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Measurement of anti-Mullerian hormone: preliminary evaluation of an ABEI-based fully automated immunoassay

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Key words:
fertility, infertility, biomarker, AMH, immunoassay

ABSTRACT

Background

The added value of Anti-Müllerian hormone (AMH) measurement is recognized for several clinical applications such as assessment of the ovarian reserve, monitoring of *in vitro* fertilization protocol or in the field of oncofertility. Our study objective was to determine the performances of a novel fully automated chemiluminescent assay for AMH testing.

Methods

We evaluated the performances of the Maglumi® 800 AMH chemiluminescent immunoassay that applies N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) labels. Assay imprecision was assessed with two levels of control materials. Method comparison was performed with an ultrasensitive AMH ELISA assay (Ansh Laboratories, Inc, Webster, TX, USA) with 88 patients' samples.

Results

The within-run and between-run coefficients of variation (CVs) were below 3% for both low and high internal quality controls. The automated and ELISA methods were significantly correlated. Bland-Altman plot evidenced a bias between the methods with a mean bias of 0.6 ng/mL.

Conclusions

Our preliminary evaluation showed overall good analytical performances for the Maglumi® AMH fully automated immunoassay and good concordance with a routinely used assay.

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INTRODUCTION

The Anti-Mullerian hormone (AMH) is a dimeric glycoprotein that belongs to the transforming growth factor-β (TGF-β) family and a key regulator of sexual differentiation and folliculogenesis (1,2). Measurement of AMH is helpful in several clinical situations (1–3). AMH is a widely used marker of functional ovarian reserve in the assessment and treatment of infertility (1,2). AMH testing also offers the advantage to detect ovarian reserve of those follicles that are not visible by ultrasound like small pre-antral follicles. Circulating levels of AMH help to establish patient profiles and predict ovarian response to stimulation in assisted reproduction techniques (1,2). AMH has recently been identified as an early predictor of ovarian follicle loss and menopause onset (1,2). AMH is also emerging in the field of oncofertility to understand the effects of different cytotoxic agents on ovarian function (4). Finally, AMH participates in the diagnosis of certain diseases such as granulosa cell tumors or Polycystic Ovary Syndrome (PCOS) (1–3,5).

Several AMH immunoassay methods are now commercially available ranging from manual Enzyme Linked Immunosorbent Assay (ELISA) methods to fully automated assays (2,6). According to the recognized clinical value of AMH testing, it is important to determine the performances of novel assays before their use in clinical practices.

The objective of our preliminary evaluation was to evaluate the performances of a novel chemiluminescent ABEI-based AMH automated immunoassay.

METHODS AND MATERIALS

We assessed the performances of the Maglumi® 800 (Snibe diagnostics, Shenzhen, China) AMH chemiluminescent immunoassay that applies ABEI labels. ABEI, *N*-(4-Aminobutyl)-*N*-ethylisoluminol, is a non-enzyme small molecule with a special molecular formula that enhances stability in acid and alkaline solutions. The chemical reaction process of ABEI using sodium hydroxide (NaOH) and hyperoxide (H_2O_2) finishes in three seconds (6). The lowest detection limit for this assay is 0.1 ng/mL.

The imprecision of the Maglumi® AMH was assessed by repeatedly measuring two different levels of Internal Quality Control (IQC). IQC at low concentration and IQC at high concentration were tested 3 times a day for 5 consecutive days according to the EP 15-A3:2014 protocol from CLSI guidelines. A method comparison was performed with ultrasensitive AMH quantitative three step ELISA method (Ansh Laboratories, Inc, Webster, TX, USA) by measuring 88 patients' serum samples (6). The limit of quantification for this assay is 0.06 ng/mL. Blood was taken by venipuncture from the antecubital vein and collected into dry serum tubes (S Monovette® 7.0 mL tubes, Sarstedt, Nümbrecht, Germany) and both methods were performed according to the manufacturer's specifications.

Data were analyzed with the Medcalc 7.2.1.0 package (Medcalc Software, Belgium). Passing and Bablock regression analysis was performed

for method comparison and Pearson's coefficient of correlation were calculated. Bland Altman plots were used to calculate the mean bias between methods.

RESULTS

Imprecision and accuracy

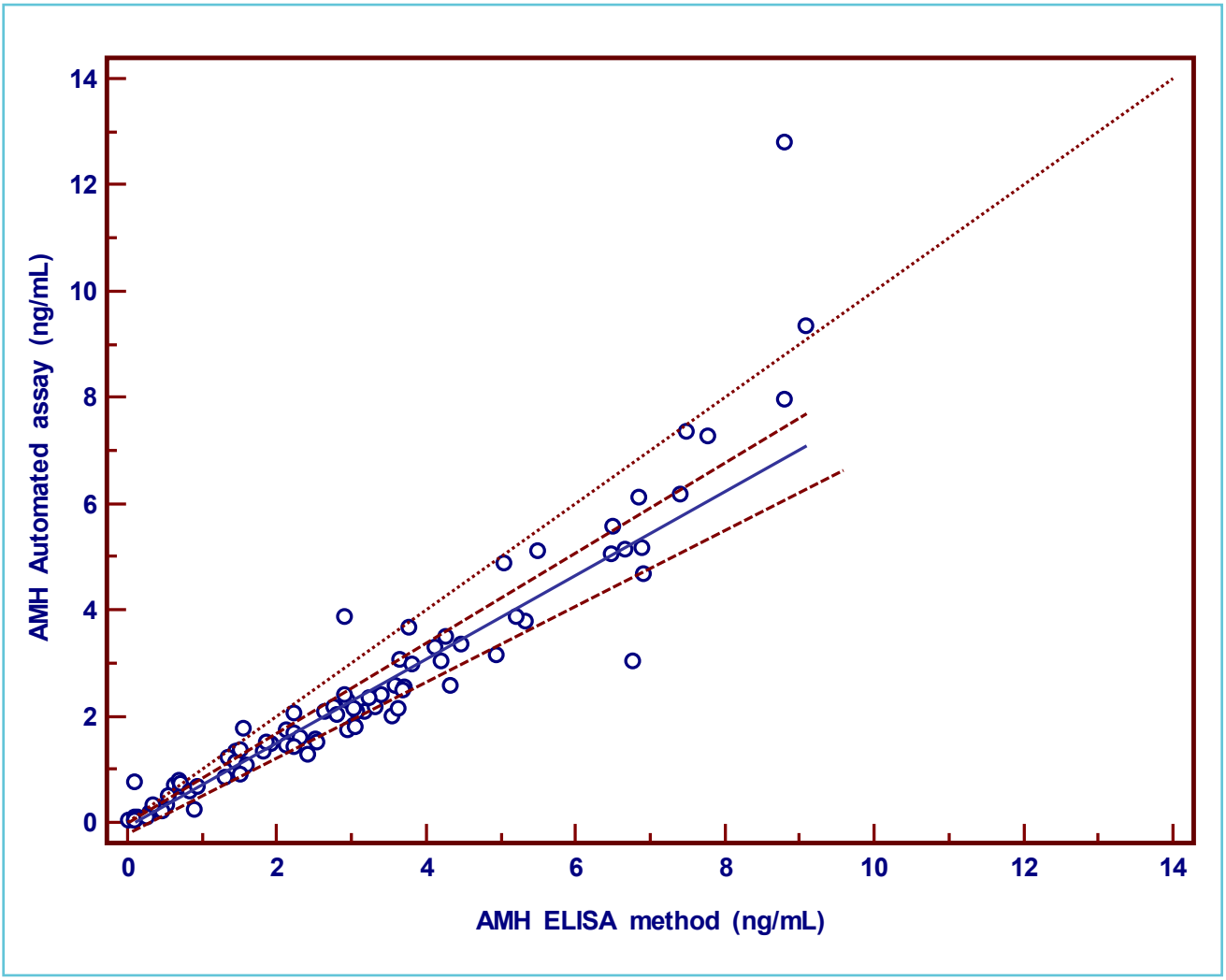
The within-run coefficients of variation (CVs) of the Maglumi® AMH assay were 2.2% and 1.4% for concentrations of 3.9 ng/mL and 15.9 ng/mL, respectively. For the same concentrations, the between-run CVs were 2.5% and 2.4%, respectively. Accuracy was also determined based on the targets IQC concentrations, and bias were 2.99% for the low IQC and 0.29% for the high IQC.

Comparison with the ELISA assay

The median AMH levels were 2.0 ng/mL (range: 0.1 – 12.8 ng/mL) with the Maglumi® assay and 2.9 ng/mL with the ELISA method (range: 0.1 – 9.1 ng/mL).

The correlation between both methods was good (r = 0.95, p<0.001). Passing-Bablok regression analysis showed a slope of 0.79 (95%

Figure 1 Passing and Bablok regression analysis between the automated and the ELISA AMH immunoassays



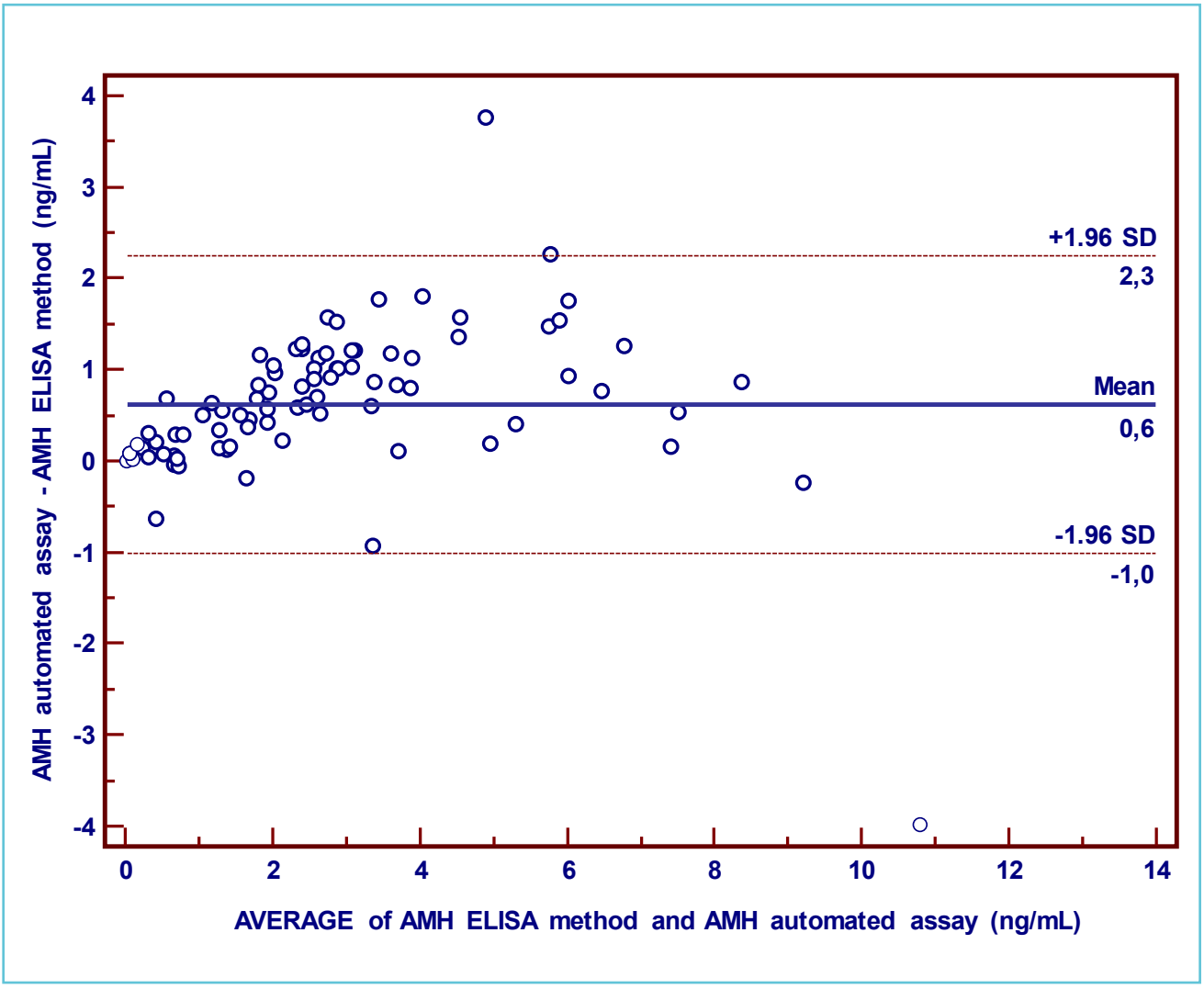
confidence interval (CI): 0.74 to 0.85) and an intercept of -0.07 (95% CI: -0.17 to -0.02) and no significant deviation from linearity (Figure 1).

The Bland Altman Plots revealed a mean difference of 0.6 ng/mL (95% CI: -1.0 to 2.3) between the two AMH immunoassays and a bias proportional to the concentration (figure 2). Looking at the lower AMH concentrations, 32 samples had concentrations below 2 ng/mL with the ELISA method. Eleven of these samples had AMH concentrations between 1 and 2 ng /mL with the ELISA. Two of these 11 had concentrations below 1 ng/mL (0.82 ng/mL and 0.88 ng/mL). Of the remaining 21 samples, 7 were with AMH concentrations below 0.1 ng/mL. Six of these samples were also below 0.1 ng/mL with the automated and the seventh one had a concentration of 0.74 ng/mL. We can conclude an overall good concordance between the two methods for lower AMH levels.

DISCUSSION

Our preliminary evaluation showed good analytical performances for the Maglumi® AMH ABEI-

Figure 2 **Bland Altman Plot between the automated and the ELISA AMH immunoassays**



based automated immunoassay, confirming that this method meets the expectations of clinical laboratories for use in routine practices.

AMH has emerged as value-added biomarker in clinical applications like the assessment of ovarian reserve, companion testing in in vitro fertilization, prediction of menopause, diagnosis of PCOS, serving as tumor marker for some cancers and monitoring the return of fertility in women with cancer treated with chemotherapy (1–5).

Because of this wide range of clinical indications, the measuring range of AMH assays needs to cover low concentrations in the case of low ovarian reserve but also high concentrations as in the case of PCOS. The imprecision of the assay should also be low to optimize monitoring of patients in the case of repeated measurements.

The advantage of automated immunoassay like the Maglumi® AMH method is the ability to integrate routine laboratory automated workflows and a faster delivery of results to the physicians for diagnosis purpose or monitoring of treatment efficiency. However, performance evaluation is necessary before routine diagnostic use. The analytical performance of the Maglumi® AMH ABEI based immunoassay was demonstrated by assessment of imprecision and by method comparison. Our results showed a very good precision of the ABEI based immunoassay with low CV for both low and high AMH concentrations. The coefficients of variation observed in our study agree with those reported in the literature for other automated immunoassays and was below 5% (8,9). Our study showed that Maglumi® AMH significantly correlated with a widely used ELISA assay with good overall agreement. A good concordance between the two methods was also observed for lower (below 2 ng/mL) AMH concentrations. However, a bias was observed with the Bland Altman analysis. This bias is not surprising, and is frequently reported in AMH methods comparison studies (6,8,10), and reflects the current lack of standardization that exists for AMH immunoassays (10). Differences are attributed to different assay formats, and also the different antibodies used by the manufacturers. The Ansh ELISA assay is based on a capture antibody specific to the pro region of AMH (Clone 39/6C) and a detection antibody specific for the mature region of AMH (Clone 39/30A), these are not used by other manufacturers. The antibodies used by Snibe for the Maglumi® AMH was not disclosed.

The commercially available AMH assays are, therefore, still not commutable and some important discrepancies have been also reported in the literature for low AMH concentrations (10). Clinical laboratories need therefore to establish specific reference limits for every individual assay to guide clinical decision-making. The recent development of a reference preparation by World Health Organization might help to standardize AMH immunoassays and to improve AMH measurement and interpretation (11).

CONCLUSION

Our preliminary evaluation showed overall good analytical performances of the Maglumi® AMH ABEI-based automated immunoassay. This assay offers an additional automated solution for AMH testing, a value based biomarker with increasing clinical applications in fertility medicine.

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Conflict of interest and funding

Reagents for the evaluation were kindly provided by Snibe diagnostics.

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The influence of COVID-19 disease on pre-analytical blood sample haemolysis rates in three acute medical units: an interrupted time series analysis

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Key words:
COVID-19 disease, pre-analytical, haemolysis, interrupted time series

ABSTRACT

The COVID-19 pandemic impacted delivery of health services. The aim of our study was to determine the impact of COVID-19 disease on pre-analytical blood sample haemolysis by modelling the daily haemolysis rates variations pre and post COVID-19 infections. Ethics approval was obtained prior to study commencing.

Interrupted Time Series data analysis was conducted on UK National Health Service Acute Admissions Unit 25-month (1 February 2019 to 28 February 2021) biochemistry (total and haemolysed) blood sample dataset. Interruption was set on 23 March 2021, the start of the first UK lockdown. Daily haemolysis rate (% samples haemolysed) data were fitted with a spline curve to determine influence of haemolysis rates on short or medium-term temporal trends.

Linear regression was performed so as to determine long-term temporal trends pre- and post-intervention.

There were 32,316 biochemistry blood sample results: 19,058 pre and 13,258 (342 days) from the post-intervention period. Overall median daily haemolysis rate was 7.3% (range: 0-30.6%), 7.7% pre-intervention versus 6.5% post-intervention ($p<0.0001$). The proportion of haemolysis cases negatively correlated with the number of samples processed (*rho*=0.09; p=0.01). The pre-intervention slope was -1.70 $\%.y^{-1}$, y intercept 9.04%; post-intervention slope was -1.88%.y^{-1}, y intercept was 10.2%; with no difference in either the slope (p=0.87) or intercept (p=0.16).

There was no association between short-term variation in haemolysis rates with changes in practice due to COVID-19 disease and the disease itself. The negative correlation between haemolysis rate and the number of samples processed highlights the importance of continued venepuncture practice to facilitate haemolysis rate reduction.

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INTRODUCTION AND BACKGROUND

COVID-19 disease is a novel severe acute respiratory syndrome caused by coronavirus 2 (1). The disease was first reported in Wuhan, China, where pneumonia cases of unknown aetiology were observed (2). The preliminary epidemiological investigations confirmed that sufferers were mostly people that worked at or were handlers and frequent visitors to the Huanan Seafood Wholesale (2). The first UK COVID-19 cases were identified in the North of England on the 29 January 2020 with confirmed COVID-19 infection reported on 31 January 2020 (3). On the 30 January 2020, the World Health Organisation declared this disease as a public health emergency of international concern (2). This implied that the infection would affect global health security and required an international coordinated response (4). As the infection continued to spread throughout the world, the virus started to mutate. For example, the identification of the Alpha (previously known as Kent) variant was made, through viral genomic sequencing, on 14 December 2020 (5). Figure 1 summarises the timeline of the infection from China to the UK during the study period.

