENCY LABORATORIES

The final debate was performed with the collaboration of the in vitro diagnostics industry, who outlined potential technological solutions for emergency laboratories.

Initially, each representative of the in vitro diagnostics industry explained their technological proposal for stat test laboratories. All speakers agreed that each laboratory and hospital is different and that flexible solutions should be sought for each model.

Currently, three laboratory models are available: ordinary and stat tests fully integrated into automated chains; stat test laboratories independent from ordinary test laboratories; and an intermediate option in which stat test analyses are semi-integrated in an automated core chain with routine samples, all of which are processed at the same time.

Many laboratories are currently considering the installation of total laboratory automation systems for routine clinical chemistry and laboratory haematology testing. The advantages of total laboratory automation include cost reduction and improved turnaround time. Such automated pipelines integrate pre-analytics (check-in, sorting, centrifugation and aliquoting) and post-analytics (storage and disposition), offering the possibility of bulk input, volume detection, aliquoting and storage. All speakers stated that their companies are attempting to find solutions with which to prioritise the processing of stat test samples within automated chains.

The moderator and attendees asked the industry representatives to promote the development of computer-based tools that would allow the calculation of turnaround time and allow users to determine exactly how long each process lasts, including centrifugation, aliquoting,

processing and report global delivery time. They also advised that the industry focus their efforts on achieving improvements in pre-analytical processes. Computer enhancements should also be made available for communicating critical results either through mobile telephony or messaging via tablets and other devices. All speakers agreed that they encounter many problems regarding the protection of data when installing such systems in hospitals. Finally, they were asked to work to offer technological and other solutions so that stat test laboratories can obtain accreditation, according to UNE-EN ISO 15189, for the analyses carried out.

Finally, the in vitro diagnostics industry representatives acknowledged that it is necessary to work together to solve both current needs and those that may arise in the future.

## CONCLUSIONS

We are moving towards a new model of health-care system, in which every patient has a critical pathology and treatment is “emergent” for all. In this scenario, laboratories must consider every test a stat test, so that the separation between routine and stat processes is abolished. Automation, and particularly total laboratory automation, represents a formidable tool with which to both meet increasingly demanding critical needs and, even more importantly, improve patient outcomes.

In conclusion, in laboratory medicine, although technology can be used to improve clinical effectiveness and patient outcomes, it must be managed by qualified laboratory professionals.

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# Diamond Blackfan Anemia: genetics, pathogenesis, diagnosis and treatment

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## ABSTRACT

Diamond Blackfan Anaemia (DBA) is a sporadic inherited anemia with broad spectrum of anomalies that are presented soon after delivery. It is inherited mainly in autosomal dominant inheritance manner and caused by mutations and deletions in either large or small ribosomal protein genes that results in an imbalance between the biosynthesis of rRNA and ribosomal proteins, eventually the activation and stabilization of p53. Diagnosing DBA is usually problematic due to a partial phenotype and its wide inconsistency in its clinical expression; however, molecular studies have identified a heterozygous mutated gene in up to 50% of the DBA cases and corticosteroid drugs are the backbone treatment options of DBA. Anomalies in bone marrow function in DBA cases are broadly associated with both congenital and acquired bone marrow failure syndromes in human. In this review different literatures were searched in Medline (eg. PubMed, PMC, Hinari, Google scholar), OMIM, EMBASE by using search engines (Google, Yahoo, Baidu Ask.com) and searching was performed by using search key words (DBA, ribosomopathies, Bone Marrow Failure Syndromes, pure red cell aplasia). Only human studies were included. This review is summarizing the current understandings of DBA.

## INTRODUCTION

Diamond Blackfan Anemia (DBA) is a sporadic heterogeneous genetic disorder characterized by red blood cell aplasia in association with skeletal anomalies and short stature that classically appear soon after birth (1-4). Although the prominent feature of DBA is anemia (5), clinically it is a broader disorder and is manifested by growth retardation and congenital malformations of the head, heart, neck, upper limbs, and urinary system, which are present in approximately 30% to 50% of the DBA patients (6-9).

As shown by many studies, the incidence of DBA was estimated to be 1-4 cases per 500,000 live births in a year and it seems to be constant over time. No seasonal disparity has been observed as a function of the date of conception. The tendency of the disorder across the ethnicity of the people and in both genders, is almost comparable (10,11).

Chronic macrocytic-normochromic anemia, low reticulocyte count, and decreased or totally absent erythroid precursors characterized by failure of erythropoiesis with normal production of leukocytes and platelets in the bone marrow, are the main hematological features of DBA (2,3,12). Furthermore, a majority of the patients have laboratory findings of increased mean corpuscular volume (MCV), elevated erythrocyte adenosine deaminase activity (eADA) and persistently elevated fetal hemoglobin (Hgb F). However, these laboratory findings may not be observed in some DBA cases; and even in the same families, signs and symptoms may vary among affected family siblings (13,14).

After a succession of studies, by now DBA has been shown to be associated with both ribosomal and non-ribosomal mutations in genes located on more than 11 chromosomes which are responsible for encoding the ribosomal proteins (RP) (12,15).

Molecular mechanisms underlying the causal consequence between RP haploinsufficiency and anemia have not yet been clearly elucidated. A generally documented pathogenetic hypothesis implies that a defective ribosome biosynthesis leads to apoptosis in erythroid progenitors which in turn is leading to erythroid failure. This mechanism has been named "ribosomal stress", and there are indications that it may be signaled through p53. All genes identified to be mutated in DBA encode ribosomal proteins which are involved in either the small (RPS) or large (RPL) subunits of these proteins and the scarcity of these proteins can cause the development of the disease (16). The disorders of ribosome synthesis or associated genes that lead to disrupted ribosomal biosynthesis (ribosomopathy) are more than one independent ribosomal protein mutation i.e., all patients of DBA may not show the same RP mutations (6). Corticosteroids, transfusion therapy and stem cell transplantation are the current options for the treatment of DBA (17,18).

The main aim of this narrative review is to provide the reader with a comprehensive knowledge on DBA genetics, pathogenesis, diagnosis and treatment. Different literatures were searched in Medline (e.g., PubMed, PMC, Hinari, Google scholar), OMIM, EMBASE by using search engines (Google, Yahoo, Baidu Ask.com) and searching was performed by using search key words (Diamond Blackfan Anemia, Ribosomopathies, Pure red cell aplasia, Ribosomal proteins, Bone marrow failure syndromes). Only human studies were included.

## MOLECULAR PATHOPHYSIOLOGY OF DIAMOND BLACKFAN ANEMIA

### *Impaired ribosomal biogenesis in DBA*

Recently, DBA is a well-recognized inherited bone marrow failure syndrome, frequently caused by alterations in ribosomal protein (RP)

genes. Otherwise it rarely results from the mutation of the hematopoietic transcription factor gene, GATA1. After the preliminary descriptions of heterozygous RPS19 mutations in a subset of DBA cases, a substantial progress has been made over the past decades for improved explanation of the genetic causes of DBA (13). With a growing emphasis on the RP genes, the search for DBA-related genes, which was initially based on classic genetics techniques including cloning of cytogenetic abnormalities and extended linkage analysis, has shifted to target resequencing of the known RP genes (19-21).

These advanced studies have recognized both large and small subunits of RP gene anomalies which currently includes but is not limited to RPL5, RPL11, RPL35A, RPS7, RPS10, RPS17, RPS19, RPS24, and RPS26 as a mutated gene in multiple families of DBA patients (8,22,23). Changes in larger number of RPs have also been identified in isolated patients or families, including RPL3, RPL7, RPL9, RPL14, RPL19, RPL23A, RPL26, RPL35, RPL36, RPS8, RPS15, RPS27A, RPL18 and RPL35 (24-26).

Mutations in RP genes have been confirmed to be the direct cause of faulty erythropoiesis and consequently anemia, and are found in more than half of DBA cases (27). The RPS19 gene was the formerly known mutated small ribosomal protein and is still the most frequently mutated gene in DBA patients which accounts about 25% of total DBA patients (13). More than 50% of RPS19 mutations are either deletions of one RPS19 allele, or insertional, frame shift, splice site, or nonsense mutations, which lead to an untimely termination of RPS19 protein synthesis. This results in a deficiency of RPS19 protein which is termed as “haploinsufficiency” in human cells (28,29). Again, greater than half of the missense mutations termed as “class I” which cause the RPS19 protein to be folded improperly and rapidly aimed at premature degradation

of ribosomal proteins consequently leading to RPS19 haploinsufficiency (30).

RPS19 protein plays an important role in 18S rRNA maturation and 40S synthesis in human cells (29,31,32). Mutations associated with RPS19 can disrupt the pre-rRNA processing of the 18S rRNA and pre-40S subunits, leading to reduced production of 40S ribosomal subunits. Finally, the decreased expression of RPS19 is able to imitate many aspects of the DBA phenotype (21,35). As a result, this knockdown of RPS19 by RNA interference can cause a severe defect that alters the normal primary human hematopoietic progenitor differentiation and proliferation of the erythroid progenitor (EP) cells (33). Furthermore, the defective erythropoiesis in DBA caused by RPS19 deficiency can be rescued by ectopic overexpression of exogenous RPS19. All of these strongly support the notion that RPS19 haploinsufficiency resulted from RPS19 modifications could be the central pathogenic mechanism in the underlying DBA pathology suggesting the feasibility of RPS19 gene augmentation to treat DBA cases (31).

Thus, RPS19-lacking cells suffer from a relatively shortage of 40S rRNA and has a reduced ability for translation initiation. RPS19 deficiency can lead to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of RPS19 prevents cell proliferation and early erythroid differentiation, but not late erythroid maturation in RPS19-deficient DBA cell lines. Haploinsufficiency of RPS19 has been shown in a subset of patients and appears to be sufficient to cause DBA (20,22).

Advanced studies showed that the RPS19Dsk3 mouse recapped the human DBA phenotype insofar as a hypoproliferative, pro-apoptotic anemia with growth hindrance. Given existing data, it is now generally believed that DBA resulted from an intrinsic cellular flaw in which erythroid progenitors and precursors are greatly sensitive to demise

by apoptosis (36,37). The phenotypes observed in zebrafish and mouse DBA models DBA can be partly or entirely rescued by alterations in p53, robustly proposed that p53 stabilization and activation plays a noteworthy role in the pro-apoptotic phenotype of cells with RP haploinsufficiency (38).

DBA have led to the formulation of “ribosomal stress” hypothesis in which reduced RP synthesis activates p53 that induces the downstream events and leads to cell cycle termination or apoptosis. Finally, this phenomenon results in the DBA phenotype of anemia, deprived growth and results in congenital abnormalities (16).

There are numerous potential mechanisms by which a faulty ribosome assembly or nucleolar stress might signal to p53 stimulation. One interesting mechanism in the pathophysiology of DBA is the interaction of ribosomal proteins with Murine Double Minute (MDM2) which is a powerful controller of p53 level and its activity. MDM2 is a ring finger ubiquitin ligase that interacts with and enhances the degradation of p53. In this interaction, the large proteins of the 60S subunit namely RPL5, RPL11, and RPL23 have been presented to bind to MDM2 and reduces the activities of MDM2 which in turn results in p53 stabilization (39).

The heterozygous DBA mutations result in loss of function in a single copy of ribosomal protein gene. Thus, the pathophysiology of DBA is now attributed to ribosomal malfunction and these mutations in ribosomal genes have been identified in approximately 50% of DBA patients (40). The large number of ribosomal proteins mutated in DBA fail to cluster in any specific region of the ribosome. Haploinsufficiency or reduced expression of a ribosomal protein results in decreased levels of the cognate 40S or 60S subunit and a defect in processing of the ribosomal RNA precursors (33,41,42).

Although DBA is considered as a ribosomopathy, it can be also caused by non-RP gene mutations.

GATA1 encodes a transcription factor which is essential for erythroid differentiation and therefore it is not surprising to state that this gene is involved in DBA pathogenesis (43). The mutation in the GATA1 gene is a Guanine-Cytosine (G→C) transversion at location 48,649,736 on the X chromosome. By applying whole-exome sequencing, aberration in the zinc-finger transcription factor gene “GATA1”, has been identified to be one of the possible causative agents for the development of DBA. This G→C transversion is associated with X-linked form of DBA, leading to the substitution of leucine to valine at amino acid 74 of GATA1. This aberration affects GATA1 splicing processes and leads to termination of the full-length GATA1 protein level and synthesis of a short isoform what we call GATA1 short (GATA1s) (11). While comparing the two isoforms, there is no exon 2 in GATA1s consequently the absence of a transactivation domain. Full-length GATA1 has a crucial role in humans for the differentiation of erythroid cells. During erythroid differentiation, full-length GATA1 enhances the synthesis of erythroid genes by silencing megakaryocytic or other hematopoietic lineage-specific genes. This up and down regulation of GATA1 is vital during the megakaryocyte erythroid progenitor (MEP) commitment program to megakaryocytic and erythroid differentiation (44,45).

While the specific role of GATA1s in DBA pathophysiology remains unclear, studies suggest that full-length GATA1 protein level could be an important driver of DBA pathophysiology. Researchers used short ribonucleic acids (shRNA) against several RPs in normal CD34+ cells to induce RP deficiency to confirm the role of GATA1 in DBA patients.

Thereafter they observed a reduction of full-length GATA1 protein level and erythroid maturation defects. On the other hand, increasing the GATA1 protein level could partly rescue defects in DBA associated RP haploinsufficiency (46,47).

### **Mechanisms of erythroid failure due to ribosomal protein deficiency**

DBA typically presents with erythrocyte aplasia in the first one year of life and both quantitative and qualitative defects of erythroid progenitors contribute to the abnormal erythropoiesis. Majority of the genes responsible for the development of DBA are ribosomal proteins suggesting that insufficiency in ribosomal function may be the underlying cause of red cell aplasia in DBA cases. Ribosomal haploinsufficiency leads to disrupted ribosome biosynthesis and a consequent cytoplasmic buildup of numerous free RPs which results in the stabilization and activation of p53 (38,48-50). In ribosomal stress conditions, several cytoplasmic free RPs such as RPL11, RPI5, RPL23, RPS7, and RPS27 will bind to Murine double minute (MDM2) and hinders its interaction with p53, leading to the stabilization of p53 (51-53). This pathway has been shown to be upstream of apoptosis and cell-cycle arrest, eventually which leads to DBA erythroid hypoplasia (54,55).

Homozygous inactivation of p53 in RPS19 mutant mice totally corrected the DBA erythroid hypoplasia, further corroborating the MDM2/p53-mediated DBA pathogenesis caused by RPS19 mutations and cytoplasmic free RPL26 could influence the translation of p53 mRNA (30,56). However, it is believable that permanent overexpression of RPS19, by gene transfer may result in a persistent activation of MDM2 and/or inhibition of p53 (55).

Moreover, when global translation was blocked by inhibition of the translation initiation factor Eukaryotic Transcription Initiation factor 4- Erythroid (eIF4E), the investigators observed a down regulation of full-length GATA1 while the other proteins persisted unaffected. These observations suggest that ribosome biosynthesis faults could impact translation of specific proteins, as GATA1. Thus, in addition to the

cytoplasmic free RPs, full length GATA1 level reduction may participate in the stabilization of p53 and, consequently, the DBA erythroid cell phenotype (47).

Although the mechanism by which mutations in the RP genes caused explicit defects in erythroid cell maturation is not copiously understood, several lines of evidences indicate that p53 activation induced by ribosomal malfunction may be fundamental for the pathogenesis of DBA (56). Disruption of 40S biosynthesis provoke the release of RPL11 and other RPs into the nucleoplasm results in the binding with MDM2. This phenomenon can compromise MDM2 activity; thereafter the consequent accumulation of p53. The exhibition of phenotypes such as growth retardation, macrocytic anemia with reticulocytopenia and increased apoptosis in bone marrow progenitors by RPS19 mutant mice suggested the presence of a direct link between DBA phenotypes and accumulation of p53 (51).

The pathophysiology for erythroid defect in DBA might therefore be because of the failure of a particular protein to achieve a threshold level at a critical stage; for example, by disturbing the stoichiometry of multi-protein erythroid-specific complexes or by a more selective influence on the translation of a vital protein. A global reduction in translation could also be significant for the pathophysiology of DBA (57).

In conclusion, defective erythropoiesis in DBA is mostly the consequence of either ribosome biogenesis defects due to mutations in ribosomal protein (RP) genes or as consequence of mutation in the GATA1 gene, leading to a reduction of full-length GATA1 and synthesis of a short isoform, GATA1s. In normal conditions, p53 is degraded because of its interaction with MDM2. In DBA, the ribosome biogenesis defect induces a release of several free RPs in the cytoplasm, which can inhibit p53 degradation. p53 degradation is also inhibited by the reduction

in full-length GATA1 synthesis (15). Treatment includes gene therapy strategies such as gene addition (to increase copy numbers of the wild-type form of the mutated proteins), gene silencing (leading to the degradation of the mutated mRNA) and genome editing (such as zinc-finger nucleases, transcription-activator like effector nucleases, or clustered, regulator).

## **GENETICS AND INHERITANCE OF DIAMOND-BLACKFAN ANEMIA**

### *Inheritance*

According to the report of recent studies, approximately 40 – 45% of DBA cases are hereditary which are inherited with autosomal dominant inheritance which mean that a single copy of altered gene in each cell is adequate to cause the disorder (58) whereas the remaining 55 – 60% of the DBA patients are sporadic, i.e., resulted from new aberrations in the gene which occur in people who have no history of this disorder in their family (12).

Even though autosomal dominant inheritance is the frequently observed pattern of inheritance, autosomal recessive inheritance, which is defined as the presence of DBA siblings from unaffected consanguineous parents, with a lesser frequency has been reported. DBA classically presents at 2 - 3 months of age; only 25% of affected offsprings are anemic at birth and hydrops is occasional (59,60).

### *Genetics*

As shown in the pathophysiology of DBA, it mainly arises from an abnormal ribosomal protein gene with the exception of the rare form resulting from mutation of transcriptional factor GATA1 (6,13,22,24,25,28,61-63). The genes encoding ribosomal proteins belonging to both the large and small ribosomal subunits are found to be mutated in DBA (7,11). This disorder is described by genetic heterogeneity,

disturbing different ribosomal gene loci. More than a decade of heterozygous mutations resulting in haploinsufficiency have been identified for the genes that encode ribosomal proteins (40) (Table 1).

Mutations in ribosomal genes account for 60 – 70% of DBA patients. Among these ribosomal gene mutations, about 20% of the cases involve large deletions that require analysis of copy number variation for detection. On the other hand, overall it is estimated that around 35% of the DBA cases remain yet genetically indeterminate. These ribosomal gene aberrations may be inherited in an autosomal dominant pattern or may arise spontaneously-linked mutations in the transcription factor GATA1 (11).

## **GENOTYPE AND PHENOTYPE CORRELATION**

In general, the phenotypic spectrum of DBA embraces a wider domain of severity, even within the same families, it ranges from the classical syndrome to individuals with a solitary increase in eADA. No correlation has yet been found between the identity of the DBA gene and hematological severity, including response to steroids. However, craniofacial abnormalities are more linked to mutations affecting either RPL5 or RPL11 (22,29).

Clinical figures from European and American DBA cases disclosed that the incidence of malformations in DBA patients having RPS19 mutations is 31% and this is not significantly different from that of the entire DBA population (29,64,65). RPS19 mutations are found in some first-degree families presenting only with isolated high eADA and/or macrocytosis. However, large deletions at the 19q13 locus and unbalanced translocation t (X; 19) are always linked to mental retardation, which points to a contiguous gene syndrome. Conversely, the patient with balanced translocation t(X;19)

**Table 1** Mutations of ribosomal protein genes in Diamond Blackfan Anemia

Sr. No.	Study	Year	Gene	Chromosome	Subunit	Frequency*
1	Mirabello et al	2017	RPL18 RPL35	19q13.33 9q33.3	60S 60S	2 2
2	Gazda et al	2012	RPS26	17p13	40S	6.5
3	Farrar et al	2011	RPS17	15q	40S	5†
4	Doherty et al	2010	RPS7 RPS10	2p25 6p	40S 40S	1 2-5
5	Gazda et al	2008	RPL5 RPL11	p22.1 p36.1-p35	60S 60S	7 6.5
6	Farrar et al	2008	RPL35A	3q29-qter	60S	2
7	Cmejla et al	2007	RPS17	15q	40S	5†
8	Gazda et al	2006	RPS24	10q22-q23	40S	2
9	Draptchinskaia et al	1999	RPS19	19q13.2	40S	25
10	-	-	Others	-	GATA1, RPS28	-

\* Frequency may depend on the cohort size.

† Including gene deletions.

which interrupts RPS19, without loss of other genes, has normal mental development (66). Comparatively, mutations in RPL5 and RPL11 possibly will be accompanied by a more severe phenotype than mutations in RPS19, particularly with respect to skeletal deformations (22). Current studies advise that the patients with an RPL5 and RPL11 mutation have an increased probability to present with craniofacial, thumb and heart anomalies (9,22,61).

Physical anomalies are observed in up to 50% of the cases with a wide range of severity. These are classically craniofacial, including hypertelorism, flat nasal bridge, and high arched or cleft palate. Thumb abnormalities have also been seen in 20%

of the DBA cases, including the usual triphalangal thumb (67). In RPS5 alterations, craniofacial, congenital heart, and thumb defects are more severe than those seen with pathogenic variants in RPL11 and RPS19 (22,68). Curiously, patients with RPL5 mutations incline to have cleft lip and/or palate or cleft soft palate, solitarily or in combination with other physical abnormalities.

Consistent with these reports, patients with RPL5 mutations also had physical malformations and cleft palate, and in contrary, patients without an RPL5 mutation presented with cleft palate (69). Cleft lip and/or cleft palate was also reported in almost 50% of the affected babies with RPL5 pathogenic variants (22). Small

gestational age was reported in individuals with an RPL5 and RPS19 pathogenic variant as well, which is higher in individuals with RPS5 pathogenic variant (68). Pathogenic variants in RPL11 are largely associated with thumb abnormalities (22); individuals with RPL11 pathogenic variants have been also identified with cleft lip or palate (CL/P) (68).

Individuals with mutated variants of RPS10, RPS19, RPS26 have not yet shown any genotype-phenotype correlations (24). Till now, no genotype-phenotype correlations have been also identified in persons with RPS29 pathogenic variants. DBA with mandibulofacial dystostosis were identified in mutants of RPS28 and TSR2 whereas persons with the variants of RPL27, RPL31 and RPL27 have no identified genotype-phenotype correlations (70).