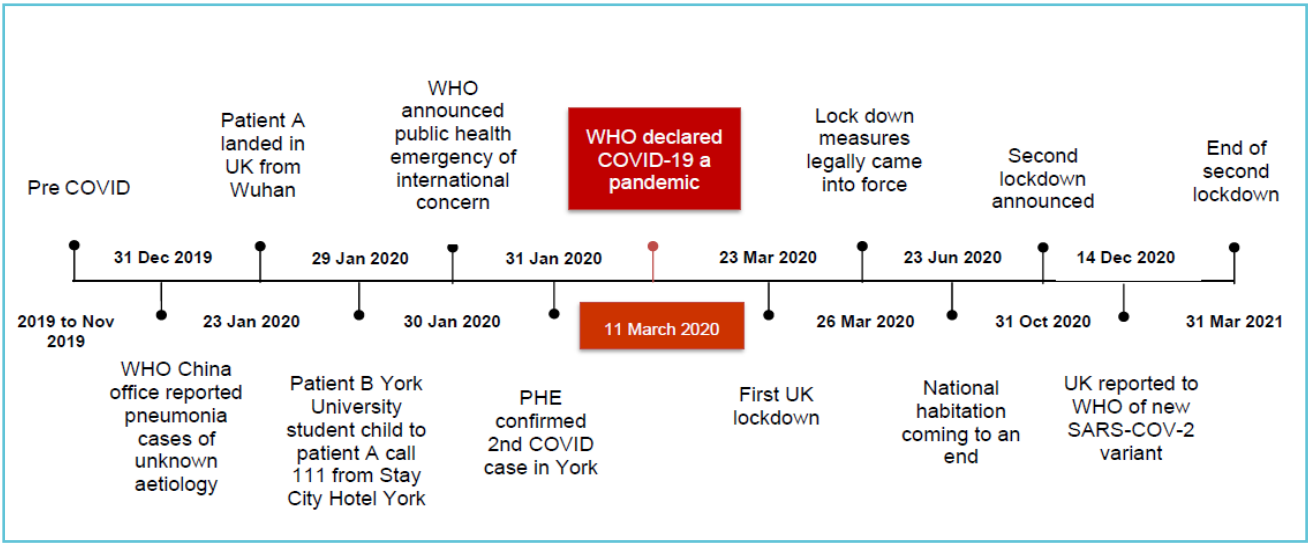
COVID-19 DISEASE

COVID-19 affects the respiratory and the immune systems (6). In severe disease, other organs such as the heart and kidneys are affected (4). Renal failure can result when kidneys are affected (6). Hypercoagulability, acute myocardial injury and myocarditis, arrhythmias, and acute coronary syndromes can follow if the heart is affected (7). Associated abnormal blood tests have also been reported in patients with COVID-19 on admission to hospital (8). Some of the blood results have indicated coagulation abnormalities and organ dysfunction (4). These have included decreased lymphocyte count, prolonged prothrombin time, increased D-dimer level, or increased aspartate aminotransferase, creatinine, creatine kinase, and lactate dehydrogenase (4). However, gradual drop in haemoglobin levels have also been identified in some cases and have been confirmed as Autoimmune Haemolytic Anaemia secondary to COVID-19 Disease (8; 9).

BIOCHEMICAL ANALYSIS

All routine biochemical analysis including the measurement of potassium and the determination of the haemolysis index was performed using the Siemens Advia 2400XPT and Atellica CH930 analysers (Siemens Healthcare Ltd, Camberley, UK). Haemolysis is assessed by diluting 5µL of specimen in 0.9% saline and measuring the absorbance at

Figure 1 **UK COVID-19 infection timeline.**
The timelime summarises COVID-19 related events in the UK from pre-COVID period to end of the study, February 2021.



571 and 596nm. The assessment of haemolysis was performed whenever any serum specimen was analysed, with the level of haemolysis being determined as being either negative or positive, with positive results flagged as "+", "++", "+++" or "++++". The positive haemolysis flags equate to cut-off values for haemoglobin of 45, 140, 235 and 445 mg/dL respectively. In routine practice, and for the purposes of this study, any specimen with a haemolysis flag of "+" or above was considered to be haemolysed and as such the numerical potassium result was not reported.

RATIONALE FOR THE RESEARCH

Autoimmune haemolytic anaemia is a rare autoimmune disorder characterised by autoantibodies that react with self-red blood cells and result in their destruction (8). Cases of autoimmune haemolytic anaemia in patients with COVID-19 have been reported in literature (8; 9; 10). This is an atypical presentation of COVID-19 that may occur during the period of infectivity (9). Nevertheless, patients with respiratory diagnosis had significantly higher (52.9%) haemolysis rate than patients with other diagnoses (11). The haemolysis rate in patients with respiratory diagnosis was second highest (16.7%) compared to 18.6% in patients with neurological diagnosis (12). The disease process and acuity of the patients at time of presentation to the hospital may be the associated factors to sample haemolysis. High haemolysis rate in patients with poor venous access such as those dehydrated have previously been reported (11). A total of 114,463,420 people worldwide were infected by COVID-19; and 2,557,524 deaths were reported by end February 2021 (2).

Older people are commonly most affected by COVID-19 infection (2). Comorbidities and low immune status could be the most common explanations for the vulnerability in this age group. Hypertension and diabetes are amongst the commonly reported risk factors for severe COVID-19 disease (7). Significantly high rates of haemolysis were reported in individuals aged 63 and above (11). The highest haemolysis rate (24%) was reported in individuals of

age 95 years and above (13). Factors such as severe COVID-19 disease, multi-organ failure and dehydration amongst the individuals with COVID-19 disease, could lead to hypovolaemia. These characteristics could in turn lead to difficulties in performing venepuncture and result in the venepuncture being performed in the smaller veins distal to the recommended antecubital fossa veins (14). Furthermore, the risk of infection amongst the staff taking care of patients with COVID-19 disease could also lead to such practice in individuals with poor venous access.

AIM AND RESEARCH QUESTIONS

The aim of this study was to investigate the influence of COVID-19 disease on pre-analytical blood sample haemolysis rate in three front of house acute admissions units (Clinical Decisions Unit, Acute Medical Unit and Acute Assessment Unit) and in one North East England National Health Service Trust. Research questions were designed as a guide to achieving the aim.

Research questions

1. Did the peak period of COVID-19 disease in the UK have any influence on the pre-analytical blood sample haemolysis rate?
2. Did any factors, such as age, interact with any COVID-19 influence on pre-analytical blood sample haemolysis rate?

HYPOTHESIS

The hypothesis to the study is based *a priori* on COVID-19 infection literature (8; 9; 10) and on previous relevant literature on haemolysis rate and respiratory infection (11; 12). The hypothesis is that there will be a temporary increase in pre-analytical blood sample haemolysis secondary to COVID-19 disease. Once the infection rates drop, haemolysis rates will revert to the pre-COVID-19 levels.

METHODS

Interrupted Time Series design was used to investigate the impact of COVID-19 disease (intervention) on pre-analytical blood sample haemolysis. A 25-month dataset (1 February 2019 to 28 February 2021) of biochemistry blood sample (total and haemolysed) was used in the analysis. Time series is a continuous sequence of observations on a population taken repeatedly over time (15). Interrupted Time Series works best with short term outcomes that are expected to change either relatively quickly after an intervention is implemented or after a clearly defined lag (15). The uncertainty of the impact of COVID-19 disease on pre-analytical blood sample haemolysis and the novelty of the infection meant that the design is appropriate. Ethics approval obtained from the Teesside University Health and Social Care Ethics subcommittee, under an Ethics Release format and from the local Trust Research Ethics and Development team. The study preparation and release of followed General Data Protection Act (GDPA) regulations.

DATA COLLECTION

Biochemistry blood results with the following specifications, were obtained through the Laboratory Information Management System:

- biochemistry blood sample results for potassium (total and haemolysed),
- results from the three front of house units of Clinical Decisions Unit, Acute Assessment Unit and Acute Medical Unit of the local National Health Service Foundation Trust,
- results from the samples collected from 1 February 2019 to 31 January 2020,
- and from 1 February 2020 to 28 February 2021.

Virology reverse-transcriptase polymerase chain reaction or lateral flow test results were not

requested for the study due to them not being available for all patients being admitted to hospital earlier in the pandemic. Some incidental diagnoses were made through chest x-rays and computerised tomography scans. Such variability in reaching the diagnosis made it impossible for x-ray and scan results to be included in the requested dataset. However, the Acute Assessment Unit was set aside for potential and confirmed COVID-19 cases. Therefore, a comparison of haemolysis rate by diagnosis was made based on this knowledge.

DATA ANALYSIS

A 25-month dataset of biochemistry blood results collected from the three front of house units of a local Trust was used in the data analysis. The blood results were from pre and post COVID-19 infection (after first UK lockdown). The interruption was defined as the start of the first UK lockdown, 23 March 2020. The pre-intervention phase (before COVID-19 infection) was set from 1 February 2019 to 22 March 2020. The post intervention (after COVID-19 infection) was set from 24 March 2020 to 28 February 2021.

The blood results were uploaded into Excel and checked for completeness (e.g., sample collected, and results released date and the results). Descriptive data analysis was performed to summarise patterns and trends in the data (15). These included measures of central tendency (mean, median, and measures of variance such as range, minimum and maximum and percentile). The daily, weekly, fortnightly, and monthly haemolysis rate proportions were analysed in Excel. Thereafter, the data was imported in GraphPad Prism software for further analysis. The main aim was to model the daily variations in pre-analytical haemolysis rates pre and post COVID-19 infections. 'The day' was the main unit of analysis with the time points based on the day of the release of test results as opposed to the blood sample collected day. Test of normality (Kolmogorov-Smimov) was performed to determine the characteristic of the data prior to further statistical analysis. The outcome was haemolysis rate pre (coded 0) and post (coded 1). Kruskal-Wallis test was used to determine the proportion of haemolysis cases affected by the day of the week. Mann Whitney U test was applied to compare haemolysis rate pre and post COVID-19. Spearman correlation test was performed to determine the association between haemolysis rate and the number of samples processed. Paired t-test was then performed to compare the mean haemolysis rate pre and post COVID-19 infection with significance level set at 0.05.

To establish whether haemolysis rates were influenced by short- or medium-term temporal trends, the data were fitted with a spline curve, which is a number of different polynomial curves that are joined smoothly end-to-end to cover the full period (16). In generating the spline curve, it is necessary to decide how many 'knots' (join-points) there should be, which governs how many end-to-end curves will be used and therefore how flexible the curve will be. If there are too few knots, the spline curve will be a poor fit, and fail to capture the main long-term patterns closely; whereas too many knots will result in a spline curve which fits short-term trends too closely, preventing further analysis of these trends. In our case, the long-term data were best described by 25 knots. Linear regression was then performed pre- and post-intervention to determine whether there were long-term temporal trends in haemolysis rates.

RESULTS

A total of 32,316 biochemistry blood samples were reported from the three acute admissions units during the study period. Of which 19,058

were from the period prior to the 23 March 2020 lockdown, pre intervention (1 February 2019 to 22 March 2020) and 13,258 from the post intervention period (24 March 2020 to 28 February 2021). There were 16,271 blood samples results in 2019, 14,196 in 2020, and 1,849 in January and February 2021. A total of 351 blood results were excluded due to missing or results labelled 'old'.

There were only 3 days (26 to 28 February 2021) in the final week of the study, the data in that week was omitted in the analysis of fortnightly trends. The number of results recorded per day ranged from 7 to 81 (mean: 43), with more test results before the intervention (median, per day: 50, range: 18-81) than after the intervention (median, per day: 34.5, range: 7-60) (Mann Whitney *U* test, p<0.0001). The number of haemolysed cases reported per day positively correlated with the number of samples processed (Spearman correlation; *rho*=0.50; p<0.0001) (Figure 2A), while the proportion of haemolysis cases negatively correlated with the number of samples processed (Spearman correlation; *rho*=0.09; p=0.01) (Figure 2B).

There were 416 time points in the pre-intervention phase and 342 time points in the post intervention phase. The proportion of haemolysis cases was not affected by the day of the week pre-intervention (Kruskal-Wallis, p=0.96), Figure 3A; or post-intervention (Kruskal-Wallis, p=0.59), Figure 3B. Combined results of 3A and B are presented in Figure 3D and proportions of days with number of haemolysed samples presented in 3C.

The overall median daily haemolysis rate during the entire period (1 February 2019 – 28 February 2021) was 7.3% (range: 0-30.6%). The median daily haemolysis was lower post-intervention: the pre-intervention rate was 7.7% (range: 0-30.6%), compared to 6.5% (range: 0-28.6%) post-intervention (Mann Whitney *U* test, p< 0.0001). Daily, weekly, fortnightly and monthly haemolysis rates are presented in Figures 4A, B, C and D, respectively. The January 2019 to February 2021 daily collected haemolysed samples and rates are are available upon request.

Figure 2 **Haemolysis cases (A) by number and (B) proportion, compared to the number of reports per day**

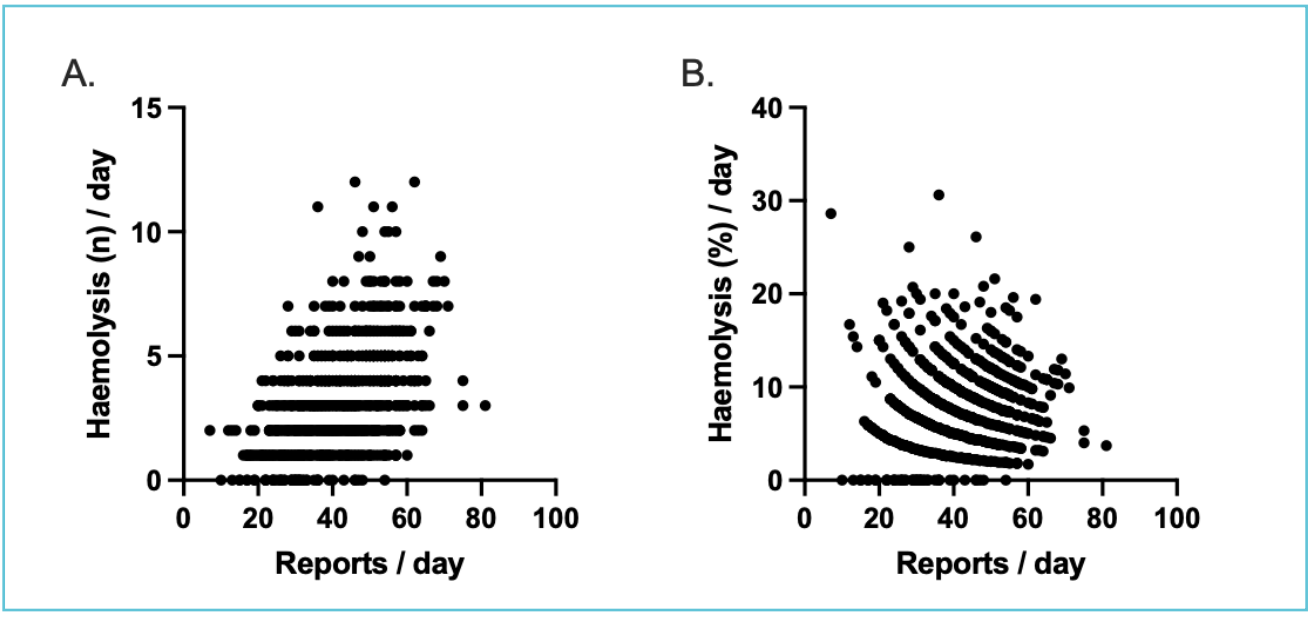


Figure 3 Daily haemolysis rates by day of the week, (A) pre- and (B) post- intervention, and (C) the proportion of occasions in which no haemodialysis cases occurred. (D) The same data as A and B, presented by day

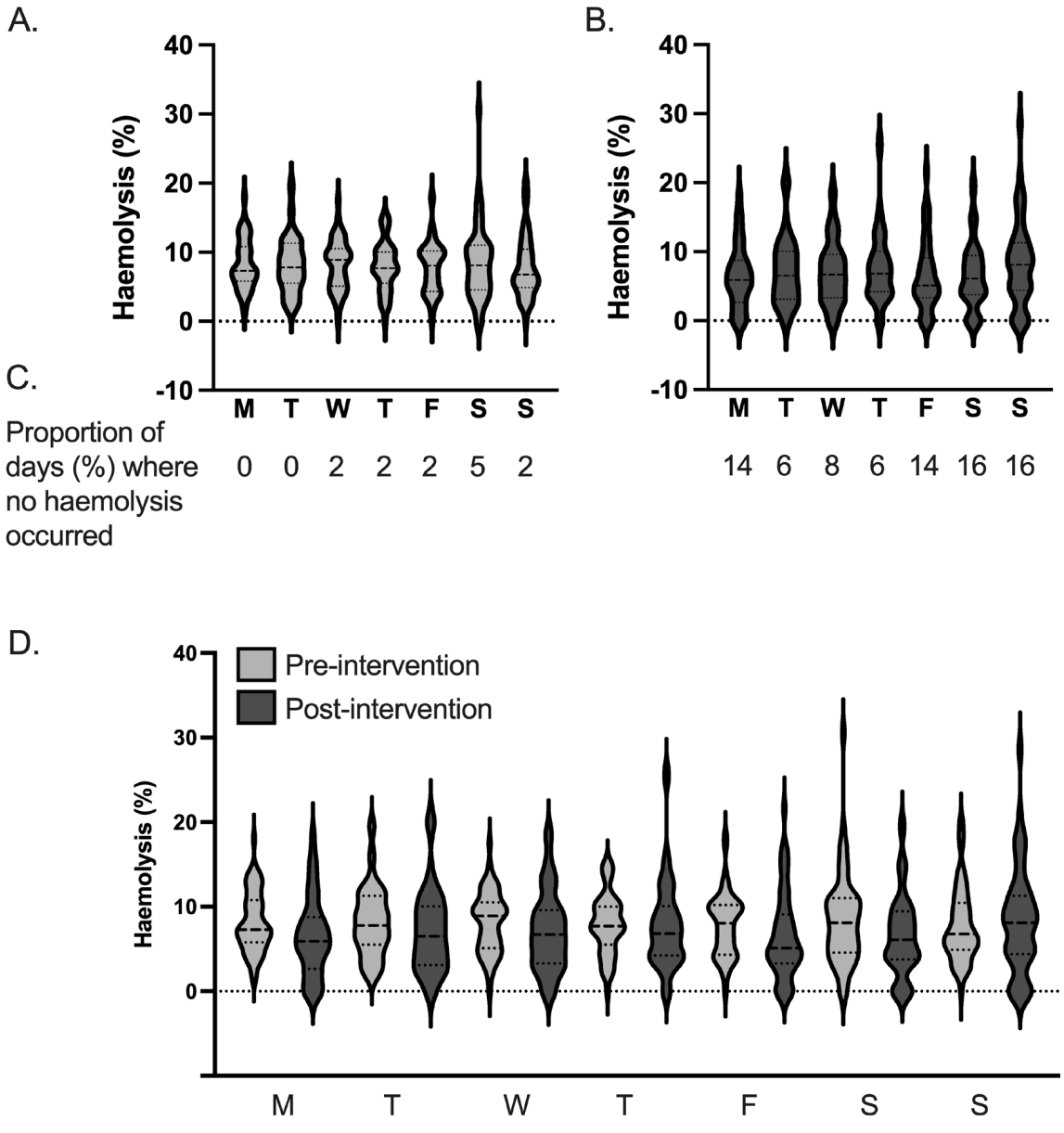
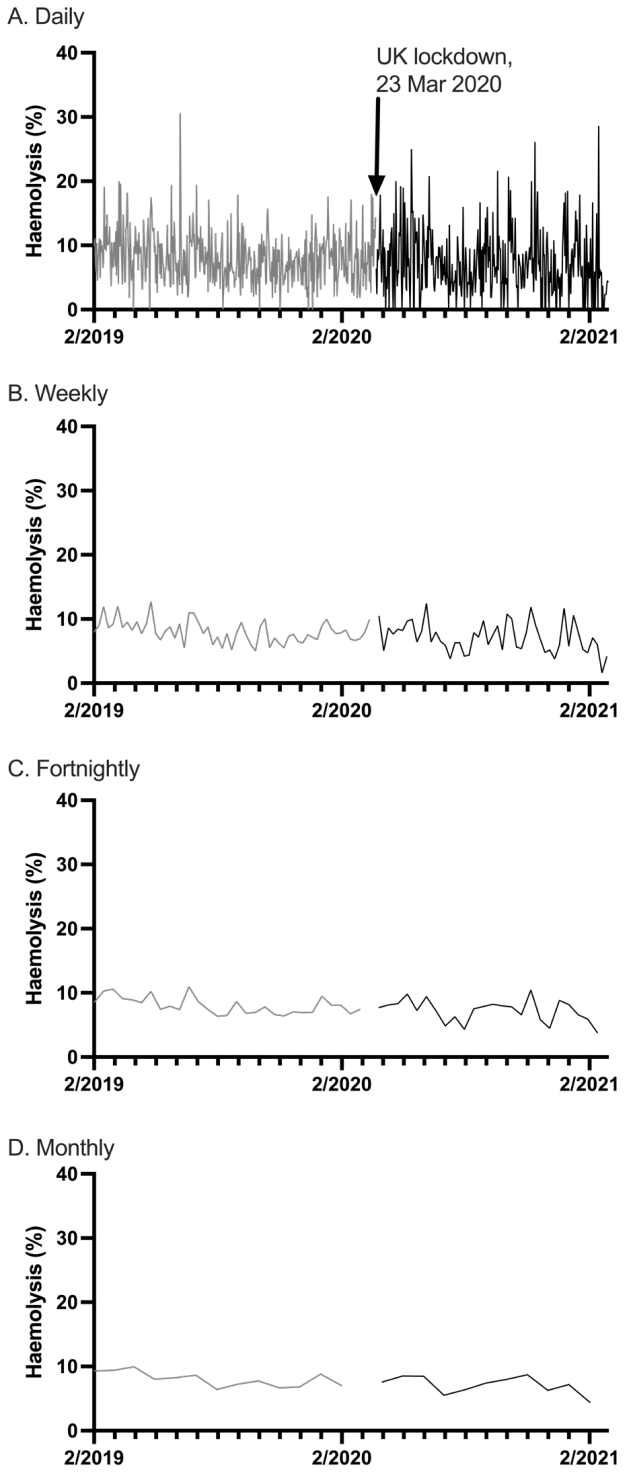


Figure 4 **(A) Daily, (B) weekly, (C) fortnightly and (D) monthly haemolysis rates pre-intervention (grey line) vs. post-intervention (black line)**



HAEMOLYSIS RATES IN DIFFERENT WARDS

The front of house assessment units admit patients from the Emergency Departments, Clinics and General Practitioners for initial/confirmation of diagnosis and treatment. Below in Table 1 is a summary of daily pre and post COVID-19 haemolysed samples, total samples processed and haemolysis rates from the three front of house units (A. Clinical Decisions Unit; B. Acute Medical Unit and C. Acute Assessment Unit) in a local North-East England Trust.