## CLINICAL PRESENTATIONS AND DIAGNOSIS

### *Clinical presentations*

#### *Hematologic features*

DBA babies typically present with an erythrocyte aplasia in the first twelve months of life, with the median age of exhibition of two months but occasionally its presentation may be delayed up to adulthood. By definition, all DBA patients are anemic (71), and it is present at birth in only 15% of patients and fetal hydrops has occasionally been reported, suggesting that erythropoiesis is spared *in utero* perhaps this is due to transiently being rescued by maternal or placental factors with a post-natal switch from effective to ineffective erythropoiesis (17,60,72).

Red blood cells are usually macrocytic; reticulocyte counts are reduced or zero but the other hematological lineages are not involved as a rule with the exception of slightly an abnormal lower leukocyte and increased platelet

counts reported at diagnosis. Bone marrow aspirates indicate isolated erythroblastopenia, (usually <5% of nucleated cells on bone marrow smears) in more than 90 percent of the patients. Another unusual bone marrow pattern is erythroid hyperplasia with maturation detention; apparently normal numbers and maturation of erythroblasts have been exceptionally described (17,73).

Bone marrow analysis also demonstrated normal cellularity and morphology except for the erythroid line in all patients (74). Erythroid entire aplasia and hypoplasia have been found in DBA cases presented with erythroid maturation arrest with an elevated number of juvenile precursors and indicated dyserythropoietic morphology (71,74,75).

The colony assessment for BFU-E confirmed totally absent/reduced growth in 83% of patients. Addition of stem cell factor (SCF) induced a noticeable increment of erythroid colonies in all the tested subjects. The activity of eADA which is a crucial enzyme in the purine salvage pathway, is usually high in DBA patients (71,74,76). A moderately increased risk of developing hematological malignancies also exists and initial clinical manifestations such as pallor, shortness of breath while suckling, failure to thrive and systolic murmur are observed during infancy (16). The risk of developing solid tumors, myelodysplastic syndrome, or leukemia is elevated in DBA patients (77).

#### *Physical abnormalities*

More than a third of the disordered persons present with a variety of associated congenital physical anomalies. Especially, thumb and upper limb malformations as well as craniofacial anomalies including short stature are common. A cute snub nose and wide spaced eyes, and other craniofacial anomalies are also seen. Other defects frequently observed include urogenital

anomalies, atrial or ventricular septal defects, and prenatal or postnatal growth retardation. A distinct facial appearance and triphalangeal thumbs have been characteristically explained in DBA (27,70,78,79).

According to some studies, the incidence and severity of physical abnormalities have not been gender-related, whereas other researchers revealed that a greater severity of abnormalities was observed among males, compared to females (66).

### Diagnostic criteria

Diagnosing DBA is usually tough due to its partial phenotypes and the wide inconsistency of clinical expressions (16,71). Having the variability, the International Clinical Consensus Conference stated diagnostic and supporting criteria for the diagnosis of DBA (71) (Table 2). The diagnosis of DBA is made when the requirements of major criteria outlined in Table 2, are fulfilled and the Parvovirus infection and Fanconi's anemia are ruled out. However, some essentials are not

**Table 2** Diagnostic criteria for DBA\*

Diagnostic criteria
<ul style="list-style-type: none"> <li>• Normochromic, often macrocytic anemia developing in the first year of life</li> </ul>
<ul style="list-style-type: none"> <li>• Profound reticulocytopenia</li> </ul>
<ul style="list-style-type: none"> <li>• Normocellular bone marrow with selective deficiency of erythroid precursors</li> </ul>
<ul style="list-style-type: none"> <li>• Normal or slightly reduced leukocyte count</li> </ul>
<ul style="list-style-type: none"> <li>• Normal or slightly increased platelet count</li> </ul>
Major supporting criteria
<ul style="list-style-type: none"> <li>• Gene mutation described in “classical” DBA</li> </ul>
<ul style="list-style-type: none"> <li>• Positive family history</li> </ul>
Minor supporting criteria
<ul style="list-style-type: none"> <li>• Elevated erythrocyte adenosine deaminase activity</li> </ul>
<ul style="list-style-type: none"> <li>• Congenital anomalies described in “classical” DBA</li> </ul>
<ul style="list-style-type: none"> <li>• Elevated HbF</li> </ul>
<ul style="list-style-type: none"> <li>• No evidence of another inherited bone marrow failure syndrome</li> </ul>

\* Accepted by the DBA working group of the European Society for Paediatric Haematology and Immunology, ESPHI (17).

included in these criteria such as the presence of typical malformations, the response to steroids and the chronic course of the anemia can also help to diagnose DBA (73).

Onset after the age of two years or the absence of isolated bone marrow erythroblastopenia induce a big caution in the diagnosis of DBA. The only identification of pathogenic mutants in one of the DBA genes conclusively establishes a diagnosis of DBA (61,80). Furthermore, molecular diagnosis enables the detection of carriers, and the exclusion of hematopoietic stem cell transplantation from sibling donors living with the mutations. However, determining the effects of missense mutations may be difficult, whereas nonsense and frameshift mutations will probably be pathogenic in the majority of cases (73). The bone marrow is classically normocellular, with a scarcity of erythroid precursors. The erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) in vitro are severely reduced, with relative sparing of the granulocyte-monocyte colony-forming units (CFU-GM) (81). The erythrocyte adenosine deaminase activity (eADA) levels are often elevated (74).

DBA may be misdiagnosed with transient erythroblastopenia of childhood (TEC). Therefore, to avoid misdiagnosing of DBA, TEC must be ruled out. TEC is a common disorder of children age greater than one year. It is an acquired, short-lived failure of red cell production usually of a month or so in duration. TEC, most likely a post infectious, transient autoimmune IgG-mediated disorder, typically occurs in children probably as the result of infections acquired through contact (82). As in DBA, children with TEC often present with profound anemia as a result of pure red cell aplasia. On the other hand, DBA is a dominantly inherited disorder observed in children younger than one year. It is characterized by presence of congenital anomalies; elevated MCV, fetal Hgb and erythrocyte adenosine deaminase activity (75,76,83,84).

## **TREATMENT AND PROGNOSIS OF DBA**

Heterogeneity of DBA is also shown in response to treatment and in the follow-up of DBA cases. In 2008, a group of veteran clinicians established a consensus for the diagnosis and treatment of DBA. This document represents a gold standard for therapeutic decisions. There are no obvious phenotypic or genotypic differences between remission and non-remission patients (71,83).

Although corticosteroid therapy is endeavored with varying regimens for almost all DBA cases, treating the patients with RBC transfusion may be used primarily in the first year of life to reduce corticosteroid associated side effects and adverse effects in neonates which may include noteworthy growth disturbances. Even so there is currently no way to select the patients who will respond or not to the treatment, around 80% of the patients initially respond to steroids but those DBA patients who initially responded to this therapy suffered from many side effects. Some DBA cases remained responsive to steroids, while efficacy vanished in others taking the treatment (18,71,83). The mode of action of corticosteroids in DBA is particularly obscure. Apoptosis at the progenitor level seems to be the cause of the anemia and corticosteroids seem to have a non-specific anti-apoptotic effect in erythroid progenitors particularly at the colony forming unit-erythroid (CFU-E) proerythroblast interface (84).

Patients who are not responsive to steroids or are unable to tolerate the treatment may require chronic RBC transfusion therapy. This therapy is given to most patients every 35 weeks with a goal of sustaining the Hb level greater than 8 g/dL. Based on the child's growth and function, Hb values may need to be higher for some patients. Since the transfusion therapy of RBC is chronic, iron overload becomes challenging, so careful monitoring of serum ferritin levels and other parameters indicative of iron overload is

mandatory (71). Pediatric hematologists with expertise in the treatment of DBA will frequently commence iron chelation with intravenous or oral agents after 15 units of red cell transfusions. Intravenous chelation is frequently needed for the establishment of nonmetallic ports that are magnetic resonance imaging compatible (84,85).

Performing allogeneic Hematopoietic Stem Cell Transplantation (HSCT) is another alternative to cure hematological aspects of DBA. This therapy is performed when DBA patients become unresponsive to repeated transfusions or to prevent iron overload and organ damage due to dependency of frequent transfusions. However, the adverse events due to HSCT may exceed from those adverse events due to iron overloading. In the future, gene therapy is presumed promising for RPS19 deficient DBA patients. Other treatments, have been used in DBA over the last three decades. However, these drugs appear to be largely ineffective and there is currently no evidence that any of these has a major role in the management of DBA (71). The prognosis is generally good. However, complications of treatment and a higher incidence of cancer may reduce life expectancy. Disease severity depends on the quality and response to treatment. For patients undergoing regular transfusions, quality of life is clearly altered (85).

## **CONCLUSIONS**

DBA is a clinically heterogeneous disorder accompanied by hypoplastic anemia and also manifested by congenital malformations. Even if Ribosomal protein haploinsufficiency have been coined as a primary causative agent of DBA, non-ribosomal proteins such as GATA1 have been identified to have a vital role in the pathogenesis of DBA. The molecular mechanisms which are fundamental for the pathogenesis of DBA are still not entirely understood. The

discovery of RPS19 as the foremost DBA gene led to an exciting scientific research in DBA and other ribosomal disorders. Even though there is convincing evidence for the involvement of p53 in the pathogenesis of DBA, no single proposed mechanism so far accounted for all facets of DBA and yet none is mutually exclusive. Even though a number of genes are responsible for DBA, mutations in RPS19 are the major causes of impairment of ribosomal protein biosynthesis which lead to nucleolar disorganization and activation of the p53 family. Many treatment options have been tried over the past several years with inconsistent success. Despite many treatments having been tried for the management of DBA, chronic red blood cell transfusion, corticosteroids and hematopoietic stem cell transplantation remained the cornerstones of therapy.



## **Abbreviations**

**CL/P:** Cleft of Lip or Palate

**DBA:** Diamond Blackfan Anemia

**eADA:** elevated erythrocyte Adenosine Deaminase Activity

**HDM:** House Dust Mites

**MDM:** Murine Double Model

**RP:** Ribosomal Protein

**RPL:** Large Ribosomal Protein

**RPS:** Small Ribosomal Protein

**TEC:** Transient Erythroblastopenia of Childhood



## **Availability of data and materials**

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

### Competing interest

The authors declare that they have no competing interests.

### Authors' contributions

MS & BE performed literature searching and drafted the manuscript. MM involved in drafting of the manuscript along with MS & BE. All authors read and approved the final manuscript.



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# Gaucher disease: an underdiagnosed pathology in the Eastern Moroccan population

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## ABSTRACT

Gaucher disease (GD) is a lysosomal storage disease. It corresponds to a congenital deficit in  $\beta$ -glucocerebrosidase. This pathology should be considered in the presence of unexplained splenomegaly, with or without signs of haemorrhage, skeletal manifestations or hepatomegaly.

The diagnosis is based on the measurement of the  $\beta$ -glucocerebrosidase activity but the preanalytical process should be respected in order to avoid the under-diagnosis of this disorder and the delay of its management. We report two cases of Gaucher disease collected at Mohammed VI University Hospital and Al Farabi regional hospital in Oujda. We have emphasized the need for a reference center for overload diseases.

## INTRODUCTION

Gaucher disease is a lysosomal storage disease, the transmission is autosomal recessive.

It follows a mutation in the GBA gene coding for a lysosomal enzyme:  $\beta$ -glucocerebrosidase, which hydrolyzes glucosylcerebroside (glycosyl ceramide) into ceramide (cerebroside) and glucose.

There are three subtypes of the disease. The most common is type 1, known as the non-neuropathic form. Pancytopenia, hepatosplenomegaly and bone lesions occur as a result of glucocerebroside accumulation in the liver, lung, spleen and bone marrow in these patients.

The two other phenotypes include a severe neurological involvement in type 2 that affects infants and is deadly; in type 3 the neurological involvement is less severe and is also associated with features of types 1 [1].

### CASE 1

A 37-year-old male, was admitted to the internal medicine department of the regional hospital of Oujda for the management of anemic syndrome with splenomegaly. The clinical examination found mucocutaneous paleness with voluminous splenomegaly, the rest of the clinical examination is without particularity. Laboratory tests revealed bicytopenia with normochromic anemia (Hb at 6.3 g/dL) and thrombocytopenia at 140 G/L, leukocytes at 5 G/L. Liver and renal functions were normal, the serologies of hepatitis B, hepatitis C and HIV were negative. The abdominal ultrasound showed a huge splenomegaly, a normal-size liver without signs of portal hypertension. The myelogram showed a marrow, with cellular abundance a hyperplasia of the erythroblastic line with the presence of many Gaucher cells. X-ray of the skull, thoracolumbar spine, and pelvis were normal. The chest CT scan revealed the presence of alveolar syndrome with a small left pleural effusion.

The abdominal computed tomography showed significant heterogeneous splenomegaly with compression of adjacent organs and a small fluid effusion in the Douglas cul-de-sac.

Subsequently,  $\beta$ -glucocerebrosidase [7.5 U/L (reference range: 6.5 - 10.5 U/L)], Chitotriosidase [2980 nmol/h/mL (normal value <120 nmol/h/mL)] and ferritin [833  $\mu$ g/L (reference range: males 20-200  $\mu$ g/L)] measurement was done.

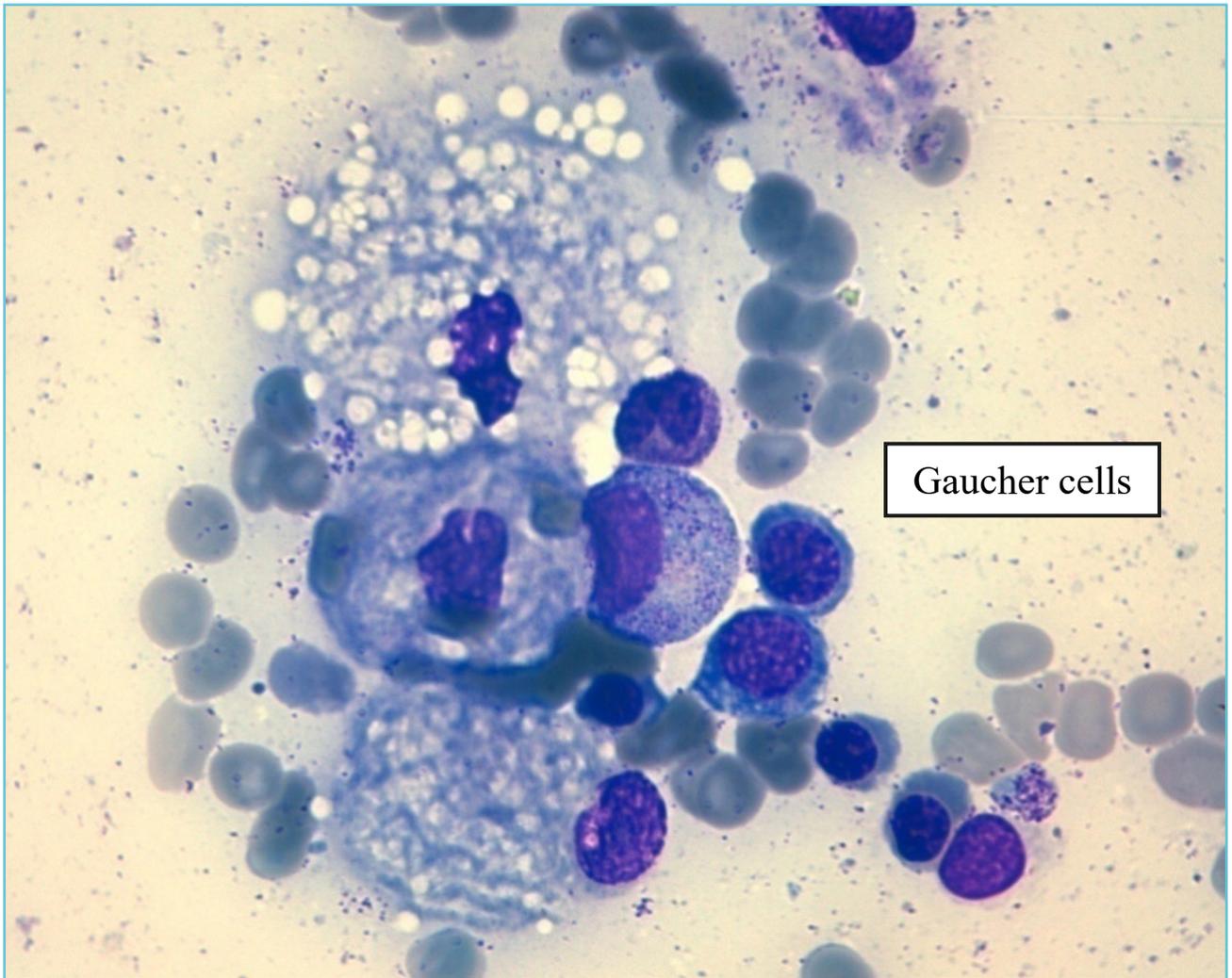
The diagnosis of Gaucher disease type 1 was based on clinico-biological data, the cytological aspect of the marrow and the results of the biomarkers, without enzymatic evidence. The patient was splenectomized; and the pathological examination of the spleen showed sub capsular splenic infarction with presence of Gaucher cells.

### CASE 2

A 26-year-old male, with no significant medical history was admitted to the Department of Internal Medicine of Mohammed VI University Hospital for the management of splenomegaly with anemic and haemorrhagic syndrome. The history of the disease dated back to 6 months before admission by the progressive appearance of anemic syndrome constituted by cutaneous pallor, asthenia and fatigability, associated with a hemorrhagic syndrome demarked by episodes of repeated epistaxis and diffuses bone pain. The symptomatology constituted hepatic, colic and left hypochondrial pain.

Clinical examination found a cutaneomucous pallor with splenomegaly exceeding the white line of the abdomen, without hepatomegaly or other associated signs. Blood test found a hemoglobin level at 12 g/dL (reticulocyte: 1.54%), platelet count at 40 G/L and leucocytes at 3940/  $\mu$ L. The serologies of hepatitis A, B and C and leishmaniasis were negative. The myelogram showed the presence of many Gaucher cells (Figure 1).

Figure 1 Gaucher cells on medullary smear



The histological aspect of the bone marrow biopsy (BMB) suggesting a storage disease (in particular Gaucher disease). Abdominal CT showed portal hypertension with huge heterogeneous splenomegaly including areas of necrosis. Standard radiography was normal and osteo-densitometry displayed osteopenia.

The result of the  $\beta$ -glucocerebrosidase assay was 6 U/L (reference range: 6.5 - 10.5 U/L). But given that the bone marrow aspiration showed typical cytological image of Gaucher disease; a new measurement was requested which showed a deficit at 1.6  $\mu$ kat/kg (reference range: 4.2 to 8.1  $\mu$ kat/kg). The levels of chitotriosidase, hexosami-

nadae and ferritin were 3380 nmol/h/mL, 1500 nmol/h/mL and 690  $\mu$ g/L, respectively. The diagnosis of Gaucher disease type 1 was retained. While waiting for enzymatic substitution treatment, the patient was treated by corticosteroids.

Beta-glucosidase activity was determined in the lymphocytes by the fluorimetric method using a synthetic substrate 4-methylumbelliferyl- $\beta$ -glucopyranoside. Taurocholic acid was used as an activator at pH 5.5. In parallel, the control activity of N-acetyl- $\beta$ -D-glucosaminidase was performed using the fluorogenic substrate 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucopyranoside to validate the quality of the specimen.

## DISCUSSION

The diagnosis of metabolic disorders could be difficult and time consuming. Pancytopenia, organomegaly (especially splenomegaly) and bone symptomatology are the most typical signs of type 1 Gaucher disease, the most common form [2]. Neurological signs are typical for types 2 and 3 of the disease [3].

For both patients, the diagnosis was made in adulthood, at an average age of 32 years. These data are consistent with previously published results [4]. However, Charrow and al [5] report, in their study of the international register containing the records of 1698 patients, an average age of diagnosis of 17.4 years.

It should be noted, however, that in this study, 50% of patients were diagnosed before the age of ten, because of severe symptoms, requiring special medical care and thus an earlier empiric diagnosis was possible [6].

When the clinical presentation is insufficient and in the absence of signs of orientation in the family, Gaucher disease remains a diagnostic challenge. It is often not included in the differential diagnosis of thrombocytopenia, and can present a challenge even to an experienced hematologist [7].

However, the presence of the most common initial symptoms, splenomegaly, cytopenia with cytological and biochemical exploration can lead to the diagnosis [8].

Demonstration of reduced enzymatic activity of  $\beta$ -glucocerebrosidase is required for definitive diagnosis of GD. However, our patients in whom enzymatic assays was carried out displayed subnormal values of the GBA activity.

By analyzing the clinical and biological contradiction, we emphasize the importance of the pre-analytical process, especially the one pertaining to the maximum sample transportation time of 48 hours.

We reiterate the importance of standardizing diagnostic methods and setting up specialized laboratories. Given the critical role of the preanalytical phase, screening tests using dried blood spots can be an optimal and alternative solution to the enzymatic activity assay on blood specimens.

Under-diagnosis of Gaucher disease in developing countries is explained among other factors by the lack of referral centers specialized in the diagnosis and management of this pathology.