Overall, results in the table 1 confirm a mean reduction in the number of sample processed post in comparison to the pre COVID-19. The greatest reduction was observed in the Acute Assessment Unit (6.23 post versus 17.07 pre-COVID-19). However, there was a slight increase in the samples processed post compared to the pre COVID-19 period in the Acute Medical Unit (20.47 versus 19.51 respectively).

Results from the Acute Assessment Unit show a slight decrease in mean haemolysis rate post-COVID-19, 7.75 (pre) and 7.53 (post), p=0.812. The mean decrease in the mean score was 0.22% at 95% confidence interval. The unit admitted patients with suspected and confirmed COVID-19 infection.

Table 1 Summary of daily pre and post COVID-19 haemolysed samples, reports, and haemolysis rates by ward. Three tables are presented under each unit: A. Clinical Decisions Unit; B. Acute Medical Unit; C. Acute Assessment Unit: the daily haemolysis cases (i), the total number of samples (ii) and the proportion (%) of haemolysed cases (i.e. i/ii) as (iii).

A. Clinical Decisions Unit

i: Number of occurrences of haemolysis per day

	mean (range)	*p*-value
Pre-	1.02 (0-5)	<0.0001
Post-	0.57 (0-4)	
Overall	0.81 (0-5)	

ii: The total number of samples analysed per day

	mean (range)	*p*-value
Pre-	11.80 (2-25)	<0.0001
Post-	7.78 (1-17)	
Overall	9.98 (1-25)	

iii: Proportion of haemolysed samples, expressed as a percentage		
	mean (range)	*p*-value
Pre-	8.73 (0-57)	0.0570
Post-	7.30 (0-100)	
Overall	8.08 (0-100)	

B. Acute Medical Unit		
i: Number of occurrences of haemolysis per day		
	mean (range)	*p*-value
Pre-	1.57 (0-9)	0.2389
Post-	1.45 (0-9)	
Overall	1.51 (0-9)	

ii: The total number of samples analysed per day		
	mean (range)	*p*-value
Pre-	19.51 (6-44)	0.0512
Post-	20.47 (3-42)	
Overall	19.94 (3-44)	

iii: Proportion of haemolysed samples, expressed as a percentage		
	mean (range)	*p*-value
Pre-	7.88 (0-33)	0.0435
Post-	6.90 (0-30)	
Overall	7.44 (0-33)	

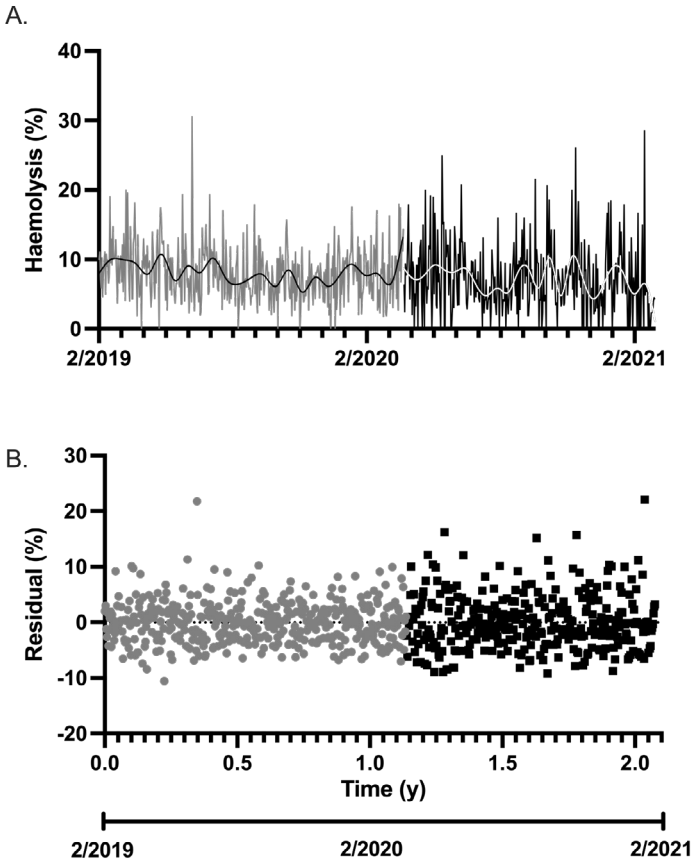
C. Acute Assessment Unit		
i: Number of occurrences of haemolysis per day		
	mean (range)	***p*-value**
Pre-	1.32 (0-6)	0.0001
Post-	0.45 (0-5)	
Overall	0.93 (0-6)	
ii: The total number of samples analysed per day		
	mean (range)	***p*-value**
Pre-	17.07 (4-30)	<0.0001
Post-	6.23 (0-23)	
Overall	12.17 (0-30)	
iii: Proportion of haemolysed samples, expressed as a percentage		
	mean (range)	***p*-value**
Pre-	7.75 (0-36)	0.8120
Post-	7.53 (0-100)	
Overall	7.67 (0-100)	

DETERMINING UNDERLYING TEMPORAL TRENDS IN HAEMOLYSIS RATE

Daily haemolysis rate was fitted with a model (spline curve) to visualise any underlying seasonality and long-term trends (Figure 5a). No clear periodicity, such as might be explained by seasonality, is evident. Figure 5b presents the residual variation in daily haemolysis after 'removing' (i.e. modelling) longer-term trends. Table 2 describes the data.

The comparison of residual haemolysis rate for the two periods of pre- and post-intervention (i.e. lockdown) allows a better understanding of variability in haemolysis. The median residual rate, which is always close to zero if seasonality and long-term trends are accurately modelled,

Figure 5 (A) Daily haemolysis rates, pre-intervention (grey line) vs. post-intervention (black line). Superimposed is a spline curve for each of the two periods: pre- (black line) and post-intervention (white line). (B) Residual haemolysis rate



is less meaningful than the comparison of the variability.

To determine whether there were temporal trends in haemolysis rates, linear regression was performed pre- and post-intervention (Figure 6). From this analysis, the slope helps us to characterise how haemolysis rates change over time, i.e. long-term changes in rates. Likewise, comparing the intercept before and after the first lockdown (in order to compare pre-COVID-19 rates with rates observed during the pandemic) allows us to see if there has been an immediate effect, i.e. a short-term change in rate, because of changes in clinical practices because of the pandemic. The pre-intervention slope was -1.70 $\%.y^{-1}$ and its y intercept, 9.04%. The post-intervention slope was -1.88 $\%.y^{-1}$ and its y intercept was 10.2%. There was no difference in either the slope (*F* test, p=0.87) or intercept (*F* test, p=0.16).

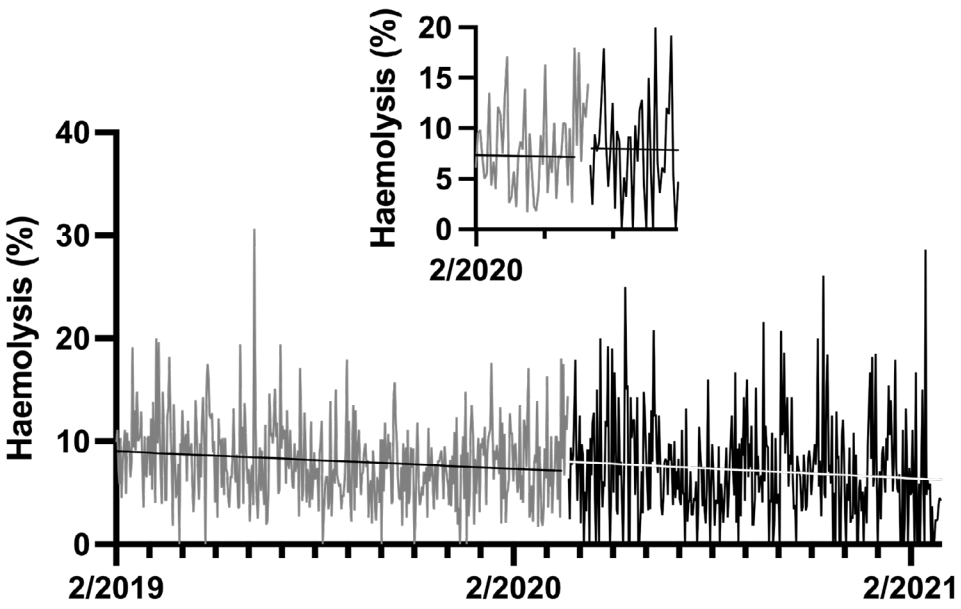
DISCUSSION

This analysis sought to determine whether short-term variation in the outcome (i.e., any change in haemolysis rates) is explained by the exposure of interest (lockdown and changes in practice due to COVID-19 disease). There were

Table 2 Residual haemolysis rate

	Pre-intervention	Post-intervention
No. values	416	343
Minimum (%)	-10.55	-9.190
25% percentile (%)	-2.747	-3.117
Median	-0.046	-0.601
75% percentile (%)	2.154	2.518
Maximum	21.76	22.08
Range	32.31	31.27

Figure 6 (A) Daily haemolysis rates, pre-intervention (grey line) vs. post-intervention (black line). Superimposed is a linear regression for each of the two periods: pre- (black line) and post-intervention (white line). The inset shows a 6-week period either side of the intervention.



no significant differences in the long-term trend, with a haemolysis rate slowly falling over time that is unaffected by the UK lockdown and the pandemic (slope: -1.70%.y^{-1} prior to the 23 March 2020 lockdown vs. -1.88%.y^{-1} in the following year). Likewise, there was no short-term effect observed, with the intercept of haemolysis rate over time with no difference for these two periods. These results confirm the initial assumption in the trend of pre-analytical blood sample haemolysis rate; where temporary increase in the rate secondary to COVID-19 Disease, followed by a drop and revert to the pre-COVID levels, was predicted.

These findings contradict previously reported findings in literature (11; 12) where high haemolysis rates were presented in patients admitted with respiratory diagnoses. COVID-19 disease as a condition affecting the respiratory system does not seem to have an impact on pre-analytical blood sample haemolysis rate in this study. Seasonal and long-term patterns in both the exposure and the outcome can dominate crude association, making the short-term association of interest hard to detect (16). Such trends could be due to genuine physiological changes when dealing with biological data as well as external factors (17). The more residuals in the post intervention phase may have been associated with patient acuteness at the time of presentation to hospital. It may have also been related to external factors such as those pertaining to blood sample collection, associated venepuncture training and competence attainment. As the current data is based on population level, the distribution of common confounders is unlikely to change (16). Therefore, the observed results should be due to the impact of lockdown and changes in practice due to COVID-19 disease.

Overall, there was a reduction in pre-analytical blood sample haemolysis over the study period. Nonetheless, the acute medical unit had a significant mean reduction in haemolysis rate post COVID-19 (7.88% versus 6.90%, p=0.0435). The unit is 30 bedded and the largest of the 3 with equally large volume of samples collected by a cohort of in-house staff. The findings may be linked to the negative correlation between haemolysis rate and the number of samples processed. The repeated conduct of venepuncture may have contributed to increased competence and confidence amongst staff. These factors may also be secondary to the knowledge acquired during venepuncture training including competence attained.

Almost 83% of the respondents to a survey conducted in the same Trust as the current study reported to have achieved venepuncture competences (22). Furthermore, there was an overall increase in confidence at the last performed venepuncture reported across all staff categories (phlebotomist, nurses, health care assistants and doctors) involved in the conduct of venepuncture (22). However, such confidence may depend on the quality of venepuncture training and the support that learners receive within the clinical area. Venepuncture training provided during the study period in the Trust included face-to-face theory and practical and competence attainment completed within the clinical area. Clinical support in achieving competences contributed to pre-analytical blood sample haemolysis reduction (18; 19; 20). Moreover, there is usually a delayed effect of an intervention such as venepuncture training on pre-analytical blood sample haemolysis rate (21). The pattern in pre-analytical blood sample haemolysis rate over time may reflect the impact of venepuncture training being delivered in the Trust.

LIMITATION AND RECOMMENDATIONS

Analysis of impact of COVID-19 disease on pre-analytical blood sample haemolysis has been done using data from one unit admitting patients

with suspected or confirmed COVID-19 infection. The data used did not contain information of the patients' presenting diagnosis. It is not known the extent to which lack of exact diagnosis may have had on the results. It is recommended that similar studies consider obtaining the patients' presenting and or confirmed diagnosis so a clear correlation data analysis between the presenting diagnosis of COVID-19 and haemolysis rate is conducted.

There were challenges brought about by the pandemic such as social distancing restrictions and low staff attendance to training including that of venepuncture. Likewise, there were less face-to-face sessions during this period than there usually are. The Clinical Decisions Unit, situated off the main Trust site was the most affected with almost all the sessions cancelled. It is not known the impact such restrictions may have had on the training internal validity and overall venepuncture practice, and on pre-analytical blood sample haemolysis. If there was any impact, it seems to have been low. However, future studies on the subject should consider the impact on the trend of the independent (e.g., venepuncture training) on dependent (e.g., pre-analytical blood sample haemolysis rate) variables when changes are implemented.

Similarly, changes introduced in some services in the hospital, such as outpatient departments, meant that staff were moved to the acute departments, such as the admissions units. Some of the staff may not have previously attended formal venepuncture training or refresher. Due to the demand posed by the pandemic, the staff may have been performing venepuncture. There is no evidence the involvement impacted in variability in the rates.

Furthermore, there was a difference of 73 data points between the pre and post-intervention phases in the dataset used in the current study. The pre-intervention phase had the most data points, 416 compared to 343 post intervention. This may have had some influence in the current results; however, this is not known. It is recommended that similar future studies consider having equal data points for pre and post intervention to minimise bias caused by such variation.

CLINICAL IMPLICATION

The study has highlighted lack of association of COVID-19 disease on pre-analytical blood sample haemolysis. Such understanding will facilitate the development of clinical pathways in the disease area. The inverse relationship between the venepuncture episodes and haemolysis rate underscores the importance of venepuncture proficiency on haemolysis rate reduction.

CONCLUSION

The current study shows the lack of impact of COVID-19 disease as a respiratory condition, on pre-analytical blood sample haemolysis. The continued reduction in pre-analytical blood sample haemolysis rate confirms consistency venepuncture processes despite the risk transmission. Furthermore, the continued pre-analytical blood sample haemolysis rate reduction confirms positive impact of a general venepuncture training.

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Conflicts of interest

NM was a clinical educator in the local NHS Trust and had previously worked as a clinical nurse in the acute medical unit. JSY has undertaken contract research for Takeda and Boston Scientific; worked with Pfizer on an EU FP7 -funded research project, 'INCOMB'; performed consultancy for TENA, Performics, Zenith and PALL Europe; and received funding from Astellas

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Discrepancies in lipemia interference between endogenous lipemic samples and Smoflipid®-supplemented samples

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lipemia, interference, endogenous lipemia, artificial lipids, intralipid, smoflipid

ABSTRACT

Background

Manufacturers evaluate lipemia-induced interference using Intralipid®, but it does not contain all lipoprotein types. The aim of this study was to evaluate lipemia-induced interference in biochemical parameters from endogenous lipemic samples and SMOFlipid® supplemented samples, in order to assess if SMOFlipid® can be used in lipemic interference studies.

Methods

Serum pools were supplemented with SMOFlipid® to achieve 800 mg/dL and 1500 mg/dL triglyceride concentration, and analyzed for 25 biochemical parameters both before and after the supplementation. In another independent phase, lipemic serum pools were prepared choosing patient samples of 800 mg/dL

and 1500 mg/dL triglyceride concentration. These lipemic serum pools were ultracentrifugated in order to remove lipids. Biochemical parameters were analyzed before and after ultracentrifugation. The bias between SMOFlipid®-supplemented samples and endogenous lipemic samples were compared. The bias between the lipemic and non-lipemic samples were compared with the reference change value.

Results

At 800 mg/dL triglyceride concentration, we found that total protein and transferrin had been affected only in endogenous lipemic serum samples. Magnesium and creatinine had been affected only in SMOFlipid®-supplemented samples. At 1500 mg/dL triglyceride concentration, we found that total protein, amylase, ferritin and glucose had lipemic interference only in endogenous lipemic samples, and chloride only in SMOFlipid®-supplemented samples.

Conclusions

The use of SMOFlipid®-supplemented samples does not provide suitable data to estimate lipemia-induced interference. Thus, interference studies should be performed using a wide variety of lipemic patient samples that represent the heterogeneity of the lipoprotein particles size.

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BACKGROUND

Lipemia in serum samples is a common problem in the daily practice of clinical laboratories. Analytical results may be perturbed by lipemia, leading to misdiagnosis and unnecessary treatments for patients. The overall frequency of lipemic samples ranges from 0.5% to 2.5%, with the higher percentage in primary care (1, 2).

Lipemia is defined as turbidity in serum samples produced by accumulation of lipoprotein particles. Turbidity in serum samples depends on the lipoproteins´ size and number. Chylomicrons are the largest lipoproteins (70- 1000 nm) and the principal cause of lipemia. Very low-density lipoproteins (VLDL) are classified as: small (27-35 nm), intermediate (35-60nm) and large (60-200 nm), but only intermediate and large VLDL contribute to the turbidity. Small lipoproteins particles such as high-density lipoproteins (HDL) (6-12.5 nm) and low-density lipoproteins (LDL) (20-26 nm) do not cause lipemia (3).