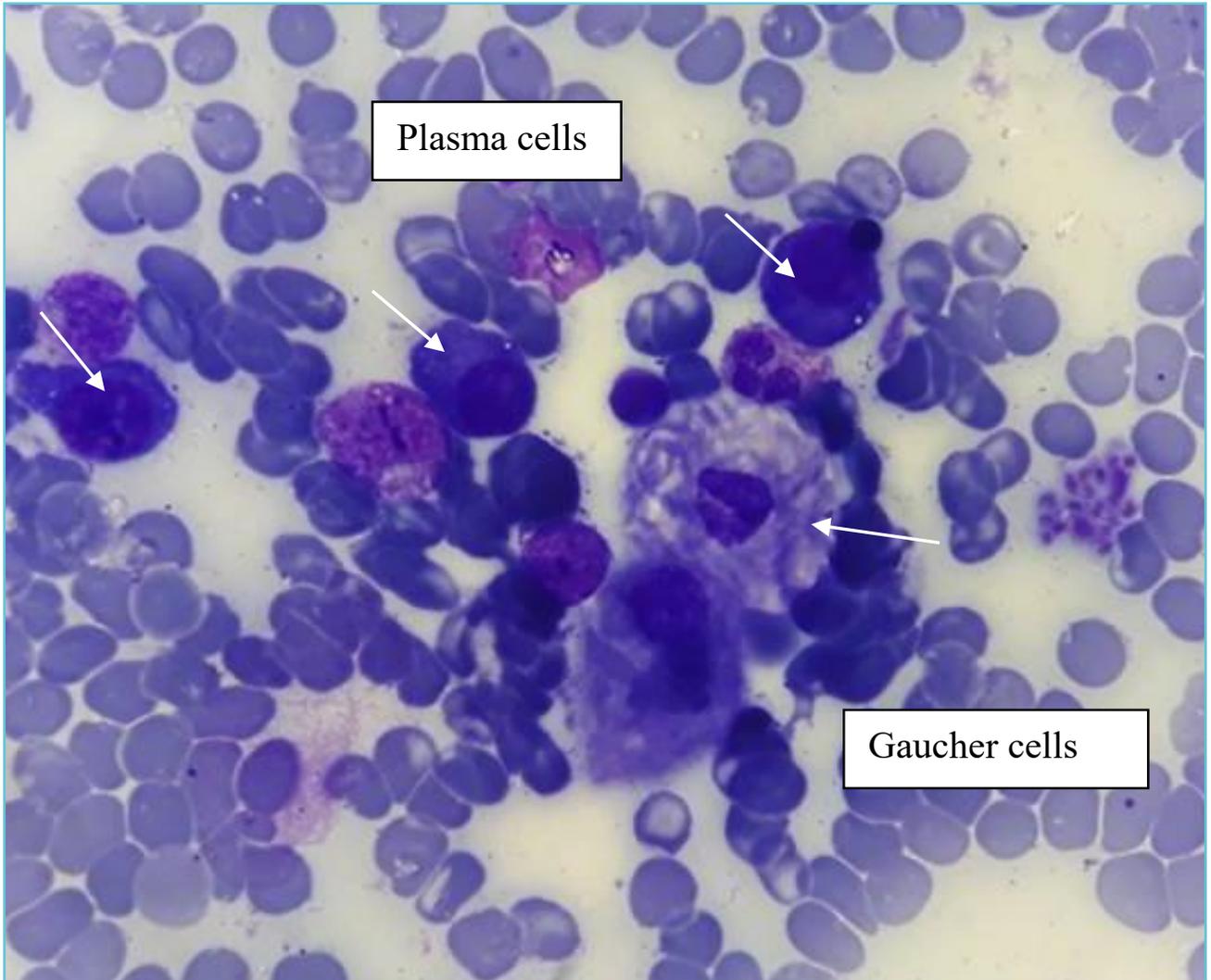
Apart from the pre-analytical and analytical problems, the case with normal  $\beta$ -glucocerebrosidase activity can be explained either by the mutation of its activator "Saposin C" (PSAP), or it is a heterozygosity resulting in a borderline enzyme result (knowing that heterozygotes have 15 to 20% of the normal enzymatic activity of  $\beta$ -Glucocerebrosidase) [9].

Chitotriosidase is a sensitive biological marker. This is a good diagnostic guide and is especially useful for monitoring the course of the disease [10]. It is also elevated during sarcoidosis, leishmaniasis and other storage diseases. Although, its rate is moderate compared to the values found in the GD [11]. The assay of chitotriosidase activity was performed in our two patients and in both cases showed high activity.

The evolution of Gaucher disease could be complicated by the appearance of haematological diseases (malignant or not) [6,11], or solid cancers [6]. We also identified a patient aged 59 years, diagnosed with type 1 Gaucher disease, who developed multiple myeloma with Ig G kappa monoclonality after 23 years, unfortunately due to lack of medical documents we did not report his case (Figure 2).

The therapeutic management has been revolutionized by enzyme replacement therapy. Because of its high cost, access to this therapy remains limited in developing countries. The clinical trials of gene therapy have given very promising prospects.

**Figure 2** Medullary smear showing Gaucher cells with plasma cells in a patient with Gaucher disease complicated by multiple myeloma



## CONCLUSION

Gaucher disease is not exceptional in our country. Type 1 is the most common. Given the frequency of consanguineous marriages, we insist on the importance of a regional registry and the need for the establishment of a reference center for Gaucher disease and metabolic diseases in general, to allow early diagnosis and adequate care.

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# A rare case of non-secretory multiple myeloma: a case report and literature review

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### **Key words:**

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## CASE REPORT

Non-secretory myeloma (NSM) is a rare form of myeloma. It is defined as monoclonal plasmocytic proliferation of the bone marrow with the same clinical and radiological manifestations of myeloma. However, plasma cells are unable to secrete immunoglobulin (serum and urinary electrophoresis are negative and free light chain measurement is unquantifiable).

This variant of multiple myeloma (MM) usually poses a diagnostic challenge to the biologist and clinician. We report a rare case of non-secretory myeloma in a 76-year-old patient who was diagnosed at the Mohammed V University Hospital Center in Oujda, Morocco.

## INTRODUCTION

Multiple myeloma is a hematological malignancy characterized by the presence of clonal plasma cells in the marrow that typically secrete an abnormal immunoglobulin causing a monoclonal gammopathy. This serum protein is often characterized by an intact immunoglobulin (heavy and light chain), or it may be characterized only by the light chain. In the urine, an intact immunoglobulin is also often present [1].

Myeloma is characterized by end-organ damage as manifested by hematologic, renal, or bone complications [2].

Myeloma may be preceded by a premalignant phase in which clonal plasma cells are present but there is no evidence of end-organ damage: this is known as “monoclonal gammopathy of unknown significance” or “smoldering myeloma” [3].

Non-secretory myeloma (NSM) is a rare clinical form of multiple myeloma with monoclonal plasmocytic proliferation of the bone marrow and the same clinical and radiological manifestations. However, in the case of non-secretory myeloma, plasma cells are unable to secrete immunoglobulin (serum and urinary electrophoresis are negative and free light chain measurement is unquantifiable) [1].

## CLINICAL-DIAGNOSTIC CASE

Mr. B.T., 76 years old, whose medical history includes:

- Chronic smoking for 25 years, weaned 35 years ago;
- Type 2 diabetes with oral antidiabetic drugs;
- Epilepsy treated with Phénobarbital, 0.75 mg/day.

The patient was admitted for mixed-type back pain, left intercostal neuralgia and left rib pain that was resistant to analgesics. Everything

evolves in a context of apyrexia and conservation of the general state.

The osteo-articular examination found pain in the palpation of the lower back spine. The rest of the clinical examination was without any particularities.

The patient has benefited from a biological assessment which did not indicate a biological inflammatory syndrome (normal erythrocyte sedimentation rate and CRP test) and the complete blood count with differential was without abnormalities. Serum protein electrophoresis showed hypogammaglobulinemia at 3.7 g/L and serum and urine immunofixations were negative with a normal Kappa/Lambda ratio. Renal and hepatic status was normal. (Table 1, Figure 1)

Magnetic resonance imaging (MRI) of the thoracic spine showed suspicious-looking D9 vertebral body compression with swollen pre-vertebral soft tissue swelling and posterior wall retraction, as well as a heterogeneous aspect of the cervical vertebrae.

The myelogram revealed 85% medullary plasmocytosis. (Figure 2)

Immunohistochemistry performed on osteomedullary biopsy showed medullary infiltration by myelomatous plasmocyte proliferation (CD138 positive) with a Kappa monotype.

Therapeutically, the patient was put on melphalan-prednisone-thalidomide (MPT)/Zometa protocol with a partial response (medullary plasmocytosis is of 18%).

## DISCUSSION

Multiple myeloma is a hematological malignancy characterized by monoclonal plasmocytic proliferation invading the hematopoietic bone marrow. Serum protein electrophoresis shows either the presence of a narrow peak migrating most often in the gamma globulin

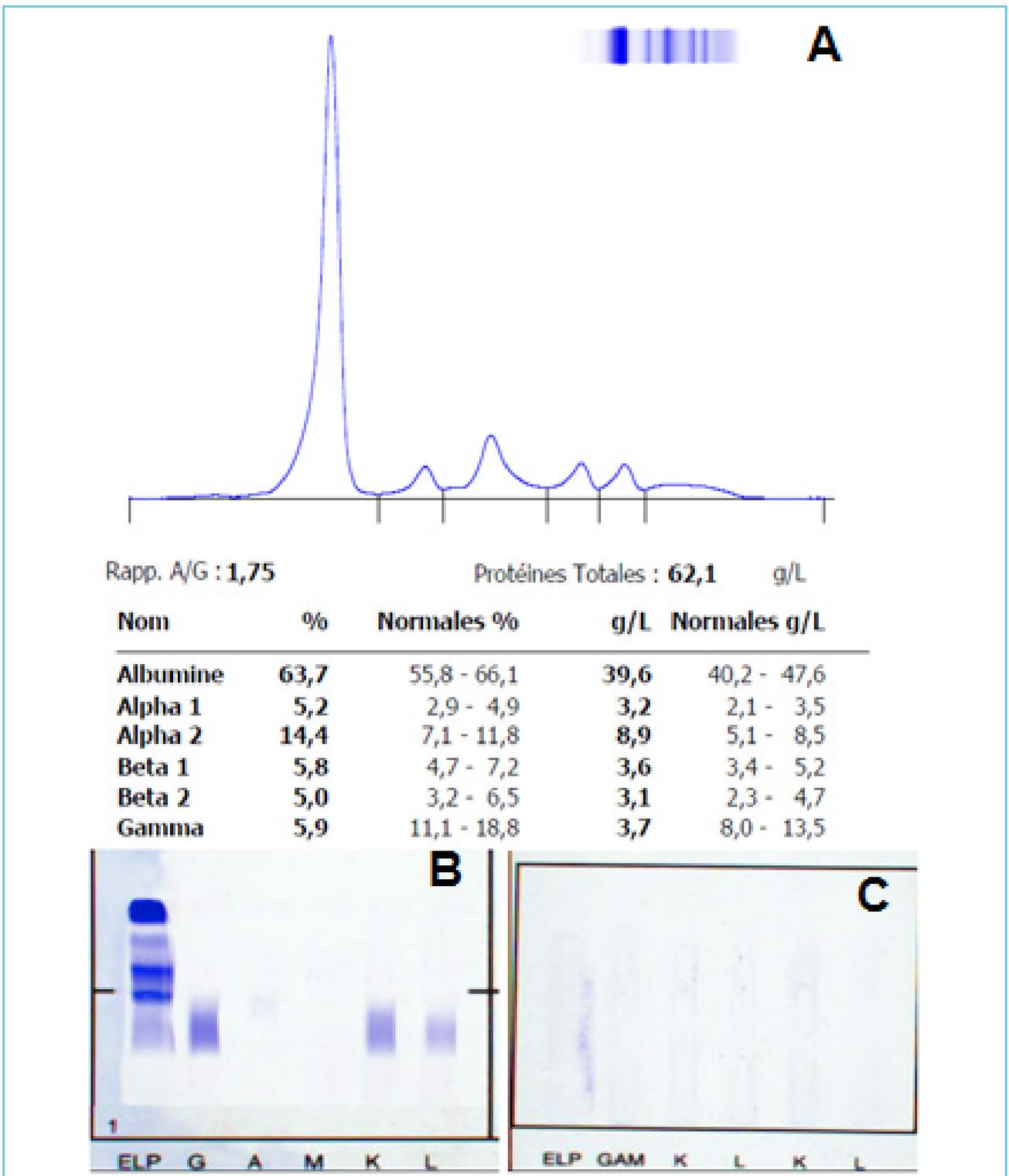
zone for secreting myelomas, or hypogammaglobulinemia associated with Bence-Jones proteinuria for light chain myelomas. The study of the myelogram shows a plasmocytosis greater than 10%. This plasmocytic proliferation is accompanied by hematological, bone and renal complications [4]. The contribution of Flow