The most frequent cause of lipemia is postprandial hypertriglyceridemia; however, lipids and lipoproteins only change minimally in response to normal food intake. Intravenous lipid emulsion is the most common cause of severe lipemia (4, 5).

Other causes include diabetes mellitus, dyslipidemias, pancreatitis, alcohol abuse, chronic renal failure, hypothyroidism, recent parenteral nutrition (6, 7) and some treatments such as protease inhibitor, oral contraceptives, diuretics, cyclosporine and glucocorticoids (8).

Three mechanisms are mainly responsible for lipemic interference: 1) Spectral interference: lipoproteins absorbs and scatters light in a wide range of wavelengths (300 to 700 nm) and consequently exert profound effects on colorimetric, turbidimetric and nephelometric assays (9). 2) Volume displacement effect: the aqueous fraction of the serum may decrease as a consequence of the high volume of the lipid fraction, causing low values in the concentration of various analytes that are distributed in the aqueous phase of the sample (such as electrolytes) (10). 3) Non-homogeneity of the sample: due to their lower density, chylomicrons and VLDL particles are located at the top of the tube after centrifugation. Hydrophobic analytes are also distributed in that phase. Most analyzers obtain sample

from the upper part of the tube, reporting false values (3, 11).

As the lipemia-induced interference is dependent on the analytical method, manufacturers often provide guidelines for acceptable maximum lipemia established with interference studies based on spiking serum samples with commercial lipid emulsions such as Intralipid®. These interference studies are carried out without considering each parameter's individual biological variability, and using, as criterion of acceptability, only an arbitrary variation set at a 10% bias (12, 13).

Intralipid® is a commercial lipid emulsion used as a component of intravenous nutrition. Its particles' size ranges from 200 to 600 nm and lacks the sizes that mimic large VLDL, as well as the lower and upper ranges for chylomicrons size (12, 14), whereas patient samples contain a complex mixture of macromolecular lipid and protein structures (15). Therefore, lipemia induced by Intralipid® is not identical to lipemia in patient serum samples (16, 17).

Lipemia-induced interference should be verified by all clinical laboratories. SMOFlipid® is commercial available, and is a lipid emulsion of 200 mg/dL for intravenous infusion that contains soybean oil, medium chain triglyceride, olive oil, and fish oil. SMOFlipid® is an electrolyte free solution, it only contains small amounts of sodium (5 mmol/1000 mL emulsion) (18). To the best of our knowledge, there is no study that has determined SMOFlipid®'s lipoprotein size range.

The aim of this study is to determine if SMOFlipid® could be used by manufacturers to evaluate lipemia-induced interference in the analysis of biochemical parameters.

METHODS

Serum samples were taken from those routinely analyzed and frozen at −20 °C until use. Individual serums were mixed to prepare different pools using two methods:

A) Artificial lipids

50 different serum pools were prepared excluding hemolyzed, icteric and lipemic (HIL) samples based on a negative semi-quantitative HIL index. They were analyzed on AU5800 (Beckman Coulter Inc. Brea, CA, USA). Serum pools were divided into two aliquots and SMOFlipid® was added to both of them in order to achieve a final triglyceride concentration around of 800 mg/dL and 1500 mg/mL. The SMOFlipid® volume was calculated to reach the desirable triglycerides concentration, no additional diluent was added. The 800 mg/dL triglyceride concentration pools were prepared by adding 0.5 mL of SMOFlipid® to a 15 mL serum pool. To achieve the 1500 mg/dL triglyceride concentration, 1.1 mL of SMOFlipid® was added to a 15 mL serum pool. Then, biochemical parameters were remeasured to assess lipemia-induced interference. The results were multiplied by the dilution factor (1.03 for 800 mg/dL triglyceride concentration and 1.07 for 1500 mg/dL triglyceride concentration).

B) Endogenous lipids

Lipemic serum pools were prepared collecting patient samples with triglyceride concentrations around 800 mg/dL and 1500 mg/dL from routine clinical care, and rejecting hemolyzed samples based on a negative semi-quantitative haemolysis index. All collected samples had a milky or turbid appearance due to a high triglycerides concentration. To prepare each pool, 8-10 patient samples were used in order to have all lipoprotein size range. Lipemic serum pools were classified into two groups: 25 serum pools with triglyceride concentrations of 800 mg/dL and 20 serum pools with triglyceride concentrations of 1500 mg/dL. All pools were analyzed on AU5800 (Beckman Coulter Inc. Brea, CA,

USA). Consecutively, lipemia was removed by ultracentrifugation (Sorvall™ WX100+, Thermo Scientific) at 108,200x*g* for 20 minutes at 4°C. The clear infranatant was transferred into a clean tube and biochemical parameters were remeasured.

Biochemical parameters analyzed: Albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase, aspartate aminotransferase (AST), total bilirubin, calcium, chloride, creatine kinase (CK), creatinine, ferritin, iron, phosphate, gamma-glutamyl transferase (GGT), glucose, lactate dehydrogenase (LDH), lipase, magnesium, C-reactive protein (CRP), potassium, total proteins, sodium, transferrin, urate and urea. Measurements were analyzed in duplicate. Biochemical parameters, analytical methods and lipemia-induced interference with Intralipid® provided by manufacturers are presented in table 1.

Table 1 Biochemical parameters, analytical methods and lipemia-induced interference (Intralipid®) reported by manufacturers

Biochemical parameters	Analytical methods	Lipemia-induced interference (Intralipid®)
Albumin	Bromocresol green – 600/800 nm	<10% to 800 mg/dL
Alkaline phosphatase (ALP)	p-nitrophenyl phosphate – 410/480 nm (IFCC)	<3% to 1000 mg/dL
Alanine aminotransferase (ALT)	Enzymatic spectrophotometry - 340 nm (IFCC)	<3% to 300 mg/dL
Amylase	p-nitrophenol – 410 nm (IFCC)	<3% to 1000 mg/dL
Aspartate aminotransferase (AST)	Enzymatic spectrophotometry - 340 nm (IFCC)	<5% to 300 mg/dL
Total bilirubin	Dichlorophenyldiazoniumtetrafluoroborate (DPD) – 540/660 nm	<10% to 1000 mg/dL
Calcium	Arsenazo III – 660/700 nm	<10% to 1000 mg/dL
Chloride	Indirect potentiometry	<5% to 500 mg/dL
Creatine Kinase (CK)	NADPH – 340/660 nm (IFCC)	<3% to 1000 mg/dL
Creatinine	Enzymatic spectrophotometry – 600/700 nm	<10% to 1000 mg/dL
Ferritin	Immunoturbidimetry	<10% to 1000 mg/dL

Iron	Tripyridyl-5-triazine – 600 nm	<10% to 100 mg/dL
Phosphate	Phosphomolybdate – 340/380 nm	<10% to 800 mg/dL
Γ-glutamyl transferase (GGT)	Gamma-glutamil-3-carboxilo-4-nitroanilida – 410/480 nm (IFCC)	<5 % to 1000 mg/dL
Glucose	Hexokinase – 340 nm	<10% to 700 mg/dL
Lactate dehydrogenase (LDH)	NADH – 340 nm (IFCC)	<3% to 1000 mg/dL
Lipase	4-aminophenazone – 540 nm	<10% to 500 mg/dL
Magnesium	Xylidyl blue – 520 nm	<10% to 500 mg/dL
C-reactive protein (CRP)	Immunoturbidimetry	<10% to 1000 mg/dL
Potassium	Indirect potentiometry	<5% to 500 mg/dL
Total proteins	Cupric ion – 540 nm	<10% to 1000 mg/dL
Sodium	Indirect potentiometry	<5% to 500 mg/dL
Transferrin	Immunoturbidimetry	<10% to 1000 mg/dL
Urate	Uricase – 660/800 nm	<5% to 1000 mg/dL
Urea	Urease, glutamate-deshydrogenase (GLDH) – 340 nm	<3% to 500 mg/dL

STATISTICAL ANALYSIS

Normality of distributions were analyzed using normal distribution tests. Parametric and non-parametric data for each parameter´s concentration was presented as mean ± standard deviation (mean ± SD) or median with interquartile range (median ± IQR), respectively.

The percentage differences (bias) between the lipemic and non-lipemic samples were calculated, for each parameter, according to the following formula:

$$\textbf{Bias} = (Cx - Cn) / Cn \times 100$$

where Cn represents the arithmetic mean or median from the non-lipemic samples parameters and Cx represents the arithmetic mean or median from the lipemic samples parameters (endogenous lipids or spiked with artificial lipids).

Bias were compared using the independent t-test or Mann-Whitney test depending on population distribution (significance threshold: $p<0.05$).

The reference change values (RCV), defined as the critical differences that must be exceeded between sequential results for a significant change to occur, were calculated for lipemia-induced interference in biochemical parameters, considering unilateral Z statistic with 95% confidence (Z=1.65), according to the following formula:

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

CVa: analytical coefficient of variation. The analytical variation must be less than Cvi/2 (desirable quality specification).

Cvi: within-subject biological variation. Cvi values for the parameters were taken according to the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation database (19).

The difference between Cx and Cn is not affected by biological variation. Therefore, Cvi is considered as null. The proposed equation to define acceptance criteria by lipemia-induced interference was:

$$RCV = 1.65 \cdot 2^{1/2} \cdot (Cvi/2)$$

RCV calculated using Cvi/2 may be inappropriate depending on biological variation. It´s not possible to assume that the Cva meets the desirable quality specification for some analytes with small Cvi (for example sodium, Cvi= 0.5). Instead, RCV should be calculated using Cva extracted from the quality control level that is closest to the reference interval. Thus, the equation for these parameters was:

$$RCV = 1.65 \cdot 2^{1/2} \cdot (Cva)$$

Bias and RCV were calculated and compared for each biochemical parameter, in order to assess lipemia-induced interference. When bias exceeds RCV, the provided measurements should not be reported, as the error caused by lipemia exceeds the acceptance criteria.

Statistical analyses were performed using MedCalc for Windows version 19.6 (MedCalc Software, Ostend, Belgium).

RESULTS

Triglyceride concentrations in SMOFlipid® supplemented samples were 854 (790 – 919) mg/dL and 1462 (1427 - 1488) mg/dL.

Triglyceride concentrations in lipemic patient samples were 816 (800 - 846) mg/dL and 1520 (1481 - 1553) mg/dL. Results are represented either as: median (interquartile range) or mean ± standard deviation depending on normal distribution tests.

The biochemical parameter results, both before and after adding SMOFlipid® to the samples, are presented in table 2. Moreover, bias and RCV for each parameter are also presented in table 2. Bias exceeded RCV in SMOFlipid® supplemented samples for creatinine, lipase and magnesium at 800 mg/dL triglyceride concentration; and for all previously mentioned parameters as well as chloride and transferrin at 1500 mg/dL triglyceride concentration.

The results for the biochemical parameters from lipemic patient samples both before and after ultracentrifugation are presented in table 3. Bias and RCV can also be found in table 3. Bias exceeded RCV in lipemic patient samples for lipase, total proteins and transferrin at 800 mg/dL triglyceride concentration; and for all previously mentioned parameters as well as amylase, creatinine, ferritin, glucose and magnesium at 1500 mg/dL triglyceride concentration.

The bias in SMOFlipid-supplemented samples and bias in serum samples with endogenous lipemia were compared (table 4). At 800 mg/dL triglyceride concentration we found differences in all parameters, except albumin (p=0.1453),

Table 2 Results for the biochemical parameters both before and after adding SMOFlipid® to the serum samples at 800 mg/dL and 1500 mg/dL triglyceride concentration

Biochemical parameters	800 mg/dL triglyceride concentration			1500 mg/dL triglyceride concentration			Cvi	Cva	RCV (%)
	Mean or median before adding SMOFllipid® (SD or IQR)	Mean or median after adding SMOFlipid® (SD or IQR)	Bias (%)	Mean or median before adding SMOFlipid® (SD or IQR)	Mean or median after adding SMOFlipid® (SD or IQR)	Bias (%)			
Albumin (g/L)	39 (38 - 40)	40 (39 - 41)	2.6	42 (41 - 42)	43 (42 - 43)	2.4	2.5	1.6	2.9
Alkaline Phosphatase (U/L)	85 (78 – 91)	84 (79 - 92)	1.2	77 (73 - 82)	79 (73 - 82)	2.6	10.0	4.5	11.7
Alanine aminotransferase(U/L)	21 (19 - 24)	21 (19 - 23)	0	*	*	*	10.1	3.3	11.8
Amylase (U/L)	80 ± 17	79 ± 17	1.3	75 ± 14	74 ± 13	1.3	6.6	2.2	7.7
Aspartate aminotransferase (U/L)	24 (22 - 25)	24 (22 - 25)	0	*	*	*	9.6	2.8	11.2
Total bilirrubin (mg/dL)	0.6 (0.5 - 0.6)	0.6 (0.5 - 0.6)	0	0.7 (0.6 - 0.7)	0.7 (0.6 - 0.8)	0	21.8	3.2	25.4
Calcium (mg/dL)	9.3 (9.1 - 9.3)	9.2 (9.1 - 9.3)	-1.1	9.8 (9.6 - 9.8)	9.6 ± 0.3	2.0	2.1	1.4	2.5
Chloride (mmol/L)	104 ± 1	103 ± 2	1.0	105 (104 - 106)	103 ± 2	1.9 [b]	1.1	0.7	1.3
Creatine Kinase (U/L)	89 (75 - 113)	89 (73 - 109)	0	90 (84 - 96)	88 (83 - 96)	-2.2	15	3.0	17.5
Creatinine (mg/dL)	0.89 (0.83 - 1.01)	0.79 (0.73 - 0.89)	11.2 [a]	0.92 (0.86 - 0.96)	0.75 (0.71 - 0.78)	18.5 [b]	4.5	3.6	5.3
Ferritin (ng/mL)	185 (141 - 273)	182 (136 - 268)	1.6	143 (113 - 182)	149 (120 - 190)	4.2	12.8	1.1	14.9

Iron (µg/dL)	73 ± 18	58 ± 19	20.6	72 ± 19	55 ± 15	23.6 [b]	26.5	2.4	30.9
Phosphate (mg/dL)	3.6 ± 0.3	3.5 ± 0.3	-2.8	3.8 (3.7 – 4.0)	3.5 (3.4 - 3.6)	7.9 [b]	8.2	2.7	9.6
Γ-glutamyl transferase (U/L)	33 (27 - 46)	32 (26 - 47)	-3.0	29 (26 - 34)	28 (25 - 32)	3.45	9.1	2.6	10.6
Glucose (mg/dL)	114 ± 15.92	110 ± 16	-3.5	108 (105 - 111)	103 (101 - 106)	4.6	5.0	2.5	5.8
Lactate Dehydrogenase (U/L)	192 (182 - 199)	190 (178 - 196)	-1.0	195(183-206)	182 (177 - 188)	6.7	5.2	3.4	14.5
Lipase (U/L)	32 (28 – 38)	46 (41 - 51)	43.8 [a]	29 (26 - 32)	45 (41 - 47)	55.2 [b]	9.2	5.5	10.7
Magnesium (mg/dL)	2.0 ± 0.1	2.1 ± 0.1	5.0 [a]	3.5 (2.1 - 3.8)	2.2 ± 0.1	37.1 [b]	3.6	2.1	4.2
C-reactive protein (mg/L)	13 (8 – 21)	13 (8 - 21)	0	5 (4 - 6)	5 (4 - 6)	0	34.1	2.5	39.8
Potassium(mmol/L)	4.6 ± 0.3	4.6 ± 0.3	0	4.8 (4.7 - 4.9)	4.6 ± 0.3	4.2 [b]	4.1	0.7	4.8
Total proteins (g/L)	67 ± 5	67 (66 - 69)	0	70 (69 - 72)	68 (67 - 69)	2.9	2.6	1.7	3.0
Sodium (mmol/L)	141 ± 2	140 ± 3	-0.7	142 (141 - 143)	139 ± 2	-2.1	0.5	1.4	3.3**
Transferrin (mg/dL)	245 ± 31	236 ± 29	3.7	257 (245 - 267)	244 (240 - 254)	5.1 [b]	3.9	2.0	4.6
Urate (mg/dL)	5.2 ± 0.9	5.0 ± 0.9	-3.9	5.1 (5 - 5.3)	4.7 ± 0.6	7.8 [b]	8.6	2.1	10
Urea (mg/dL)	41 (37 - 44)	41 (37 - 44)	0	40 (37 - 41)	39 (37 - 41)	-2.5	13.9	3.3	16.2

RCV: reference change values; SD: standard deviation; IQR: interquartile range; Cva: analytical coefficient of variation; Cvi: intraindividual coefficient of variation.

[a] The bias exceed RCV at 800 mg/dL triglyceride concentration. [b] The bias exceed RCV at 1500 mg/dL triglyceride concentration.

**incalculable for negative values. **RCV calculated using Cva. 50 samples were analyzed for each variable*

Table 3 The biochemical parameters results from lipemic patient samples before and after ultracentrifugation at 800 mg/dL and 1500 mg/dL triglyceride concentration

Biochemical parameters	800 mg/dL triglyceride concentration			1500 mg/dL triglyceride concentration			Cvi	Cva	RCV (%)
	Mean or median before ultra-centrifugation (SD or IQR)	Mean or median after ultra-centrifugation (SD or IQR)	Bias (%)	Mean or median before ultra-centrifugation (SD or IQR)	Mean or median after ultra-centrifugation (SD or IQR)	Bias (%)			
Albumin (g/L)	43±1	44±1	2.9	43(43-44)	44±1	2.3	2.5	1.6	2.9
Alkaline Phosphatase (U/L)	81±16	85±17	3.9	90(84-100)	93(87-104)	3.2	10.0	4.5	11.7
Alanine aminotransferase (U/L)	27±8	25(22-29)	7.4	30(24-36)	32(25-34)	6.3	10.1	3.3	11.8
Amylase (U/L)	59±15	64±16	7.4	67±21	73±22	8.2 [b]	6.6	2.2	7.7
Aspartate aminotransferase (U/L)	29(26-35)	29(25-35)	0	36(31-39)	33(30-37)	9.1	9.6	2.8	11.2
Total bilirrubin (mg/dL)	0.4(0.3-0.4)	0.4±0.1	7.5	0.3(0.3-0.4)	0.3(0.3-0.4)	0	21.8	3.2	25.4
Calcium (mg/dL)	9.9(9.7-10.0)	10.1(9.9-10.3)	2.0	9.8(9.6-10.0)	10.0(9.9-10.2)	2.0	2.1	1.4	2.5
Chloride (mmol/L)	102±2	103±2	0.8	101(100-102)	102(101-104)	1.0	1.1	0.7	1.3
Creatine Kinase (U/L)	111±35	116±37	4.5	117±49	122±49	4.1	15	3.0	17.5
Creatinine (mg/dL)	0.91(0.86-0.99)	0.96(0.90-1.96)	5.2	0.79(0.73-0.94)	0.91(0.84-1.06)	13.2 [b]	4.5	3.6	5.3
Ferritin (ng/mL)	291(209-412)	327(245-481)	11.0	321±132	380±156	15.5 [b]	12.8	1.1	14.9

Iron (µg/dL)	84±24	91±26	8.2	76±18	85±21	10.6	26.5	2.4	30.9
Phosphate (mg/dL)	3.5±0.3	3.6±0.4	3.1	3.3(3.2-3.4)	3.5(3.4-3.6)	5.7	8.2	2.7	9.6
Γ-glutamyl transferase (U/L)	72(58-112)	79(61-118)	8.9	129±62	140±66	7.9	9.1	2.6	10.6
Glucose (mg/dL)	158(127-169)	163(131-176)	3.1	177±54	188±53	5.9 [b]	5.0	2.5	5.8
Lactate Dehydrogenase (U/L)	158±19	170±23	7.1	176±19	196±20	10.2	5.2	3.4	14.5
Lipase (U/L)	49(43-57)	41(36-51)	18.3 [a]	65(62-74)	50(44-55)	30 [b]	9.2	5.5	10.7
Magnesium (mg/dL)	2.0±0.2	2(1.9-2.0)	0.5	2.2(2.1-2.3)	2.0(1.9-2.0)	10 [b]	3.6	2.1	4.2
C-reactive protein (mg/L)	4(3-5)	4(2-5)	9.7	5(3-8)	4(3-6)	25	34.1	2.5	39.8
Potassium(mmol/L)	4.6(4.4-4.7)	4.7(4.5-4.8)	1.3	4.6(4.5-4.7)	4.7(4.6-4.7)	2.13	4.1	0.7	4.8
Total proteins (g/L)	72(71-73)	75(74-77)	4.5 [a]	71(70-72)	76(75-78)	6.58 [b]	2.6	1.7	3.0
Sodium (mmol/L)	140(139-141)	141(140-141)	0.9	138(137-140)	141(140-142)	2.13	0.5	1.4	3.3**
Transferrin (mg/dL)	278(265-291)	298(282-306)	6.7 [a]	270±26	293±29	7.85 [b]	3.9	2.0	4.6
Urate (mg/dL)	6.3±1.6	6.5±1.6	2.6	6.4(6.1-6.9)	6.7(6.4-7.2)	4.48	8.6	2.1	10
Urea (mg/dL)	37(33-39)	37(33-40)	0	36(33-40)	37(33-40)	2.70	13.9	3.3	16.2

RCV: reference change values; SD: standard deviation; IQR: interquartile range; Cva: analytical coefficient of variation; Cvi: intraindividual coefficient of variation

[a] The bias exceed RCV at 800 mg/dL triglyceride concentration [b] The bias exceed RCV at 1500 mg/dL triglyceride concentration.
***RCV calculated using Cva.*

25 samples with 800 mg/dL triglycerides concentration and 20 samples with 1500 mg/dL triglycerides concentration were analyzed for each variable.

Table 4 Statistical significance of bias between endogenous lipids and artificial lipids at 800 mg/dL and 1500 mg/dL triglyceride concentration

Biochemical parameters	800 mg/dL triglyceride concentration			1500 mg/dL triglyceride concentration		
	Endogenous lipids Bias (%)	Artificial Lipids Bias (%)	p	Endogenous lipids Bias (%)	Artificial Lipids Bias (%)	p
Albumin (g/L)	2,9	2,6	0.1453	2.3	2,4	0.9662
Alkaline Phosphatase (U/L)	3,9	1,2	0.0001	3.2	2,6	0.0391
Alanine aminotransferase(U/L)	7,4	0	0.4584	6.3	*	*
Amylase (U/L)	7,4	1,3	<0.0001	8.2	1,3	<0.0001
Aspartate aminotransferase (U/L)	0,0	0	0.3551	9.1	*	*
Total bilirrubin (mg/dL)	7,5	0	0.1319	0	0	0.4378
Calcium (mg/dL)	2,0	-1,1	0.0100	2,0	2,0	0.0005
Chloride (mmol/L)	0.8	1.0	0.0005	1.0	1.9	<0.0001
Creatine Kinase (U/L)	4.5	0	0.0001	4.1	-2.2	0.0229
Creatinine (mg/dL)	5,2	11,2	<0.0001	13.2	18,5	0.0439
Ferritin (ng/mL)	11,0	1,6	<0.0001	15.5	4,2	0.0001

Iron (µg/dL)	8,2	20,6	<0.0001	10.6	23,6	0.0001
Phosphate (mg/dL)	3,1	-2,8	0.0001	5.7	7.9	<0.0001
Gamma-glutamyl transferase (U/L)	8,9	-3,0	<0.0001	7.9	3,45	0.0017
Glucose (mg/dL)	3,1	-3,5	0.065	5.9	4,6	0.0007
Lactate Dehydrogenase (U/L)	7,1	-1,0	0.0001	10.2	6,7	<0.0001
Lipase (U/L)	18,3	43,8	<0.0001	30	55,2	<0.0001
Magnesium (mg/dL)	0,5	5,0	0.0202	10	37,1	0.0376
C-reactive protein (mg/L)	9,7	0	0.0001	25	0	<0.0001
Potassium(mmol/L)	1,3	0	0.5693	2.13	4,2	0.0591
Total proteins (g/L)	4,5	0	<0.0001	6.58	2,9	<0.0001
Sodium (mmol/L)	0,9	-0,7	0.7356	2.13	-2,11	0.0001
Transferrin (mg/dL)	6,7	3,7	<0.0001	7.85	5,1	0.0231
Urate (mg/dL)	2,6	-3,9	<0.0001	4.48	7,8	0.0054
Urea (mg/dL)	0,0	0	0.0480	2.70	-2,5	0.0268

significance threshold: p<0.05.

ALT (p=0.4584), AST (p=0.3551), total bilirubin (p=0.1319), glucose (p=0.065), potassium (p= 0.5693), and sodium (p=0.7356). At 1500 mg/dL triglyceride concentration we found differences in all parameters, except albumin, total bilirubin (p=0.4378), potassium (p=0.5693).

DISCUSSION

We analyzed whether the lipemia-induced interference was different depending on the method used to induce lipemia: artificial lipids (SMOFlipid®) or endogenous lipids (lipemic serum samples). At 800 mg/dL triglyceride concentration, we found that total protein and transferrin had been affected only in endogenous lipemic serum samples. Magnesium and creatinine had been affected only in artificial lipemic samples (SMOFlipid®). At 1500 mg/dL triglyceride concentration, we found that total protein, amylase, ferritin and glucose had lipemic interference only in endogenous lipemic samples and chloride only in artificial lipemic samples (SMOFlipid®).

Some biochemical parameters have not shown lipemia-induced interference in any assay: albumin, ALP, ALT, total bilirubin, calcium, CK, iron, phosphate, GGT, LDH, CRP, potassium, sodium, urate and urea. Lipase has shown interference induced by both artificial and endogenous lipemia at 800 and 1500 mg/dL triglyceride concentration.

Using artificial lipemic samples it is not possible to calculate bias for ALT and AST at 1500 mg/dL triglyceride concentration due to the fact that the analyzer reports negative values. However, when employing endogenous lipids, there is no lipemia-induced interference for ALT and AST.

Table 1 shows lipemia interference reported by manufacturers, they evaluate the interference using Intralipid® without considering the biological variability of the magnitudes under study. We believe that biological variability is crucial to establish acceptance criteria in many parameters. In addition, manufacturers should perform lipemia interference studies with endogenous lipids and include them in package inserts.

Previous studies have shown discordant interference results between endogenous lipemia and lipemia induced by artificial lipids. Lipemia-induced interference was not observed in some biochemical parameters when artificial lipids were used. Bornhorst et al. compared lipemia interference both using lipemic patient serum and interference induced by Intralipid® supplementation (16). Lipemia interference was evaluated in α1-antitrypsin, ceruloplasmin, haptoglobin, prealbumin and transferrin. Results showed that concentrations of ceruloplasmin, prealbumin and transferrin were significantly different in patient samples and in Intralipid®-supplemented samples (16). Koch et al. compared lipemic interference for sodium using diferents methods: direct ISE and free interference method (indirect ISE) (17). Their results show that endogenous hyperlipidemic samples have significant deviations in sodium concentration compared with Intralipid®-supplemented samples (17).

These studies evaluated lipemia-induced interference using Intralipid®. One of the strengths of the present study is that we used SMOFlipid® instead of Intralipid®. In addition, published studies have evaluated the interference for limited number of parameters (16,17,20), whereas we have evaluated the most common biochemical parameters.

Currently, lipemia interference is being evaluated using a lipemic index instead of using the triglycerides concentration. This may be inappropriate because lipemic index have limitations, they don't correlate with triglycerides concentration (21). Hunsaker et al. evaluated the lipemic index using endogenous lipids and Intralipid®, and concluded that those limits that were defined using endogenous lipids could be different from those derived from spiking studies using Intralipid® (22).

Therefore, in the present study, the endogenous lipemic samples were collected based on

triglycerides concentration instead of lipemic index. We used many lipemic patient samples to create serum pools. This is important because lipemia-induced interference depends on different sizes and types of lipid particles and, consequently, a representative sample must be chosen to ensure that all kinds of lipid particles are represented.

CONCLUSIONS

Lipemia-induced interference studies performed with artificial and endogenous lipids show discrepancies. Laboratories should verify lipemia-induced interference using endogenous lipids. These endogenous lipids should be obtained from a wide variety of lipemic patient samples that represent the heterogeneity of the lipoprotein particles size. This study is useful for laboratories that do not have the possibility of verifying manufactures´ data of lipemia-induced interference, especially in laboratories with low number of lipemic samples.

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A combination of inflammatory and hematological markers is strongly associated with the risk of death in both mild and severe initial disease in unvaccinated individuals with COVID-19 infection

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ABSTRACT

Background

Inflammatory and hematological markers are used extensively for early prognostication and monitoring in COVID-19.

We aimed to determine whether routinely prescribed laboratory markers can predict adverse outcome at presentation in COVID-19.

Methods

This retrospective observational study was performed on 401 samples collected between July to December 2020 from COVID-19 positive subjects, admitted at All India Institute of Medical Sciences, Delhi, India. Clinical details and laboratory investigations within 3 days of COVID-19 positivity were obtained. Clinical outcomes were noted from patient medical records, till discharge or death. Laboratory parameters, with individually defined cut-offs, were used, either singly or in combination to distinguish survival and death for those having severe and non-severe disease at initial presentation.

Findings

Total Leukocyte count, Absolute neutrophil count, Neutrophil to Lymphocyte ratio, C-Reactive Protein (CRP), Interleukin-6 (IL-6), Lactate Dehydrogenase, Ferritin and Lymphocyte to CRP ratio (LCR) were significantly altered at presentation in severe COVID-19 as compared to non-severe cases; and, also in those who died due to COVID-19 compared to those who survived. A combination of four markers, CRP (≥3.9mg/dL); IL-6 (≥45.37pg/ml); Ferritin (≥373ng/mL); 1/LCR ≥0.405 was found to strongly predict mortality in cases with non-severe presentation as also in severe cases.

Conclusion and Interpretation

The combination of routinely used markers, CRP, IL-6, Ferritin and 1/LCR can be used to predict adverse

outcomes, even in those presenting with mild to moderate disease. This would identify subset of patients who would benefit from closer monitoring than usual for non-severe disease.

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INTRODUCTION

Coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 500 million people worldwide and has caused more than 6 million deaths (1). The clinical presentation of COVID-19 varies from asymptomatic cases to mild flu-like symptoms, to high fever and severe respiratory illness (2). Often, cases have rapid respiratory deterioration, shock, and/or multiple organ dysfunction or failure. Timely diagnosis of impending complications has become most important to contain the severity of disease as well as prevent fatality or any other adverse outcomes. Real-time polymerase chain reaction (RT PCR) has been the mainstay for COVID-19 diagnosis (3), however, routine tests such as hemogram and inflammatory marker levels have been used extensively to facilitate the assessment of disease severity and prognostication (4,5).

The serum levels of acute phase reactants (APRs) including serum C-reactive protein (CRP), ferritin and Interleukin 6 (IL-6), are known to exhibit significant changes due to infectious and non-infectious conditions including COVID-19 (4-6). Studies have also investigated serial evaluation of ferritin and other markers to help in prognostication (7). Other markers like d-dimer, lactate dehydrogenase (LDH), neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR), lymphocyte to CRP ratio (LCR) have also been studied. Amongst these, CRP, although a non-specific marker of inflammation, has emerged as the most widely used single marker in COVID-19 patients (8,9). Recent studies have even advocated the use of multiple markers like ferritin-transferrin ratio, NLR and WBC counts to be used together (10,11). However, no objective criteria for prediction of mortality could be evolved till date and no study till date explored the idea of assessing simultaneous derangement of multiple inflammatory and hematological markers for prognostication of adverse outcomes in mild and severe COVID-19 disease. The novelty of this study is to use a combination of regularly prescribed laboratory parameters in combination, with individually defined cut-offs, to define the risk of death at initial presentation, in both non-severe and severe COVID-19 disease.

METHODS

This study is a retrospective, observational analysis of hospital and laboratory reports of 401 COVID-19 positive patients, confirmed by RT-PCR, admitted in COVID-19 care facility of a tertiary teaching hospital in New Delhi, India between July 2020 to December 2020 and includes unvaccinated individuals. The patient records were compiled as a part of a sample repository to facilitate retrospective research. (Institutional Ethics Committee approval: Ref No. IEC-578/19.06.2020, RP-03/2020). Blood samples for baseline laboratory assessment as per clinicians' requests were collected within three days of positive RT-PCR report. The clinical details regarding the severity of the disease at initial presentation and outcome in terms of survival (S) or death (D) were noted from the patient medical records comprising their entire hospital stay till discharge or death. The patients were categorized into mild to moderate or non-severe (NSD), and severe (SD) groups as per the Indian Council of Medical Research (ICMR) and Ministry of Health and Family Welfare guidelines (12). Patients with history of recent surgery or hematological malignancies

were excluded from the study. All the patients were treated as per institutional protocol.

Complete blood count (CBC) parameters were run on Sysmex hematology analyzer (XN-9000) Kobe, Japan. Total leukocyte count (TLC), absolute lymphocytes (ALC) and absolute neutrophil count (ANC), platelet count (PC) were noted. NLR, PLR and LCR were calculated using absolute neutrophil and lymphocyte counts. Serum samples were analyzed on Roche Cobas 8000 series c702 and e801 analyzers for CRP (Tinaquant C-Reactive Protein IV - Roche Diagnostics), ferritin (Elecsys® Ferritin - Roche Diagnostics) and LDH (Lactate Dehydrogenase - Roche Diagnostics). IL-6 was estimated using Beckman DXI CLIA system.

For analysis, patients were divided into 4 subgroups based on their initial disease severity and final outcome: (a) Mild to moderate COVID-19 illness who survived (NSD-S); (b) Mild to moderate COVID-19 illness who succumbed to death (NSD-D); (c) Severe COVID-19 illness who survived (SD-S) and (d) severe COVID-19 illness who succumbed to death (SD-D). The Spearman correlation was calculated among the inflammatory markers and represented using correlation matrix graph. The comparison of laboratory parameters between NSD and SD, and between S and D was analyzed using t-test for parametric data and the Mann-Whitney U test for non-parametric data. Laboratory parameters were compared between all four subgroups (a to d) using analysis of variance (ANOVA) or Kruskal-Wallis test. Continuous data were reported as mean ± standard deviation (SD) and categorical data were reported as number (percentage). Non-parametric data were reported as median (range). A receiver operating characteristics (ROC) curve analysis was carried out to assess the discriminative ability of various laboratory parameters between survival and death in both, severe and non-severe disease. The cut-off and its sensitivity and specificity along with area under the ROC curve was reported. Further, with a sensitivity of 80% the cut-offs for four parameters which had maximum AUC in the ROC analysis were decided. An unadjusted and adjusted (for age and gender) likelihood of death for patients presenting with any three or all four out of the four elevated inflammatory markers (CRP ≥3.9 mg/dL, IL-6 ≥45.37 pg/mL, ferritin ≥373 ng/mL and 1/LCR ≥0.4052), and clinical severity was calculated using univariate and multivariate logistic regression models respectively. The results were reported as odds ratio and 95% CI. A p-value (two-sided) of less than 0.05 was considered statistically significant. All the statistical analyses were carried out using Stata 16.0 (StataCorp LLC, Texas, USA) and GraphPad ver.9.

RESULTS

In total, 401 adults (267 males and 134 females) were recruited in the study. Table 1(a) represents the distribution of patients as per severity and outcome whereas Table 1(b) summarizes the laboratory results amongst all patient subgroups: COVID-19 patients having severe (SD) and non-severe disease (NSD) at presentation, as well as, among those who survived (S) and those who succumbed to COVID-19 (D). TLC, ANC, NLR, CRP, IL-6, LDH, and ferritin were seen to be significantly higher in SD as compared to NSD. A similar trend was also obtained for D v/s S patients. In contrast, LCR was significantly lower in SD compared to NSD; and also in D with respect to S patients. However, PLR was not significantly different amongst subgroups of severity or mortality.

The median (range) of laboratory parameters is shown as box plots (Figures 1a for SD and NSD patients and 1b for outcome of patients with S and D). Table 2 compares the different laboratory parameters amongst the 4 subgroups (a-d) as described in Methods section and several

Table 1a Clinical characteristics of patients on basis of severity and mortality due to COVID-19 (n=401)

Severity	Overall (n= 401)	Non-severe disease (NSD, n=234, 58.3%)		Severe disease (SD, n=167, 41.7%)	
Gender		M (154)	F (80)	M (113)	F (54)
Age, years (mean ± SD)	52.2 ± 15.6	51.03 ± 15.6	54.95±15.5	51.3±15.7	53.6 ± 15.7
Survivors (S)	277(69.1%)	145	77	36	19
Non-survivors (D)	124 (30.9%)	9	3	77	35
Total No. (%)	401 (100%)	154 (38.4%)	80 (19.9%)	113 (28.1%)	54 (13.6%)

Data presented as n (%) and mean±SD.

Table 1b Laboratory parameters of COVID-19 patients (n=401)

Parameter (unit)	Non-severe (NSD, n=234)	Severe (SD, n=167)	p-value	Survived (S, n=276)	Death (D, n=124)	p-value
Hemoglobin (g/dL)	11.25 (3.60-18.8)	10.5 (5.3-20)	0.160	10.5 (3.60-18.8)	11.3 (5.6-20)	0.29
TLC ($x10^3/\mu L$)	9.67 (0.90-40.8)	12.91 (0.34-47.5)	<0.001	10.04 (0.71-40.8)	13.05 (0.34-47.57)	<0.001
Platelet Count ($x10^3/\mu L$)	196.5 (14-905)	180 (10-591)	0.067	201.5 (11-905)	169.5 (10-591)	0.002
ANC ($x10^3/\mu L$)	7.91 (0.05-36.68)	11.31 (0.03-43.91)	<0.001	8.1 (0.05-36.6)	11.47 (0.03-43.91)	<0.001
ALC ($x10^3/\mu L$)	0.94 (0.01-6.01)	0.90 (0.10-9.8)	0.88	0.94 (0.01-9.8)	0.091 (0.12-6.84)	0.057
NLR	7.82 (0.53-79.58)	10.5(0.09-157.17)	0.006	7.7 (0.5-120.12)	12.30 (0.09-157.17)	<0.001
PLR	217.15 (8.62-12400.79)	184.26 (4.9-2026.5)	0.256	200.7 (6.1-12400.7)	201.07 (4.93-2026.5)	0.861

CRP (mg/dL)	6.6 (0.10-132)	11.4 (0.10-46.7)	<0.001	6.65 (0.10-132)	13.4 (0.1-46.7)	<0.001
IL-6 (pg/mL)	49.09 (0.61-1624)	111.87 (1.15-1624)	<0.001	51.5 (0.61-1624)	165.03 (4.34-1624)	<0.001
LDH (U/L)	548.5 (44-6705)	616 (243-3742)	<0.001	545.5 (44-6705)	665.5 (243-3742)	<0.001
Ferritin (ng/mL)	585 (19.7-13638)	856 (8-25755)	0.004	553 (7.9-13638)	1099 (18.9-25755)	<0.001
LCR	1.72 (0.01-300)	0.73 (0.04-91)	<0.001	1.66 (0.01-300)	0.58 (0.04-38)	<0.001

Data presented as median (range). TLC: total leukocyte count; ANC: absolute neutrophil count; ALC: absolute lymphocyte count; NLR: neutrophil to lymphocyte ratio; PLR: platelet to lymphocyte ratio; CRP: C-Reactive protein, IL-6: Interleukin 6; LDH: lactate dehydrogenase; LCR: lymphocyte CRP ratio.