Cytometry (CMF) in the initial evaluation is limited. However, it plays a more important role in the differential diagnosis of MM, where it can be a useful ancillary tool in identifying unusual morphologic variants of myeloma, cases of prominent reactive plasmacytosis, or B-cell non-Hodgkin lymphomas (NHLs) with extreme

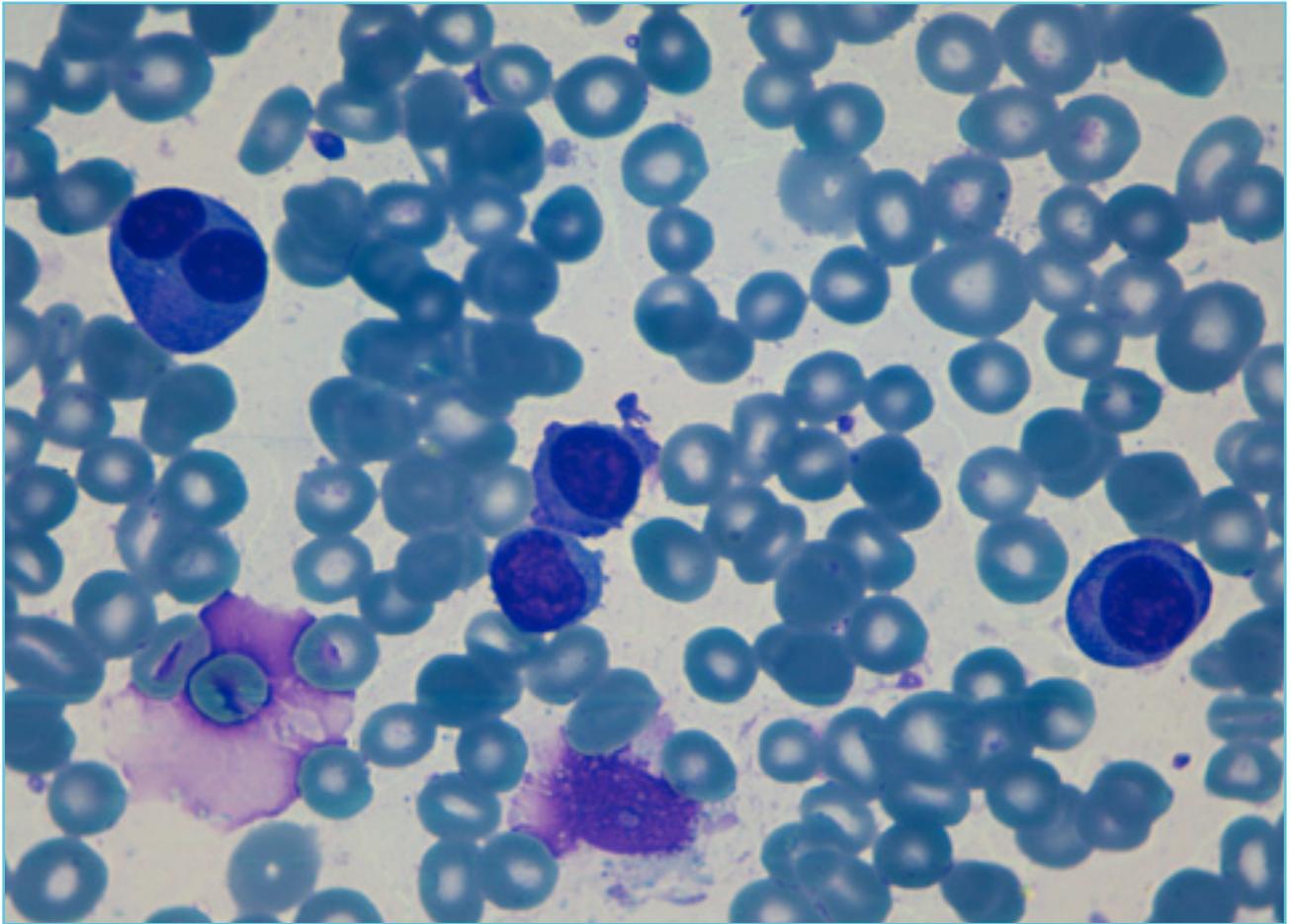
**Table 1** Laboratory results

Parameter	Case	Reference range
Hemoglobin	14,6 g/dL	13-18 g/dL
Erythrocyte sedimentation rate	24	-
CRP	8,82 mg/L	0-5 mg/L
ALT	22 UI/L	0-55 UI/L
AST	25 UI/L	5-34 UI/L
Gamma-GT	25 UI/L	12-64 UI/L
LDH	383 UI/L	125-243 UI/L
Creatinine	8,44 mg/L	7,2-12,5 mg/L
Ca <sup>++</sup>	93 mg/L	88-100 mg/L
24-h proteinuria	48,98 mg/24h	<500
Total protein	54 g/L	60-78 g/L
IgG	3,82 g/L	5,4-18,22 g/L
IgM	<0,20 g/L	0,22-2,40 g/L
IgD	<7 mg/L	7,7-132,10 mg/L
Free light chains Kappa-serum	6,57 mg/L	3,30-19,40 mg/L
Free light chains Lambda-serum	5,41 mg/L	5,71-26,30 mg/L
Kappa / Lambda Free light chains ratio	1,21	0,26-1,65

**Figure 1** A: Serum protein electrophoresis showing hypogammaglobulinemia  
 B: negative serum immunofixation  
 C: negative urine immunofixation



**Figure 2** Myelogram showing a medullary plasmocytosis



plasmacytic differentiation, among others [5]. CMF has also shown interest in accurately quantifying medullary plasmocyte infiltration and the proportion of pathological plasma cells relative to total medullary plasma cells (ratio) [6].

MM is almost always preceded by an asymptomatic premalignant stage termed monoclonal gammopathy of undetermined significance (MGUS) [7-9]. MGUS is defined as a serum monoclonal protein (non-IgM type) <30 g/L, Clonal bone marrow plasma cells <10%, and absence of end-organ damage such as hypercalcaemia, renal failure, anaemia, and bone lesions (CRAB) or amyloidosis that can be attributed to the plasma cell proliferative disorder [9]. About 80% of multiple myeloma originates from non-IgM immunoglobulin MGUS

(non-IgM MGUS), and 20% from light-chain immunoglobulin MGUS (LC-MGUS). In the event of progression, IgM immunoglobulin MGUS (IgM MGUS) usually evolves into Waldenstrom macroglobulinaemia, but in rare instances IgM MGUS can progress to multiple myeloma (IgM myeloma) [9-14].

Non-secretory myeloma is a rare clinical form of multiple myeloma (2% to 4% of all cases) [1] with monoclonal plasmocytic proliferation of the bone marrow and the same clinical and radiological manifestations. However, in the case of non-secretory myeloma, plasma cells are unable to secrete immunoglobulin (serum and urinary electrophoresis are negative and free light chain measurement is unquantifiable). (Table 2)

**Table 2** Criteria for Non-Secretory Myeloma [1]

Biopsy-proven clonal plasma cells
<0,5 mg/dl serum protein by serum protein electrophoresis
<200 mg/24hrs of light chain proteinuria by urine protein electrophoresis
Unquantifiable free light chain measurement
Evidence of CRAB criteria (hypercalcemia, renal dysfunction, anemia, bone disease) for symptomatic myeloma (which must be attributable to clonal plasma-cell infiltration)

This rare entity (NSM) must be distinguished from oligosecretory myeloma, in which proteins are produced but at very low levels that make reliable measurement more challenging. Oligosecretory multiple myeloma is often characterized by serum protein <10 mg/dL, urine protein <200 mg/24 hrs, and free light chain values <100 mg/L (or 10 mg/dL) [15].

Two distinct types of NSM have been described. The first group consists of patients who are “non-producers.” These are patients whose tumors may have defects in immunoglobulin synthesis. These tumors are not able to synthesize or secrete a protein even though they might have all the features of a plasma cell disorder [16]. In this category, we include the patients who have no measurable protein in the blood or urine, yet who still have a significant plasma cell burden in the marrow and evidence of end-organ damage. In addition, even the dosage of the free light chain will not reveal measurable disease as currently defined. The next category of non-secretory myeloma patients consists of those whose tumors produce a protein but have defects in secretion, possibly due to a mutation of the immunoglobulin gene thus explaining the absence of secretion in a patient with non-secretory myeloma.

According to the recommendations established by the International Myeloma Workshop[3], the

workup for all newly diagnosed myeloma patients includes: routine chemistries including LDH and beta-2-microglobulin, complete blood cell count with differential, serum protein electrophoresis with immunofixation, quantitative immunoglobulins (including IgD or IgE if suspected), 24-hr urine test with protein quantification and immunofixation, serum free light chain assay, skeletal survey and positron emission tomography scan[1].

Response assessment in myeloma is typically based on the absence of a detectable protein in the blood or urine and a normal free light ratio. Even with a response that meets these criteria, a more in-depth assessment using molecular techniques, such as multi-parameter flow cytometry (MPF) [1,17,18], is able to evaluate the minimal residual disease (MRD) that likely will contribute to relapse. Therefore, the IMWG included the evaluation of the residual disease of MM by MPF in the therapeutic response criteria. However, MPF alone is probably not sufficient to assess total body myeloma burden. For this reason, the pairing of imaging and more sensitive marrow assessment represents an optimal method by which to assess response to therapy and MRD, and will likely be applied to all myeloma patients, not just non-secretory patients in whom the inability to use SPEP or UPEP limits methods of response assessment [1].

## CONCLUSION

In conclusion, the absence of a monoclonal protein in serum and/or urine does not rule out the diagnosis of MM. Indeed, the diagnosis of non-secreting myeloma should be made in patients with clinico-biological and radiological characteristics of MM with a ratio of normal Kappa/Lambda free light chains. As a result, this report has now become a standard for the diagnosis of non-secreting myeloma.

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# Increased bleeding risk in a patient with oral anticoagulant therapy and concomitant herbal intake – a case report

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**Key words:**

hemoptysis, ginger, rivaroxaban, herbal intake

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## CASE REPORT

We report the case of a 36-year old male, under stable rivaroxaban therapy for 18 months, who was admitted to our emergency room with sudden onset of hemoptysis.

Anticoagulant therapy was given after recurrent spontaneous deep vein thrombosis (DVT) and a heterozygous Factor-V-Leiden mutation was present. There was no co-medication reported, however, the patient reported a constant intake of three liters of home-brewn ginger tea per day in the last month. The patient was hospitalized to further investigate the reason of hemoptysis.