Figure 1 **Levels of laboratory parameters in: (a) severe v/s non-severe disease; (b) survival v/s death**

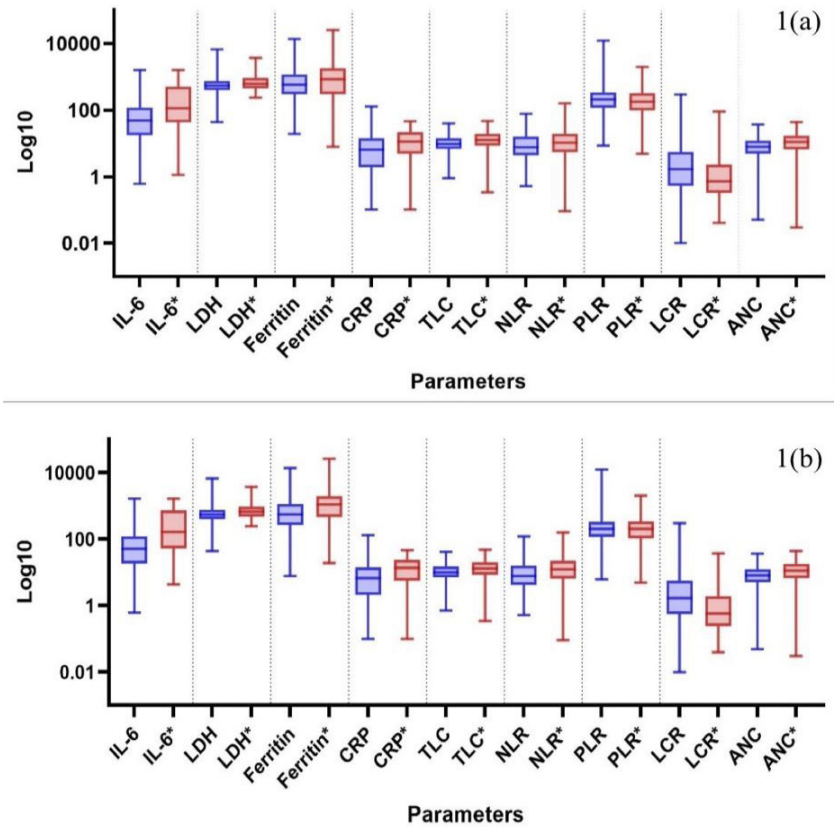


Table 2 Laboratory parameters by subgroups

	a (NSD-S)	b (NSD-D)	c (SD-S)	d (SD-D)	p-value
Number (%)	222 (55.4)	12 (3)	55 (13.7)	112 (27.9)	-
Age*, years	52.2±15.6	54.7±16.1	48.5±17.5	53.7±14.5	0.22
Hb (g/dL)	11.13±2.7	11.2±1.9	9.9±2.6	11.2±2.9	0.024
TLC ($x10^3/\mu L$)	9.6 (0.9-40.8	9.2 (5.8-26.8)	12.4 (0.7-35.5)	13.1 (0.3-47.5)	<0.001
PC ($x10^3/\mu L$)	199 (14-905)	172.5 (15-298)	221 (11-570)	169.5 (10-591)	0.015
ANC ($x10^3/\mu L$)	7.9 (0.05-36.7)	8.09 (4.8-23.8)	10.2 (0.4-32.9)	11.5 (0.03-43.9)	<0.001
ALC ($x10^3/\mu L$)	0.9 (0.01-6.0)	0.83 (0.3-1.4)	1.02 (0.1-9.8)	0.9 (0.12-6.8)	0.03
NLR	7.7 (0.5-79.5)	9.5 (5.7-28.2)	8.5 (0.7-120.12)	12.5 (0.09-157.2)	0.001
PLR	217.2 (8.6-12400)	219.1 (21.6-562.6)	169.9 (6.1-1855.9)	196.5 (4.9-2026.5)	0.49
CRP (mg/dL)	6.4 (0.1-132)	20.85 (1.8-37.4)	10 (0.1-41.1)	13.15 (0.1-46.7)	<0.001
IL-6 (pg/mL)	46 (0.6-1624)	213 (13-1528)	73.6 (1.1-1528)	153.9 (4.3-1624)	<0.001
LDH (U/L)	537.5 (44-6705)	644 (421-934)	578 (241-3490)	667 (243-3742)	0.002
Ferritin ng/mL)	558 (20-13638)	1068 (428-2000)	500 (8-5787)	1099 (19-25755)	<0.001
LCR	1.76 (0.01-300)	0.52 (0.09-5.3)	1.05 (0.08-91)	0.56 (0.04-38)	<0.001

Data represented as median (range) and mean ± SD.*

a: (NSD-S) Mild to moderate COVID-19 who survived; b: (NSD-D) Mild to moderate COVID-19 who succumbed to death; c: (SD-S) Severe COVID-19 who survived; d: (SD-D) severe COVID-19 who succumbed to death.

Hb: Hemoglobin; TLC: total leukocyte count; PC: Platelet count; ANC: absolute neutrophil count; ALC: absolute lymphocyte count; NLR: neutrophil to lymphocyte ratio; PLR: platelet to lymphocyte ratio; CRP: C-Reactive protein, IL-6: Interleukin 6; LDH: lactate dehydrogenase and LCR: lymphocyte CRP ratio.

parameters showed significant differences between the subgroups. The correlations between some of the laboratory parameters were statistically significant namely: IL-6 vs LDH; Ferritin vs CRP; TLC vs NLR; NLR vs PLR; PLR vs LCR; IL-6 vs CRP; IL-6 vs LCR; LDH vs TLC; Ferritin vs LCR; CRP vs LCR; TLC vs LCR; NLR vs LCR, and is as depicted using scatter plot matrix in Figure 2.

Table 3 summarizes the ROC analysis with individual parameter-wise cut-offs, specificity, and AUC for a fixed sensitivity of 80.6%. It was observed that the AUC of individual parameters to distinguish between death and survival varied between 0.710 and 0.614 which are generally considered to be poorly discriminatory. To improve the discriminatory power, we tried a combination approach with two, three and four parameters together. The parameters which had the highest point estimates of AUC amongst all parameters (CRP, IL-6, ferritin and 1/LCR with AUC of 0.659, 0.710, 0.654 and 0.674 respectively), were chosen. We derived the individual parameter-wise cut-offs keeping a sensitivity of 80.6%. Next we defined subsets of patients who had these parameters elevated as per our derived cut-offs (as shown in Table 3). It is evident that the percentage of patients succumbing to death increased with the increased number of

Figure 2 Correlations between laboratory parameters using scatter plot matrix

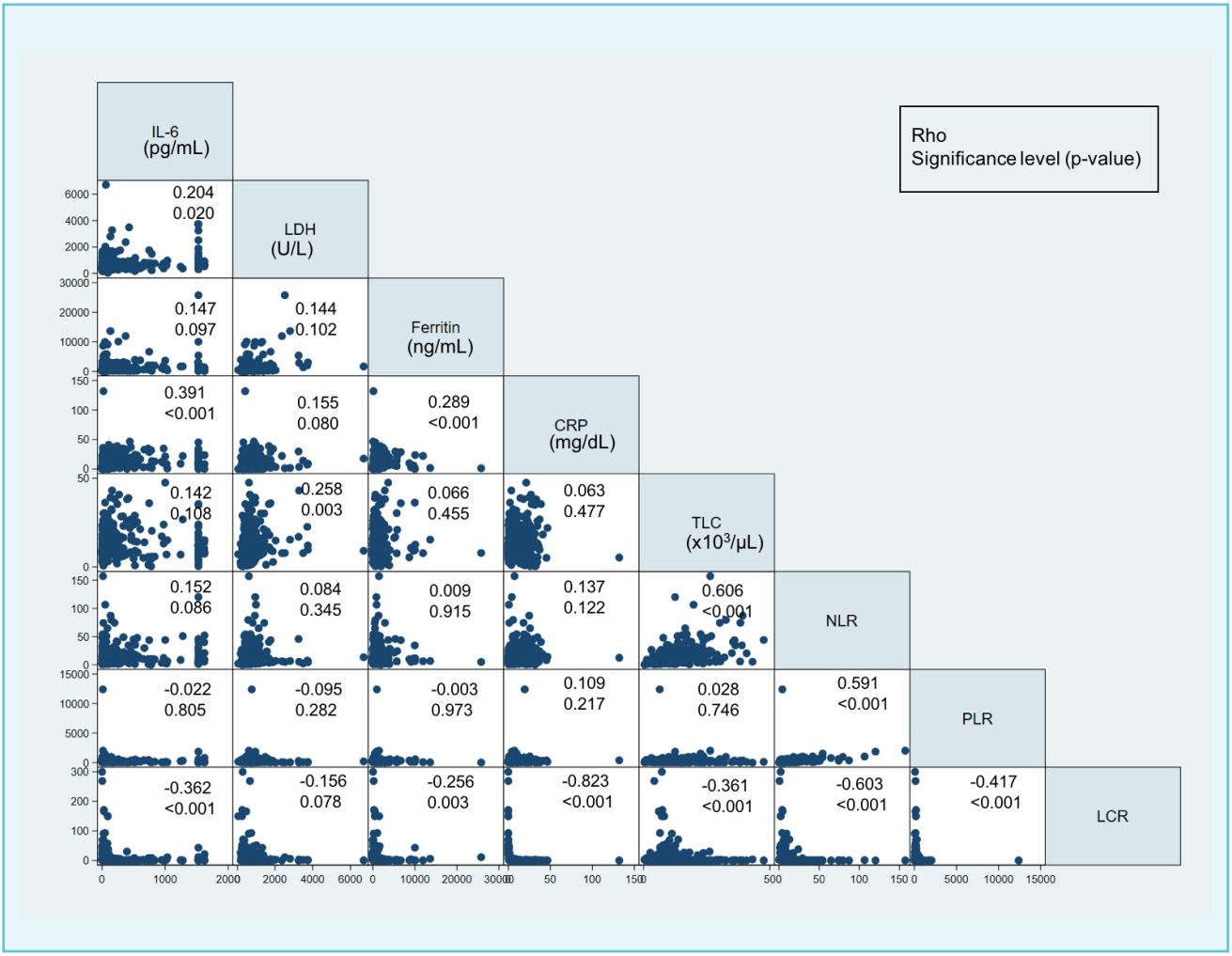
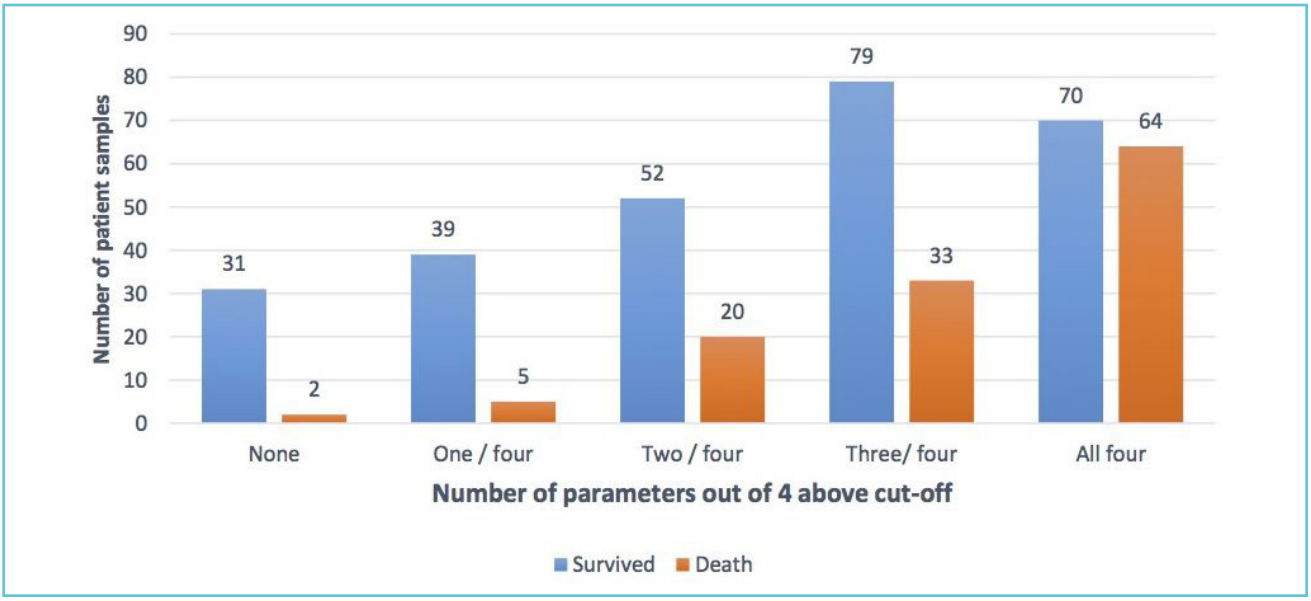


Table 3 Receiver Operating Characteristics curve for different laboratory parameters against death (n= 395#)

Parameter	Cut-off	Specificity$	Area under curve$
IL-6 (pg/ml)	≥45.37	46.13%	0.710
LCR1*	≥0.405	42.07%	0.674
CRP (mg/dL)	≥3.9	33.21%	0.659
Ferritin (ng/mL)	≥373	35.06%	0.654
ANC (x10^3/µL)	≥6.15	30.87%	0.627
NLR	≥5.76	35.06%	0.624
LDH (U/L)	≥421	31.00%	0.616
TLC (x10^3/µL)	≥7.55	29.89%	0.614

**LCR 1 is inverse of LCR (1/LCR), $ Specificity and AUC given keeping the sensitivity fixed at 80.6% for all parameters, #Samples from patients with age≥18 years included.*

Figure 3 Distribution of patients with abnormal laboratory parameters among death and survival groups



Legend: Bar diagram shows the number of patients with one or more abnormal laboratory parameters amongst the four selected using designated cut-offs: CRP (≥3.9 mg/dL); IL-6 (≥45.37 pg/ml); Ferritin (≥373 ng/mL); LCR1 ≥0.405 (LCR1 is 1/LCR).

parameters (out of our four defined parameters) having values above the decided cutoff by the ROC analysis (Figure 3).

Next, we analyzed the different subgroups based on clinical severity at onset, with reference to the presence of number of abnormal laboratory parameters as per our defined criteria. These were assessed to determine the likelihood of death with adjustment for age and gender (Table 4). NSD patients with three or more elevated markers, had an adjusted risk of death (95% CI) of 2.62 (0.69-10.00) times when compared to NSD with two or less markers. For SD with three or more elevated markers, the adjusted risk of death was 88.76 (26.25-300.08), compared to SD with two or less markers having, the adjusted risk of death of 47.64 (12.91-175.77). Further, the combination of all four of the chosen inflammatory or hematological parameters versus three or less of these parameters were also analyzed similarly. The striking feature of this analysis is that when all four

Table 4 Age and sex adjusted Odds ratios (OR) for death v/s survival using clinical and laboratory characteristics (n= 395#)

Severity of disease at onset	No. of parameters selected$	Survived (S)	Death (D)	Total Number	Unadjusted OR (95% CI)	P-value	Adjusted OR* (95% CI)	P-value
Non severe (NSD)	2 or less@	104 (97.2)	3 (2.8)	107	1.0	-	1.0	-
	3 or more	114 (92.7)	9 (7.3)	123	2.73 (0.72-10.38)	0.139	2.62 (0.69-10.0)	0.157
Severe (SD)	2 or less	18 (42.8)	24 (57.2)	42	46.22 (12.59-169.6)	<0.001	47.64 (12.91-175.77)	<0.001
	3 or more	35 (28.5)	88 (71.5)	123	87.16 (25.91-293.1)	<0.001	88.76 (26.25-300.08)	<0.001
Non severe (NSD)	3 or less@	167 (97.7)	4 (2.3)	171	1.0	-	1.0	-
	All 4	51 (86.4)	8 (13.6)	59	6.54 (1.89-22.6)	0.003	6.26 (1.80-21.71)	0.004
Severe (SD)	3 or less	34 (37.8)	56 (62.2)	90	68.76 (23.36-202.3)	<0.001	70.20 (23.75-207.47)	<0.001
	All 4	19 (25.3)	56 (74.7)	75	123.05 (40.15-377.1)	<0.001	128.33 (41.62-395.64)	<0.001

*Data presented as n (%), Odds Ratio (OR) with 95% Confidence Interval (CI), $ No. of parameters selected out of four having values above cut-off, * Adjusted for age and gender, @ Reference category, # Samples from patients with age ≥18 years included.*

parameters are deranged (as per our cut-offs) in the NSD group, there is a 6.26 (1.80-21.71) fold increase in the likelihood of death (95%CI) when compared with those having three or less deranged parameters. In SD patients with all 4 elevated markers, the adjusted risk of death was 128.33 (41.62-395.64), compared to SD with 3 or less markers having, the adjusted risk of death of 70.20 (23.75-207.47).

The results were further analyzed to find the independent effect of severity of disease and elevation of all four laboratory parameters adjusting for age and sex. The patients having SD were having 37.97 (19.15-75.27) times higher adjusted risk of death compared to NSD and all four elevated parameters had 2.48 (1.36-4.51) times higher adjusted risk of death as compared to 3 or less elevated parameters. The predictive ability of this model as assessed by AUC of the ROC for this model was 0.89.

DISCUSSION

It has been widely reported that in COVID-19, despite the presence of mild to moderate symptoms initially, many patients go on to develop complications such as acute respiratory distress syndrome (ARDS) and may succumb to death (13,14). Several laboratory markers like, TLC, ANC, NLR, CRP, IL-6, LDH, Ferritin, d-dimer etc. have been studied for prognostication and the importance of these markers used individually, has been reported. However, not enough literature is available on the combined use of these markers to facilitate prognostication in the initial phase of the disease. The novelty of our approach lies in using routinely prescribed inflammatory and hematological parameters, that were assessed and compared between patients presenting with varying severity of COVID-19 disease.

In our study, TLC, ANC, NLR, CRP, IL-6, LDH, and Ferritin were found significantly higher and LCR significantly lower in those with SD compared to NSD and in those who succumbed to death due to COVID-19 compared to them who survived. These markers are routinely done as a part of COVID-19 workup but are produced as a result of different pathophysiological processes and work independently. Their serum levels do not necessarily correlate strongly with each other as has been observed in the correlation matrix. Even the ROC analysis revealed poor discriminatory performance of individual parameters. We then used a combination of four markers (CRP, IL-6, Ferritin and 1/LCR) with cut-offs defined at 80.6% sensitivity and we found that these together were valuable in predicting mortality even in the cases that were non-severe at presentation. Next we took a novel combination approach, where, to improve the discriminatory power, we evaluated two, three and four parameters together. Further evaluation was done on the subset of patients who had these parameters (CRP, IL-6, ferritin and 1/LCR) elevated as per our derived cut-offs.

Studies on inflammatory markers including serum ferritin, procalcitonin (PCT), CRP, and IL-6 have reported these markers to be significantly associated with the high risks of the poor prognosis and development of severe disease with COVID-19 (5,15). There is a higher risk of ARDS and lung damage in those having elevated CRP levels (16,17). Recent literature has shown that IL-6 levels are significantly higher in the initial phase of the disease or at admission in those who develop more severe symptoms or succumb to the disease or have interstitial lung involvement (13,14).

A few studies including the current one showed the association of increased ferritin levels with severity and mortality in COVID-19 (18,19), however, some studies report higher ferritin to be a bystander rather than being a true characteristic of COVID-19 (20).