## **INTRODUCTION**

The use of Novel oral anticoagulants (NOACs) for the treatment of patients with venous thrombotic events (VTE) is now standard of care and demonstrated clinical efficacy and safety in numerous clinical studies (1,2).

Usually, these substances have a low risk for interaction with other medications compared to vitamin K antagonists (VKA), the standard medication given for the treatment of VTE events in the decades before. However, little is known concerning interaction of NOACs with herbal medicinal products (HMP).

The use of HMP is frequent among the population (3). Ginger (*Zingiber officinalis*) is a very common spice used worldwide because of its aromatic taste (4). Zingerone (ZGR), a phenolic alkanone found in *zingiber officinalis*, has been reported to have various pharmacological activities so that many people even use it as a medicine (4,5).

However, little is known about concomitant use regarding potential interactions associated with new oral anticoagulants like rivaroxaban or other factor Xa inhibitors (4,5,6). This may become relevant since the prescription of NOACs significantly increased in the last years (6,7,8).

We report a case of a 36-year old male under rivaroxaban therapy who developed hemoptysis after drinking about 2 to 3 liters of ginger tea per day over a period of one month.

## **CLINICAL - DIAGNOSTIC CASE**

A 36-year old male under continuous rivaroxaban therapy for 18 months was admitted to our emergency room with a sudden onset of hemoptysis appearing for the first time. The patient reported that two hours before he produced about 20 mL of sputum containing significant blood spots. He was free from respiratory tract infections the last month and did not

report any personal or family history consistent with a bleeding diathesis. No other anticoagulant drugs, platelet aggregation inhibitors including NSAIDs, or any other pharmaceutical drugs were taken.

After reviewing a long and detailed medical history, it has been ascertained that the patient has been consuming about 2 to 3 liters of home-brewn ginger tea on a daily basis for over a period of one month.

Anticoagulant therapy was prescribed to prevent recurrence of a VTE event, after he had two spontaneous DVT events in the lower extremities, together with a heterozygous factor V Leiden mutation status. The patient has been under stable NOAC treatment for 18 months without signs of bleeding. The patient was hospitalized to further investigate a reason for hemoptysis.

Laboratory testing showed no abnormalities—renal function was within reference range. The HAS-BLED Score was 0 points, Chest - X-ray was normal, a CT scan of the thorax was also unsuspecting, and ECG was found normal. The Ear Nose Throat (ENT)-specialist excluded a possible bleeding source in the Naso-oro-pharyngeal region.

Since increased consumption of ginger may also affect platelet function, platelet function testing (PFT) was performed 3 days after hospitalization. A PFT is mainly used to detect hereditary platelet function disorders, also to test for efficacy of antiplatelet therapy such as acetylsalicylic acid (ASA) or clopidogrel.

For testing platelet function, the Multiple-Electrode-Analyzer (MEA) using a multiplate system was used. The reference ranges supporting a sufficient antiplatelet effect of ASA are defined between 10 - 50 units using the ASPI test, and 19 - 46 units for clopidogrel in the ADP test, respectively.

In our patient, values in the ASPI test were found to be 69 units and in the ADP test 47 units. The value for the ADP test can be interpreted as a relevant antiplatelet effect caused by ginger, which is almost comparable to the effect of clopidogrel on the platelet function.

Because of the 2-fold thrombotic event and the heterozygous Factor-V-Leiden mutation, the patient was advised to continue rivaroxaban therapy as a long term medication. However, due to the initial hemoptysis, the NOAC treatment of rivaroxaban 20 mg was paused for 48h.

Furthermore, the patient was advised to significantly reduce ginger consumption because of its obvious additional antiplatelet effect when used in such high concentrations. The patient was discharged after 4 days in a good general condition, rivaroxaban was re-started, however in a reduced maintenance dose.

## **DISCUSSION**

The use of NOACs for several indications significantly increased in the last years. Compared to VKA, NOACs have some beneficial effects. In various clinical trials lower bleeding risk and less hospitalization were reported. Furthermore no routine laboratory monitoring is necessary (6). However, little is known about concomitant use of herbal medication and potential interactions with NOAC medication (4,5,6).

Our patient was admitted to our emergency room with hemoptysis appearing for the first time under stable rivaroxaban therapy. The patient has been on rivaroxaban treatment 20 mg for 18 months because of recurrent VTE events. Bleeding complications are possible side effects of anticoagulant treatment.

In the recent medical history no plausible causative reason for the hemoptysis was found. The patient had no personal or familial history consistent with a bleeding diathesis. Neither had

he exposure to anticoagulant drugs other than rivaroxaban, nor to platelet aggregation inhibitors including NSAIDs. The HAS-BLED score, reflecting the bleeding risk of the patient (9), was 0 points under stable anticoagulation therapy for 18 months - it seems unlikely that the anticoagulant treatment alone could have caused the hemoptysis.

After obtaining an extensive medical history, it has been ascertained that the patient increased his intake of homebrew ginger tea up to 2-3 liters daily in the last month.

There are various effects of ginger reported on the human organism, also affecting the coagulation system towards bleeding; an increased use of ginger in combination with the NOAC treatment could therefore have perpetuated hemoptysis in our patient (3,4,5,6). A platelet function testing was performed on the third day after cessation of ginger intake. The result of the PFT showed a markedly decreased platelet aggregation pattern after ADP stimulation, revealing a possible effect of ginger on platelet function which is almost comparable to the effect of treatment with clopidogrel.

Since recurrent spontaneous VTE events demonstrate an indication for long-term anticoagulation. Therefore, our patient received a subsequent dose reduction of Rivaroxaban. This was considered to avoid further bleeding events as it was recently reported to be meaningful in the so called EINSTEIN-CHOICE Study (10).

Furthermore, the patient was advised to lower his ginger consumption.

To summarize, despite the delayed testing of platelet function after three days of withdrawal of ginger intake, we conclude that extensive ginger intake together with rivaroxaban therapy enhanced bleeding risk in our patient, this should be considered in obtaining medical history in patients with unclear bleeding events under DAOK therapy.

## **TAKE HOME MESSAGES/ LEARNING POINTS**

- The use of herbal medicinal products is common among the population. However, little is known about its concomitant use and the potential interactions associated with direct oral anticoagulants.
- Platelet function testing in such situation seems meaningful and may help to support the hypothesis drawn from medical history in patients with unclear bleeding events.

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# Unusually low serum alkaline phosphatase activity in a patient with acute on chronic liver failure and hemolysis

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### **Key words:**

ACLF, autoimmune hepatitis,  
hemolytic anemia, low ALP,  
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## CASE REPORT

A 28-year-old male with acute on chronic liver failure (ACLF) and hepatic encephalopathy had deranged liver function with curiously low level (0-15 IU/L) of serum alkaline phosphatase (ALP). Peripheral smear examination suggested hemolytic anemia. The finding of persistent low ALP, after ruling out pre-analytical causes, in ACLF has been reported in Wilson's disease (WD) with/ without autoimmune hemolytic anemia (AIHA). Definitive evidences of WD were not seen in our case. Positive DCT and histological features suggest a diagnosis of autoimmune hepatitis with secondary hemochromatosis and cholangitis. Low ALP might not always be a determinant of bile duct pathology in patients of ACLF with AIHA.

## INTRODUCTION

Acute on chronic liver failure (ACLF) is acute deterioration of liver function in patients with pre-existing liver disease. The American Association for the Study of Liver Diseases defined ACLF as: “acute deterioration of pre-existing chronic liver disease usually related to a precipitating event and associated with increased mortality at 3 months due to multi-system organ failure” (1). Common causes of ACLF include active alcohol consumption, reactivation of hepatitis B virus infection, superinfection with hepatitis E virus, autoimmune hepatitis flare, sepsis and superimposed drug or toxic injury.

In our case the patient presented with acute liver failure and hemolytic anemia. However, he was detected with chronic liver disease (CLD) two years back. Most causes of ACLF were ruled out. However, an unusual finding of low serum alkaline phosphatase (ALP) values (<5-15 IU/L) was observed. Possible causes for the same in the setting of ACLF were looked for.

## CLINICAL – DIAGNOSTIC CASE

A 28-year-old man presented to the emergency with jaundice for two weeks and progressively increasing abdominal distension. Two days prior to admission he developed malena, decreased urine output and drowsiness. His past records revealed that he was diagnosed with CLD, however the etiology was unknown. He recovered spontaneously during that episode and was asymptomatic till the present admission. His relatives denied any history of blood transfusion, intravenous drug abuse, tattooing, promiscuity, consumption of alcohol or smoking.

On assessment in casualty, the patient was found to be icteric and hypotensive. His abdomen was distended; without any guarding or rigidity. Neurological examination suggested the presence of grade III hepatic encephalopathy. Kayser–Fleischer ring was not visualized on slit

lamp microscopy. Chest and cardiovascular examination was within normal limits.

On admission he was suspected to be a case of ACLF, which was confirmed in the subsequent two days. His blood investigations revealed: blood urea nitrogen (BUN): 59 mg/dL, serum creatinine: 3 mg/dL, serum total bilirubin: 24.1 mg/dL, direct bilirubin: 17.8 mg/dL, aspartate aminotransferase (AST) levels: 205 IU/L, alanine aminotransferase (ALT): 23 IU/L and ALP: 43 IU/L. Blood ammonia level was elevated to 135  $\mu$ mol/L. Details of the serial liver function are shown in Table 1. It was observed that serum ALP values decreased to very low (5-15 IU/L) to even undetectable levels over the next few days.

Prothrombin time, INR and activated partial thromboplastin time were prolonged (Table 1). Hemoglobin and platelet counts were low- 6.4 g/dL and 50,000/mm<sup>3</sup> respectively. Leucocytes were normal in count and morphology. Peripheral smear examination was suggestive of hemolytic anemia. Direct Coomb’s test (DCT) was positive and indirect (ICT) was negative.

Serum markers for viral hepatitis (HBsAg, Anti HCV, Total anti-HBcAb, IgM HAV, IgM HEV and HEV RNA) were negative. Autoimmune markers including anti-nuclear antibody, anti-smooth muscle antibody, antibody for liver-kidney microsomal type-1 were negative. Total IgG level was 1590 mg/dL (RI: 840 -1700 mg/dL). Serum copper was 53.5  $\mu$ g/dL (RI: 70-140  $\mu$ g/dL) and serum ceruloplasmin 17 mg/dL (RI: 20-60 mg/dL), both marginally on the lower side. 24 hours urine copper estimation could not be done. Urine routine microscopy showed the presence of 12-15 RBC/HPF and 6-8 WBC/HPF. Bile pigments were present in urine. However, urine culture was sterile. His esophagogastrosocopy showed grade-2 esophageal varices.

The patient was managed with broad-spectrum antibiotics and other supportive measures.