In a meta-analysis of 28 studies LDH levels reported in severe vs. non-severe groups showed mean difference of 154.49 (95% CI 121.24 - 191.73, p < 0.001) (21). On comparison a statistically significant and raised level of LDH was also seen in patients admitted to ICU vs. non-ICU patients (mean difference = 272.98; 95% CI: 195.46, 350.51; p < 0.001), and in patients who could not survive vs. survived patients (mean difference = 259.21; 95% CI: 166.91, 351.51; p < 0.001) (21).

Lymphopenia is caused by multiple factors including direct viral injury due to expression of ACE 2 on surface of lymphocytes (15), cytokine storm causing lymphocyte apoptosis induced by interleukins, as well as atrophy of lymphoid organs leading to reduced turn over. In the initial part of the disease, peripheral blood leukocyte and lymphocyte counts are normal or slightly reduced (22). With progression, significant lymphopenia occurs along with deterioration in medical condition and increase in inflammatory mediators in the blood. In our study, samples were collected within 3 days of positive RT-PCR for COVID-19, hence, significant difference in ALC between SD and NSD (p=0.88), and between S and D (p=0.057) was not observed. However, TLC and ANR were significantly increased in those with SD compared to NSD as seen in other studies. It has been reported that lymphopenia on admission (lymphocyte count < 1,100 cells/µl) is associated with a three times higher risk of poor outcomes, in younger vs older patients (22). Lymphocyte counts were lower in patients with ARDS, severe disease requiring ICU care, and in non-survivors (23). Interestingly, NLR, PLR, and LCR (all having Lymphocyte count as a component) have been reported extensively as prognostic biomarkers in COVID-19 studies (24-31). The increased mobilization of neutrophils due to increased release of pro-inflammatory cytokines leads to higher neutrophils in peripheral blood and relative lymphopenia, as a manifestation of severe infection. Lymphopenia along with stress-mediated neutrophilia, leads to a high NLR value and increased NLR was found to be associated with poor clinical outcome in severe COVID-19 (24). Another similar biomarker, LCR, which has previously been studied and used as a prognostic marker for various cancers, including colon and gastric carcinomas (25,26) serves as a marker for the systemic inflammatory process due to interaction of tumor and host immune cells (27). Since COVID-19 also leads to a systemic inflammatory response, LCR helps in prognostication and management of patients. NLR and LCR have thus been studied as inflammatory markers that reflect systemic inflammatory response, and both are easily available in almost all the laboratories (24,26,28). In our study, NLR was found to be higher and LCR was found to be significantly lower in SD compared to NSD and in D compared to S. Yang et al, 2020 reported that 46.1% of their COVID-19 patients with age ≥ 49.5 years and NLR ≥ 3.3, were likely to become severe, within a mean duration of 6.3 days; thus, close monitoring for progression of the disease is required (29).

From all the previous studies no single predictive laboratory parameter has emerged for prognostication. In our study we report that if four markers with decided cut-offs: CRP (≥3.9 mg/dL); IL-6 (≥45.37 pg/mL); Ferritin (≥373 ng/mL); 1/LCR ≥0.405 are used together it acts as a significant predictor of adverse outcome even in cases of clinically non-severe disease as well as sever disease. The novelty, of this study is the use of routinely prescribed laboratory parameters being used to do so. Thus, it provides a cost-effective solution for prognostication in COVID-19 cases.

The outcome of COVID-19 may depend upon multiple factors like strain of virus, host factors, comorbidities, vaccination status etc. This study was carried out during the first wave of COVID-19 in India when all the individuals

were non-vaccinated and had no previous exposure to the virus. Thus the findings of the study though interesting need to be validated in the current scenario. Another limitation of the study was non availability of data regarding the co-morbidities of the patients, if any. Overall, this study presents an interesting outlook for prognostication using routinely available and prescribed diagnostics.

CONCLUSION

The combination of routinely used markers, CRP (≥3.9 mg/dL), IL-6 (≥45.37 pg/mL), Ferritin (≥373 ng/mL) and 1/LCR ≥0.405 can be used to predict adverse outcomes, even in those presenting with mild to moderate disease. This would identify a subset of patients who would benefit from closer monitoring than usual for non-severe disease. The authors however feel, that at this juncture it is difficult to predict which patients would have derangements of which parameters, hence routine screening for all the above-mentioned tests would be the appropriate strategy. However, endeavors to use machine-learning tools to develop artificial intelligence-based algorithms using larger data sets could be taken up to develop more cost-effective and accurate predictors for prognostication.

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Conflict of interest disclosures

None declared.

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Glucose interference in serum and urine samples with various creatinine concentrations measured by the Jaffe kinetic method

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Key words:
glucose interference, creatinine, Jaffe kinetic method, concentration, dilution

ABSTRACT

Background

The effect of glucose interference on creatinine measurement by Jaffe kinetic method differs between serum and urine specimens. We investigated the effects of creatinine concentration and specimen dilution on glucose interference with urine creatinine measurement.

Methods

Leftover serum and urine specimens were collected and stored at -20℃ until study. Serum specimens were mixed to make 5 glucose concentrations ranging from <5.6 to 27.8 mmol/L, each group consisting of 5 levels of creatinine concentration ranging from <45 to 354 µmol/L. Urine specimens were divided into 5 groups of creatinine concentration ranging

from <1,769 to >7956 µmol/L, each sample was spiked with glucose powder to produce 5 aliquots with glucose concentrations ranging from 0 to 666 mmol/L. Urine samples were automatically diluted 1:20 before analysis. Percent interference of creatinine measurement by Jaffe kinetic method was calculated using enzymatic method as the reference.

Results

A total of 148 serum samples and 335 urine samples were analyzed. In serum, glucose interference with Jaffe creatinine measurement was found if creatinine concentrations were 177 µmol/L or less, corresponding to 3,540 µmol/L or less in urine specimens prior to 1:20 dilution. The degree of interference was greater when glucose concentration was higher or creatinine concentration was lower.

Conclusions

When creatinine concentration and specimen dilution were considered, the effects of glucose interference on Jaffe creatinine measurement were similar in serum and urine specimens, and was found when creatinine concentrations in serum or diluted urine were 177 µmol/L or less.

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INTRODUCTION

Creatinine measurement either in serum or urine is one of the most common routine laboratory tests. Generally, serum creatinine is used as an indicator of glomerular filtration rate while urine creatinine is used for hydration correction in spot urine sample. Even though the enzymatic method is accepted as an accurate method for creatinine measurement and deals effectively with most interfering substances, Jaffe kinetic is still the commonly used method for determining creatinine because of its simplicity and low cost. The Jaffe reaction is a colorimetric method and it is well known that non-specific chromogens, especially glucose, falsely increase the results. However, most of the data of glucose interference with Jaffe creatinine measurement were from the studies using serum or peritoneal dialysate [1-9]. There was only one study that investigated the interfering effect of glucose on creatinine measurement in urine and found that glucose did not significantly influence the measurement of urine creatinine by the Jaffe kinetic method even if the concentration of glucose was extremely high at 320 mmol/L [10]. As normal urine creatinine concentration is much higher than serum creatinine concentration, urine samples must be diluted by 20-25 folds before analysis. This dilution process also lowers the concentration of glucose in the urine and might abolish the interference effect of glucose. The discrepant results between the studies using serum and urine specimens could be explained by the difference in creatinine concentration and the dilution process of urine sample prior to measurement.

In the era of sodium-glucose cotransporter 2 (SGLT2) inhibitors, urine glucose excretion may be found at more than 100 g/day [11]. Theoretically, these extremely high glucose concentrations may interfere with urine creatinine assay by the Jaffe kinetic method even though the urine specimen is diluted 20 times prior to measurement. To prove this concept, we studied the interference of glucose on Jaffe creatinine assay in a broad range of glucose and creatinine concentrations, and in both serum and urine specimens.

MATERIALS AND METHODS

This cross sectional study was conducted in the biochemical laboratory of Songklanagarind Hospital during the year 2021. Leftover serum and urine specimens were collected and prepared in different concentrations of glucose

and creatinine. The effects of glucose interference with creatinine measurement by the Jaffe kinetic method were studied in both serum and urine samples. Since the enzymatic method is an accurate method for creatinine measurement without glucose interference, it was used as a reference method. Percent interference (%) of creatinine measurement by the Jaffe kinetic method was calculated as 100 x (creatinine concentration by the Jaffe kinetic method – creatinine concentration by the enzymatic method) ÷ creatinine concentration by the enzymatic method. The study protocol was approved by the Ethics Committee of Faculty of Medicine, Prince of Songkla University.

Samples preparation

Leftover serum specimens with various concentrations of creatinine and glucose were collected within 4 hours after obtaining the samples and stored at -20 ℃ until study. On the day of analysis, serum samples were thawed and some of them were mixed together if necessary to make the final glucose concentrations into 5 groups, namely <5.6, 5.6-11.1, 11.2-16.6, 16.7-22.2, and 22.3-27.8 mmol/L and each group consisted of 5 levels of creatinine concentration (<45, 45-88, 89-177, 178-265, and 266-354 µmol/L). We aimed to produce 5-10 samples in each cell depending on the creatinine and glucose concentrations of the collected specimens. Since the final concentrations of glucose and creatinine were not exactly the same as those expected from the calculation, the numbers in some cells were more or less than expected. All samples were analyzed in the same batch and on the same day.

Leftover urine specimens that were negative for glucose, protein, bilirubin, and blood were collected within 4 hours after obtaining the samples and stored at -20 ℃ until study. In order to get equal distribution, urine specimens were divided into 5 groups of creatinine concentration (<1,770, 1,770-3,540, 3,541-5,300, 5,301-7,960, and >7,960 µmol/L), each group consisted of at least 10 samples. On the day of analysis, urine samples were thawed and centrifuged at 3,000 rpm for 10 minutes. The supernatants were spiked with glucose powder to produce 5 aliquots with final glucose concentrations of 0, 167, 333, 500 and 666 mmol/L from each sample. All samples were analyzed in the same batch and on the same day.

Measurement method

Creatinine measurement by Jaffe kinetic method was performed by using an automated analyzer COBAS 8000 (Roche Diagnostics, Indianapolis, IN, USA). Urine samples were automatically diluted 1:20 with standard diluent before analysis. The intra-assay coefficient of variation at a mean concentration of 4,314 and 8,009 µmol/L was 2.66 and 2.87%, respectively.

Creatinine measurement by enzymatic method was performed by the same analyzer. Urine samples were automatically diluted 1:25 with standard diluent before analysis. The intra-assay coefficient of variation at a mean concentration of 4,270 and 8,000 µmol/L was 1.10 and 1.58%, respectively.

Glucose measurement by enzymatic method was performed by using an automated analyzer COBAS 8000 (Roche Diagnostics, Indianapolis, IN, USA). The samples were diluted 1:10 and 1:20 before analysis if the glucose levels were 167-333 mmol/L and 500-666 mmol/L, respectively. The intra-assay coefficient of variation at a mean concentration of 5.7 and 13.5 mmol/L was 0.98 and 1.39%, respectively.

Statistical analysis

Data were expressed as means ± SD, median (range) and percentages. Due to small numbers in each group, Kruskal Wallis test was used to analyze the differences of percent interference

among the groups followed by Bonferroni post hoc analysis. IBM SPSS Statistics for Windows, Version 28.0 (IBM corp., Armonk, NY, USA) was used for statistical analysis. The significance level was set at a p-value of 0.05.

RESULTS

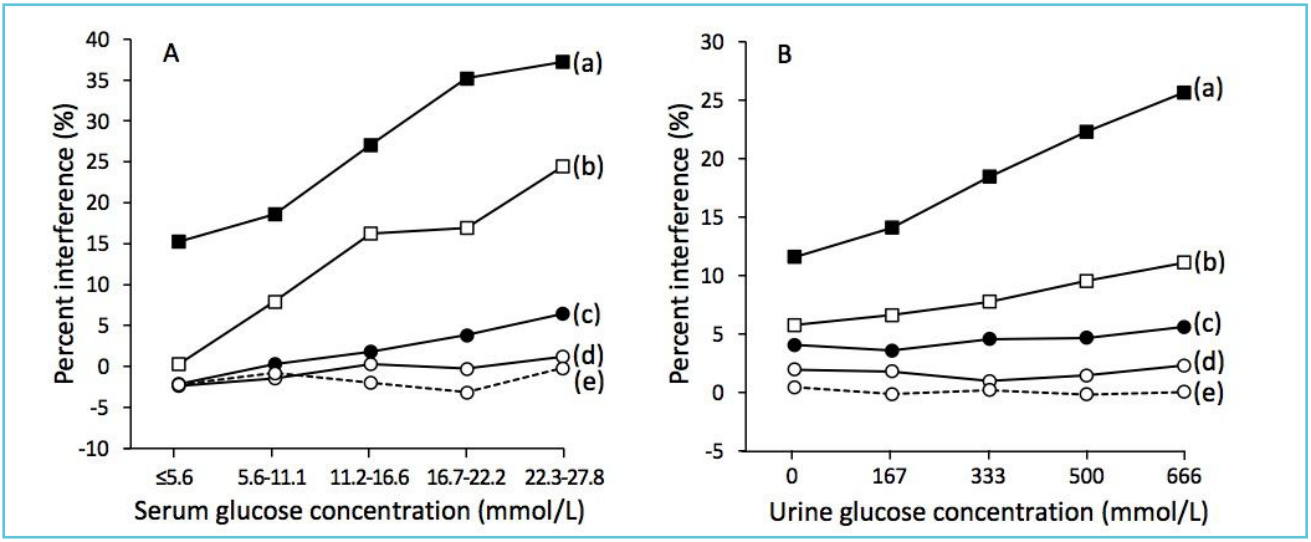
A total of 148 serum samples and 335 urine samples were analyzed. The glucose and creatinine concentrations of tested specimens in different groups are shown in Table 1. Table 2 and Figure 1A show the percent interference of glucose on serum creatinine measurement by the Jaffe kinetic method according to concentrations of creatinine and glucose. The percent interference was highest in the samples with creatinine of less than 45 µmol/L. The degree of interference was gradually decreased with increasing creatinine concentration. No glucose interference was found at serum creatinine of more than 177 µmol/L. At serum creatinine concentrations of less than 45 µmol/L, all glucose levels even less than 5.6 mmol/L interfered creatinine measurement by the Jaffe kinetic method. At creatinine concentrations of 45-88 µmol/L, glucose interference of creatinine measurement was found when glucose levels were 5.6 mmol/L or more. The effect of glucose interference with Jaffe creatinine measurement progressively increased with increasing glucose levels. As in serum specimens, the interference effects of glucose on Jaffe creatinine measurement in urine specimens showed a similar pattern (Table 3 and Figure 1B). At urine creatinine concentrations of less than 1,770 µmol/L, all glucose levels even no glucose interfered creatinine measurement by the Jaffe kinetic method. The degree of interference gradually decreased with increasing creatinine concentrations. At urine creatinine of 1,770-3,540 µmol/L, significant percent interference was found when glucose levels were 666 mmol/L. No glucose interference was found at urine creatinine of more than 3,540 µmol/L. The effect of glucose interference with Jaffe creatinine measurement progressively increased with increasing glucose levels. Although there was no glucose interference at creatinine concentration of 3,541-5,300

Table 1 Median (range) of glucose and creatinine concentrations of tested specimens in different groups

Group	Serum glucose mmol/L	Serum creatinine [a] µmol/L	Urine glucose mmol/L	Urine creatinine [a] µmol/L
1	4.7 (2.4-5.5)	37 (19-44)	0.2 (0-7.3)	1,266 (832-1,726)
2	7.9 (5.8-11.0)	57 (45-87)	178 (148-193)	2,545 (1,786-3,496)
3	13.9 (11.1-16.6)	135 (92-175)	350 (309-382)	4,517 (3,566-5,269)
4	18.9 (16.9-21.8)	220 (178-255)	527 (483-562)	6,752 (5,310-7,937)
5	24.7 (22.3-27.4)	304 (276-353)	701 (646-745)	10,096 (8,456-30,784)

[a] *Measurement by enzymatic method.*

Figure 1 Relationship between the mean percent interference of glucose on Jaffe creatinine measurement and glucose concentration



(A) in serum specimens, at 5 levels of creatinine: <45 µmol/L (a), 45-88 µmol/L (b), 89-177 µmol/L (c), 178-265 µmol/L (d), and 266-354 µmol/L (e); and

(B) in urine specimens, at 5 levels of creatinine: <1,770 µmol/L (a), 1,779-3,540 µmol/L (b), 3,541-5,300 µmol/L (c), 5,301-7,960 µmol/L (d), and >7,960 µmol/L (e).

Table 2 Mean ± standard deviation of percent interference of serum creatinine measurement by Jaffe kinetic method according to concentrations of creatinine and glucose

Serum creatinine (µmol/L)	Serum glucose (mmol/L)					*p* value (in row)
	<5.6	5.6-11.1	11.2-16.6	16.7-22.2	22.3-27.8	
<45	15.3±7.6[1,a]	18.6±8.8[1,2,a]	27.1±4.7[1,2,3,a]	35.2±11.5[2,3,a]	37.2±11.8[3,a]	0.01
45-88	0.3±1.7[1,b]	7.9±4.4[1,2,b]	16.2±4.3[2,3,b]	17.0±5.6[2,3,b]	24.4±13.5[3,a]	<0.001
89-177	-2.1±2.5[1,b]	0.3±1.8[1,2,b,c]	1.8±1.8[1,2,3,c]	3.8±5.4[2,3,c]	6.4±2.6[3,b]	0.001
178-265	-2.3±1.1[b]	-1.4±3.4[c]	0.3±2.2[c]	-0.3±1.4[c]	1.2±2.9[b]	0.212
266-354	-2.2±2.4[b]	-0.8±0.8[c]	-2.0±2.1[c]	-3.2±1.6[c]	-0.2±1.6[b]	0.112
p value (in column)	0.003	<0.001	<0.001	<0.001	<0.001	

Superscript numbers indicated intergroup Bonferroni post hoc comparisons in the same row. Values within rows not having a superscript number in common differ significantly (effective p < 0.05).
Superscript letters indicated intergroup Bonferroni post hoc comparisons in the same column. Values within columns not having a superscript letter in common differ significantly (effective p < 0.05).

Table 3 Mean ± standard deviation of percent interference of urine creatinine measurement by Jaffe kinetic method according to concentrations of creatinine and glucose

Urine creatinine (µmol/L)	Urine glucose (mmol/L)					*p* value (in row)
	0	167	333	500	666	
<1,770	11.6±4.3[1,a]	14.1±3.8[1,2,a]	18.4±5.8[2,3,a]	22.3±7.4[3,4,a]	25.6±8.5[4,a]	<0.001
1,770-3,540	5.8±3.6[1,b]	6.6±3.5[1,b]	7.7±4.2[1,2,b]	9.5±4.1[1,2,b]	11.1±4.7[2,b]	0.007
3,541-5,300	4.1±2.1[b,c]	3.6±2.1[b,c]	4.6±1.8[b,c]	4.7±2.3[c]	5.6±2.0[c]	0.192
5,301-7,960	2.0±1.9[c,d]	1.8±2.7[c,d]	1.0±1.4[c,d]	1.5±1.3[c,d]	2.3±1.8[c,d]	0.573
>7,960	0.5±1.7[d]	-0.1±13[d]	0.2±1.7[d]	-0.2±2.0[d]	0.1±1.5[d]	0.641
p value (in column)	<0.001	<0.001	<0.001	<0.001	<0.001	

Superscript numbers indicated intergroup Bonferroni post hoc comparisons in the same row. Values within rows not having a superscript number in common differ significantly (effective p < 0.05).
Superscript letters indicated intergroup Bonferroni post hoc comparisons in the same column. Values within columns not having a superscript letter in common differ significantly (effective p < 0.05).

µmol/L, the percent interference was small but significantly higher when compared with creatinine concentration of more than 7,960 µmol/L at all glucose levels.