**Table 1** Laboratory parameters at admission and follow-up

Lab parameter	Reference interval	2 months prior to admission	On admission	Day 1	Day 3	Day 4	Day 6	Day 7
TLC (x10 <sup>3</sup> /μL)	4.0-10.0	7.1	-	11	4.9	4.5	6.3	8.1
N-Neutrophils (%)	-	65	-	82	69	75	84	86
L-Lymphocytes (%)	-	30	-	08	21	10	04	09
M-Monocytes (%)	-	01	-	08	06	11	07	04
E-Eosinophils (%)	-	03	-	01	03	03	04	01
B-Basophils (%)	-	01	-	01	01	01	01	00
RBC (x10 <sup>6</sup> /μL)	4.5-5.5	-	-	1.5	1.62	2	1.9	1.13
Hemoglobin (g/dL)	13-17	7.8	-	6	6.4	7.5	7.7	6.6
HCT (%)	40-50	22.4	-	17	17.9	21.2	22.5	15
RDW (%)	11.6-14.0	19.8	-	18.5	21.1	25.3	29.6	31.1
Platelet (x10 <sup>3</sup> /μL)	150-410	118	-	60	50	80	40	20
BUN (mg/dL)	7-20	20	50	59	38	26	29	54
Creatinine (mg/dL)	0.6-1.2	1.1	2.4	3	1.5	0.8	1	2.9
Calcium (mg/dL)	9.2-11.0	7.9	-	8.5	9	8.3	8.7	9.4
Phosphate (mg/dL)	2.3-4.7	3.7	-	7.1	3.4	2.5	2.8	7.3
Uric acid (mg/dL)	4-8.5	3.3	-	4.5	1.9	1.3	1.5	4.1
Sodium (mEq/L)	136-142	120	126	-	132	137	149	-
Potassium (mEq/L)	3.8-5	5	7.6	-	4.3	4	3.5	-
Total Bilirubin (mg/dL)	0.1-1.2	4.8	24.35	24.1	26.6	29.5	37.7	38.6
Bilirubin conjugated (mg/dL)	<0.3	3.6	17.8	19	21.7	25.7	24.6	-

Total Protein (g/dL)	6-7.8	7	-	5.4	5.3	5.2	5.3	4.8
Albumin (g/dL)	3.2-4.5	2.1	-	2.1	2	2.5	2.7	2.5
Globulin (g/dL)	2.3-3.5	4.9	-	3.3	3.3	2.7	2.6	2.3
AST (IU/L)	8-40	87	-	68	205	174	88	291
ALT (IU/L)	4-45	12	-	17	23	17	10	79
ALP (IU/L)	80-270	179	-	51	43	Low	Low	Low
Arterial blood Ammonia (µmol/L)	10-35	-	-	-	121	135	-	128
Total IgG (mg/dL)	840 -1700	-	-	-	-	-	-	1590

However, his general condition worsened with progressive jaundice and grade-IV encephalopathy. He later developed shock and died 7 days after admission.

His postmortem liver biopsy showed features of cirrhosis with marked activity [Figure 1A, B, F, & G]. Features of lympho-plasmacytic bile duct injury [Figure 1C & E], interphase hepatitis, features of ascending cholangitis, ballooning of hepatocytes, significant canalicular and intra-cytoplasmic cholestasis [Figure 1D] were present. Significant steatosis or deposition of copper associated protein was not noted with orcein stain. Perl's prussian blue stain showed features of hemochromatosis, with grade-3 iron deposition in the hepatocyte cytoplasm, Kupffer cells and bile duct epithelial cells [Figure 1H]. Based on the overall features, histological possibility of chronic cryptogenic hepatitis with cholangitis and secondary hemochromatosis were suggested.

## DISCUSSION

The index case had three characteristic features: first, hemolytic anemia on peripheral smear with positive DCT; second, hemochromatosis in

histopathology; and third, very low to undetectable levels of serum ALP.

Hemolysis in the setting of liver failure can be immune mediated or non-immune mediated. Immune mediated hemolysis is seen in fulminant viral hepatitis, septicemia and autoimmune hemolytic anemia (AIHA). Non-immune mediated mechanisms are implicated in microangiopathic hemolysis - disseminated intravascular coagulation in sepsis, disseminated malignancy, fulminant Wilson's Disease (WD), viral hepatitis, etc.

In the index case, although serum IgG levels were normal and autoimmune profile was negative; clinical findings, positive DCT and histological features drive towards a possibility of an autoimmune hepatitis with secondary hemochromatosis. Features of ascending cholangitis and marked cholestasis in liver biopsy suggest acute hepatic insult. The gradual rise of AST was not accompanied with a parallel rise in ALT, which points towards a non-hepatic pathology. Due to the presence of hemolytic anemia, the patient had grade-3 iron deposition in the hepatic parenchyma, including the bile duct epithelial cells. AIHA is generally a chronic disorder

which may progress to liver failure. Extrahepatic features of autoimmunity and seroimmunologic changes may be however absent in most cases.

The third interesting finding was a persistently low serum ALP value despite presence of lymphocytic cholangiopathy, ascending cholangitis as well as iron deposition in bile duct epithelial cells. Methodological interferences in ALP assay due to anticoagulant contaminations, commonly with EDTA, were excluded on the basis of other biochemical parameters like calcium and potassium (2). A few cases have been reported in association with WD where serum ALP was undetectable (3,5). A recent report has identified two novel mutations in *ATP7B* gene which encodes for a membrane-bound copper transporting ATPase in a 42 year woman with WD and low ALP levels (4).

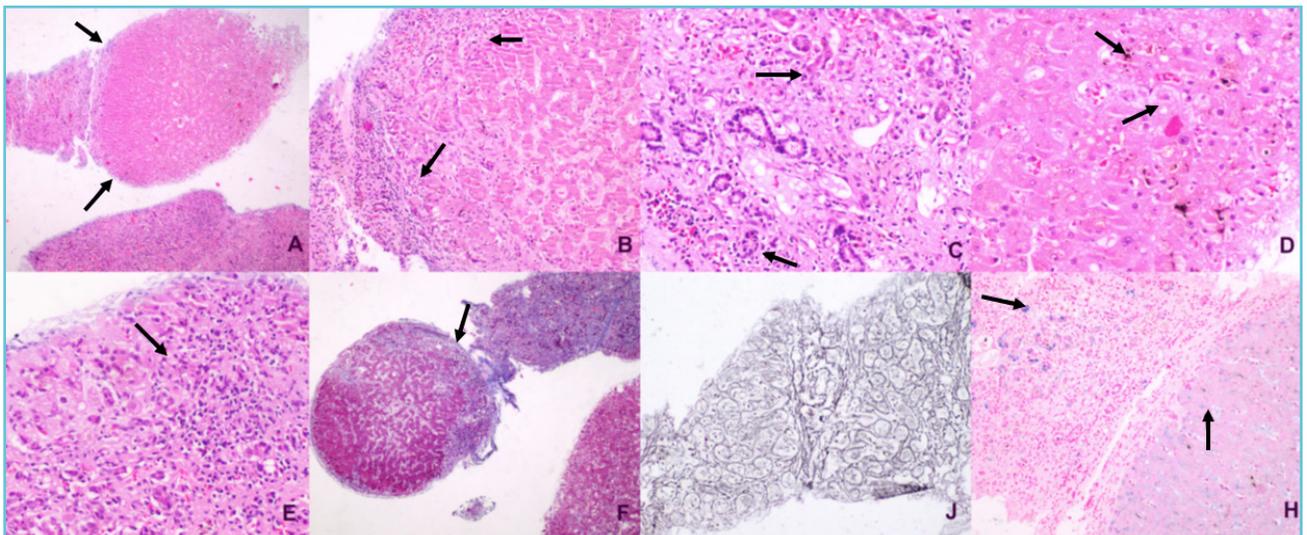
WD is manifested with impaired biliary copper excretion resulting in positive copper balance in

liver. Defective copper incorporation in apoceruloplasmin leads to low blood ceruloplasmin levels which in this case was marginally low. Serum copper was also observed to be marginally low. Hepatic decompensation frequently occurs in these patients.

Moreover, in severe liver failure, hemolytic anemia may develop as large amount of copper is released into the circulation due to hepatocellular necrosis. However, KF ring, a pathognomonic feature of WD was not visualized. In our index case, the diagnosis of WD was not considered on histological examination, as there was no macrovesicular steatosis, nuclear glycogenization or evidence of hepatic significant deposition of copper associated protein.

Besides, hypophosphatasia, a rare, genetic disease, characterized by mutations in the tissue non-specific alkaline phosphatase (TNSALP) gene is reported to lead to diminished activity

**Figure 1** Post-mortem liver biopsy photomicrograph



Photomicrograph shows distorted lobular architecture with formation of complete nodules (arrows) (A x 40).

Interphase hepatitis (arrows) and lymphocytic bile duct injury are noted (arrows) (B x 100; C x 200).

Ballooned hepatocytes and canalicular cholestasis are noted (D x 200).

At places dense septal lympho-plasmacytic cell infiltrate are seen to cross the para-septal limiting plate and destroying the hepatocytes (arrow) (E x 100).

Masson's trichrome stain highlights the collagen band (arrows) (F x 40).

Reticulin stain also highlighting the septal fibrosis (J x 100).

Perl's Prussian blue stain shows grade three iron deposit in the hepatocytes, focally in Kupffer cells and in the bile duct lining cells (arrows) (H x100).

of the TNSALP enzyme in target tissues. Clinical features include low levels of ALP in serum and bone, osteomalacia and periodontal disease.

Although, this is commonly described in children, rarely it has also been described in adulthood, when it is most commonly characterized by poor healing, recurrent metatarsal stress fractures, and bone pain. Our index case did not have any of these manifestations and was safely ruled out for the diagnosis of hypophosphatasia.

Potential interferences from any drugs in the assay of ALP were also ruled out. Drugs that are reported to interfere physiologically or analytically in ALP measurement include ibuprofen, theophylline, cefoxitin, doxycycline, amphotericin B, tetracycline, antiepileptics, anticoagulants, lipid-lowering drugs, and inhibitors of bone matrix formation and resorption. Our index patient was managed with broad spectrum antibiotics- Piperacillin with Tazobactam. In addition, he also received Rifaximin, Lactulose and other supportive measures. Piperacillin with Tazobactam is not known to cause lowering of ALP levels, rather may sometimes cause transient increase in its levels.

In summary, peripheral blood and positive DCT of the patient indicate a possible presence of AIHA. On histopathology, autoimmune hepatitis with secondary hemochromatosis was suggested. However, ALP is usually raised in cases of hemochromatosis. Hence, in the index case, whether a persistent low ALP level in the setting of hemochromatosis is caused by a sub-clinical WD or AIHA remains debated.

Two cases with WD with superimposed autoimmune features have also been reported, however definitive evidences of WD and AIH were observed in those patients (6).

Therefore, it is important to highlight that, in presence of the above two conditions; the serum alkaline phosphatase may not be a true determinant of any possible bile duct pathology.

## LEARNING POINTS

1. Autoimmune hepatitis is an important cause for ACLF and may present without the serological markers for the same.
2. Patients with AIH and WD have been reported and may present with overlapping features of both the diseases.
3. Low ALP, although reported with WD may not always be a pointer towards WD; especially in patients with overlapping features.
4. ALP might not always be a true determinant of bile duct pathology.



### Abbreviations:

**ACLF:** Acute on Chronic Liver failure

**CLD:** Chronic Liver Disease

**ALP:** Alkaline phosphatase

**BUN:** Blood urea nitrogen

**AST:** Aspartate aminotransferase

**ALT:** Alanine aminotransferase

**INR:** International normalized ratio

**DCT:** Direct Coomb's test

**ICT:** Indirect Coomb's test

**RI:** Reference Interval

**AIHA:** Autoimmune hemolytic anemia

**WD:** Wilson's Disease



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