DISCUSSION

The difference between serum and urine creatinine measurement by automated analyzers is due to the dilution process, which is required only for urine specimens. When creatinine concentration and specimen dilution were taken into account, this study found that no matter whether in serum or in urine specimens, the effects of glucose interference on Jaffe creatinine measurement were similar. In serum, glucose interference on Jaffe creatinine measurement was clearly found if creatinine concentrations were 88 µmol/L or less, which corresponds to less than 1,770 µmol/L in urine samples prior to 1:20 dilution. The effect of glucose interference was less in serum creatinine concentrations of 89-177 µmol/L, which corresponds to 1,770-3,540 µmol/L in urine specimens prior to 1:20 dilution.

The degree of interference was greater when glucose concentration was higher. The glucose interference effect was abolished when serum creatinine concentrations were more than 177 µmol/L, which corresponds to more than 3,540 µmol/L in urine samples prior 1:20 dilution. As with creatinine concentration, extremely high glucose concentrations in undiluted urine

specimens became similar to serum glucose concentrations after being 20 times diluted.

The glucose interference with Jaffe creatinine measurement in serum specimens were found consistently among studies [1-6]. The possible reason for this is that most of the serum creatinine concentrations in those studies were less than 177 µmol/L. Similar to previous studies [1,9], our study found that the higher the creatinine concentration, the less the glucose interference. An explanation is that glucose and creatinine react with alkaline picrate in Jaffe's reaction in a competitive manner. The high creatinine concentration would reduce the formation of glucose-picrate complexes which cause falsely high creatinine results. Interestingly, our study found that the interference with the Jaffe kinetic method at creatinine concentrations of less than 45 µmol/L was also found even when the glucose concentration was in normal range. Practically, serum creatinine in normal range determined by Jaffe kinetic method should therefore be interpreted with caution particularly in those with hyperglycemia as well as in those with low muscle mass, cirrhosis or malnutrition whose serum creatinine concentrations are usually low. However, Jaffe kinetic method is still a reliable tool for measuring creatinine at high concentrations without glucose interference.

There was only one study performed, by Watts and Pillay, that investigated the effect of glucose interference on Jaffe creatinine measurement in urine [10]. They studied the effects of glucose concentrations ranging from 5-320 mmol/L on 3 levels of creatinine concentrations (~2,000, ~10,000, and ~25,000 µmol/L) and found that glucose did not significantly influence the measurement of urine creatinine by Jaffe kinetic methods. Unfortunately, this study did not give the details of sample dilution during the process of creatinine measurement. If the urine samples were diluted 1:20 as in our study, their results were compatible with ours which showed that at urine creatinine concentration of 1,770-3,540 µmol/L, glucose concentrations of 500 mmol/L or less did not interfere with Jaffe creatinine measurement while the significant interferences were found at glucose concentration of 666 mmol/L. In addition, no interference effect of glucose was found at urine creatinine concentrations of more than 3,540 µmol/L.

Since serum creatinine concentrations in the general population are low, automated analyzers are designed to measure serum creatinine without dilution. In contrast, urine creatinine concentration is naturally much higher than serum creatinine concentration, therefore automated analyzers are programmed to dilute urine specimens 1:20 or 1:25 before analysis. Apart from creatinine, glucose and other substances in urine specimens that interfere with Jaffe creatinine measurement are also diluted. The dilution process can therefore explain the discrepancy of glucose interference with Jaffe creatinine measurement between in serum and in urine specimens which have similar glucose concentrations.

Although the dilution process can decrease or get rid of glucose interference with urine creatinine measurement by the Jaffe kinetic method, interpretation should be made with caution in patients receiving SGLT2 inhibitors. This class of drugs could increase urine glucose excretion up to 100 g/day [11]. After 1:20 dilution, these extremely high levels may remain high enough to interfere with Jaffe creatinine measurement, particularly in patients with urine creatinine concentration of 3,540 µmol/L or lower. Attenuation of albuminuria expressed as urine albumin to creatinine ratio in these patients may be overestimated.

This study had some limitations. As isotope-dilution mass spectrometry (IDMS) method was not available, the enzymatic method was used

as reference for studying the effect of glucose interference on Jaffe creatinine measurement. When compared with IDMS method, the enzymatic method has been found to show variation at a low creatinine concentration, which might affect the results of this study [12]. Extremely high glucose concentrations in urine samples were artificially created by adding glucose powder which might be different from urine samples collected from patients receiving SGLT2 inhibitors in terms of other interference substances. To confirm the effects of glucose interference on Jaffe creatinine measurement in urine, studies using samples collected from those receiving SGLT2 inhibitors are needed.

In conclusion, when creatinine concentration and specimen dilution were taken into account, the effects of glucose interference on Jaffe creatinine measurement were similar either in both serum and urine specimens, and was found when creatinine concentrations in serum or diluted urine were 177 µmol/L or less.

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Declaration of competing interests

All authors declare no conflicts of interest.

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Author contributions

PeC and SS designed the study, analyzed data, and wrote the manuscript. NB, PA, PhC, and YY were involved with sample collection and laboratory measurements. AB contributed to sample preparation. WS checked and approved the final data.

All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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A clinical laboratory study of a non-classical case of celiac disease: how to anticipate the diagnosis

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ABSTRACT

Celiac disease (CD) is a systemic autoimmune pathological condition caused by the intake of gluten in genetically predisposed individuals. Despite its wide prevalence, it remains an underdiagnosed disease since a large percentage of individuals who suffer from the condition do not have the classic symptoms described for the disease.

We present the case of a 43-year-old man with severe iron deficiency and asthenia. We found high levels of anti-transglutaminase and anti-endomysium antibodies, a severe intraepithelial lymphocytosis, 3A Marsh-Oberhuber classification upon gastroscopy and the presence of HLA-DQ2 and HLA-DQ8 heterodimers.

The patient was diagnosed with CD and was placed on a gluten-free diet. After 19 months, an improvement in biomarkers of CD and other biochemical parameters was observed.

A delay in the diagnosis of CD can produce nutritional deficiencies, such as iron deficiency which may not improve even with oral iron treatment. In similar clinical presentation, the laboratory can advance a diagnosis of CD.

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INTRODUCTION

Celiac disease (CD) is immune-mediated and a highly prevalent chronic enteropathy caused by the ingestion of gluten in genetically susceptible individuals. It can be present at any age, with a peak onset of adult CD between the age of 40-60 years. Celiac disease is known to be underdiagnosed because of the heterogeneous presentation of clinical signs and symptoms. Steatorrhea, weight loss, anemia, hypo-proteinemia and electrolyte imbalance are known classic symptoms and usually trigger diagnostic work-up, but patients with less common presentations are often not screened for CD. The incidence of CD varies geographically, and appears to be increasing over time in several regions of the world. Its prevalence in Europe is 1% in both children and adults. Despite the growing recognition of CD, many cases remain undiagnosed. A gluten-free diet (GFD) is currently the only effective treatment for CD [1-3].

CLINICAL-DIAGNOSTIC CASE

We present the case of a 43 years old male with a 3-month history of asthenia, with no diarrhea or abdominal pain. No familial or personal history of interest were presented by the patient.

The first analysis showed a marked deficiency in serum ferritin and iron, as well as low hemoglobin (Hb) concentration, red blood cell (RBC) count and vitamin B12. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values were within the reference range. No other alterations in hematological or biochemical parameters were found.

Possible causes of iron deficiency were ruled out and oral iron was prescribed to the patient. Intramuscular B12 vitamin treatment was prescribed too. Two months later a follow-up blood test was performed, where it was observed that ferritin levels were still at levels of <8 µg/L. After ruling out poor compliance with treatment, the study of CD biomarkers was initiated. The diagnostic work-up was according to the guidelines of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [7]. We determined immunoglobulin A (IgA) by nephelometry (Immage analyzer, Beckman-Coulter), and transglutaminase antibodies IgA (IgA tTg) (Elia Celikey IgA with Phadia-250 analyzer, Thermo Fisher) and IgA antibodies against endomysium (monkey esophagus sections) (EmA) (BioSystems) using indirect immunofluorescence (IIF). The results of the different analyses over time are shown in Table 1. The results of the EmA examination are illustrated in Figure 1.

For the gastroscopy examination, the patient was instructed not to be on a gluten restricted diet. During the examination, portions of the esophagus, stomach and duodenum were studied, the report enumerated a mucosa with normal characteristics, without apparent signs of atrophy, with vascularization and normal appearing villi. Three biopsies of the second duodenal portion and two fragments of the bulb were performed. Hematoxylin-eosin (HE) staining of the biopsy specimens showed severe intraepithelial lymphocytosis (> 30 intraepithelial lymphocytes per 100 enterocytes), crypt hyperplasia and mild villous atrophy (Figure 2). The patient was classified as having grade 3A CD according to the modified Marsh-Oberhuber histologic classification [7].

HLA DQ determination was also performed. Histocompatibility antigens were studied by sequence-specific oligonucleotide (SSO) PCR technique of HLA-DQA1 and HLA-DQB1 loci with Luminex technology.

The results were: HLA-DQA1 locus: DQA1 * 03, DQA1 * 05 and HLA-DQB1: DQB1 * 02 and DQB1 * 03. These results confirmed the presence of the CD risk factor, i.e., heterodimers DQ2 and DQ8.

DISCUSSION

According to population screening studies, the true prevalence of CD is greatly underestimated [3]. The reason for the underestimation could be that only a small portion of people affected by CD show the classical signs of the disease, while the majority have the asymptomatic form. Thus, the variability of clinical symptoms of this disease makes its diagnosis difficult [4].

Table 1 Results of the 6 serial analyses performed in our laboratory [a, b]

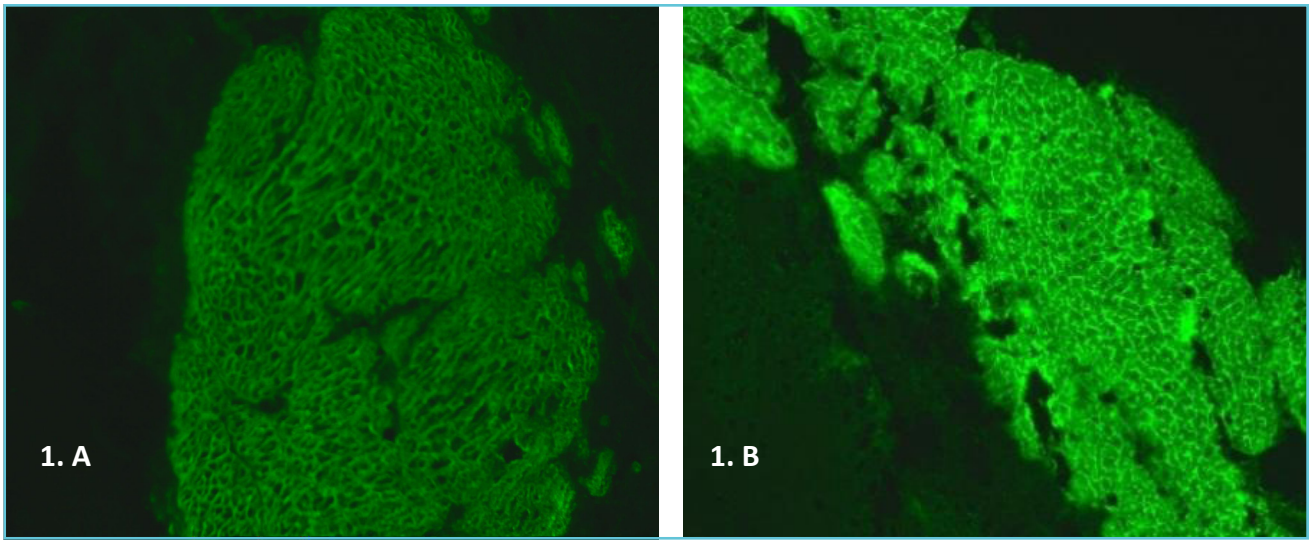
Laboratory tests (normal range)	Nov 19, 2019	Jan 31, 2020	Feb 21, 2020	Dec 30, 2020	Jun 11, 2021	Sep 27, 2021
Haemoglobin (13,5-18 g/dL)	13,6	13,1	14,4	16	15,3	15,6
Red blood cells (4.7-6.0 x10*12/L)	3,81	3,93	4,35	4,61	4,58	4,59
Mean Corpuscular Volume (MCV) (78-100 fL)	103	98	98	97,7	97,1	98,1
Mean Corpuscular Haemoglobin (MCH) (27-31 pg)	35,8	33,2	33,2	34,7	33,5	34
Mean corpuscular haemoglobin concentration (MCHC) (32-36 g/dL)	34,7	33,7	33,9	35,5	34,5	34,7
Red cell distribution wide (RDW) (11,5-14 %)	15,7	13,8	13,6	13,4	13,1	13,4
Ferritin (20.0-250.0 µg/L)	8	<8	<8	102	141	141
Iron (70.0-180.0 µg/dL)	49	60	70	56	105	90
Transferrin saturation index (25-50 %)	NP	16,7	18,1	16,2	34,8	29,5
B12 Vitamin (180.0-914.0 pg/mL)	105	117	121	329	237	216

Aspartate Aminotranferase (5.0-35.0 U/L)	NP	61	49	37	28	27
Inmunoglobulin IgG (800.0-1400.0 mg/dL)	NP	NP	1320	NP	NP	NP
Inmunoglobulin IgA (100.0-300.0 mg/dL)	NP	NP	398	NP	NP	NP
Transglutaminase antibodies IgA (<7 U/mL)	NP	NP	**186**	19	9,7	6,6
IgA antibodies endomysium (EmA)	NP	NP	**POSITIVE**	NP	NP	NP

[a] *The first (November 19, 2019) and second (January 31, 2020) tests showed severe iron deficiency, vitamin B12 values below the population reference values, and elevated AST, with normal values of MCV and MCH. The second analysis was performed after supplementation with oral iron and intramuscular vitamin B12 after 2 months. The third (February 21, 2020) examination was requested in the following month, where the requisition was extended to the serological study of celiac disease (CD) biomarkers, the result of which confirmed the diagnosis. The last three tests (December 30, 2020; June 11, 2021 and September 27, 2021) were performed after the diagnosis of CD and gluten-free diet, where a decrease in transglutaminase IgA antibody titers can be observed until normalization, as well as AST, vitamin B12, ferritin, serum iron, erythrocytes and hemoglobin.*

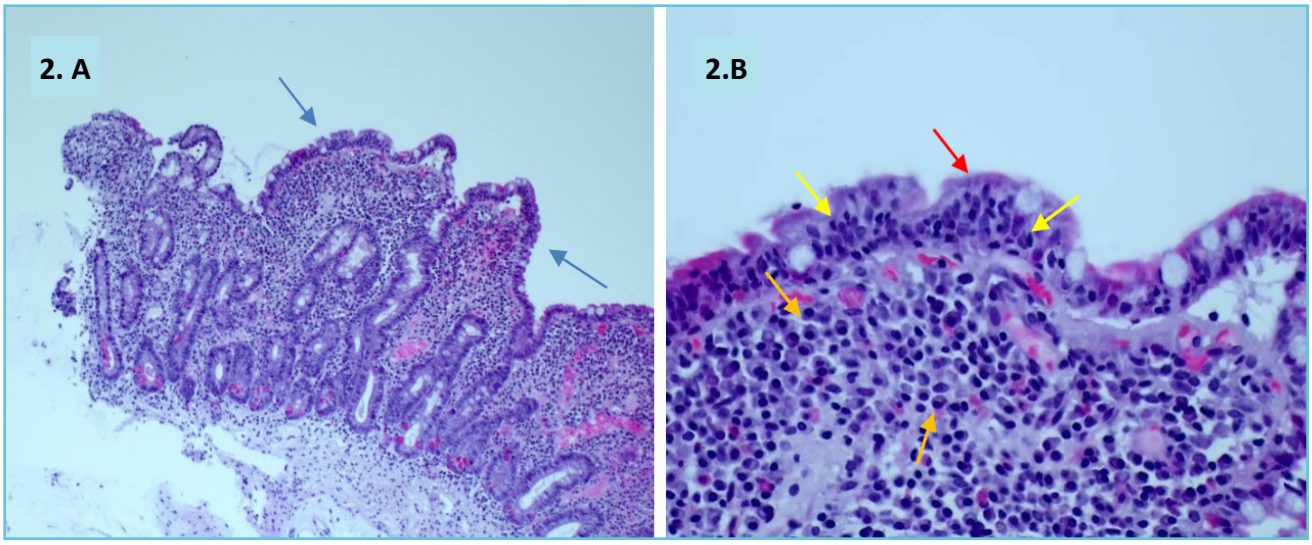
[b] *NP: not performed.*

Figure 1 **Figure 1.A (Positive control) and 1.B (patient's serum) show the results of the IIF study under fluorescence microscopy***



* *The images were obtained after a 1/20 dilution of the patient's serum and were observed at 400. In both, we can see the network-like fluorescence labeling of the layer surrounding the smooth muscle fibers of the monkey esophagus with the large fluorescence emission of **1.B** image compared to that produced in the positive control used in the technique, depicted in Figure **1.A**.*

Figure 2 **Low magnification image (100X) of the duodenum's second portion shows mucosa and submucosa***



** An increased number of intraepithelial lymphocytes and mild villous atrophy (blue arrows) (HE) can be seen (**Fig. 2.A**). Higher magnification image (200X) showing the damaged surface of the epithelium (red arrow), with numerous intraepithelial lymphocytes (yellow arrows) and an increase in plasma cells in the lamina propria (orange arrows) (HE) (**Fig. 2.B**).*

A delay in the diagnosis of CD may account for cases of adult patients manifesting severe nutritional deficiencies and iron malabsorption anemia, which is the most common extraintestinal subclinical manifestation in CD, even in patients who do not have duodenal villous atrophy [5]. Oral iron therapy may be ineffective, leading to chronic iron deficiency anemia [6, 7].

Such is the importance of iron deficiency anemia in these cases that the British Society of Gastroenterology guidelines recommend a CD work-up in every patient with iron deficiency anemia [8].

In our case, the extraintestinal manifestations (asthenia) and several altered biomarkers in the analytical tests (iron deficiency, low vitamin B12 and elevated liver enzyme levels), were the prelude to suspect possible CD [9]. In addition, the patient did not respond to oral iron therapy before the diagnostic work-up for CD.

The importance of early diagnosis is illustrated by reduction in the risk of morbidity and mortality due to complications associated with CD, such as bone abnormalities (osteopenia and osteoporosis), liver damage, anemias, neurological manifestations (neuropathies and headaches, among others), other autoimmune diseases and malignancies [10]. The prevalence of these complications is related to age and duration of gluten exposure [11].

Our patient did not present with clinically evident symptoms of CD, nonetheless, we considered initiating the study of serological markers for CD, based on similar cases in the literature [12].

In addition to the above, the examination of the alleles encoding CD risk molecules helped determine the patient's HLA status and initiate tests in first-degree relatives [13].

In conclusion, the determination of the serological markers studied by the laboratory were

particularly useful in the diagnosis of our patient [14, 15].

An early diagnosis can avoid serious complications and we recommend screening for CD in patients with findings similar to our case.

LEARNING POINTS

- CD is underdiagnosed because it presents a wide spectrum of associated symptomatology (classical and non-classical symptoms).
- The clinical laboratory can perform serological determinations of CD biomarkers to advance the diagnosis of the disease.
- In cases of patients with abnormally low ferritin levels, without an apparent cause who do not respond to oral iron therapy, it is recommended to rule out the presence of CD.
- A delay in the diagnosis of CD can lead to severe nutritional deficiencies and chronic iron deficiency anemia.

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In *silico* Copy Number Variation (CNVs) bioinformatics estimation: dream or nightmare?

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LETTER TO THE EDITOR

Decades before the availability of next generation sequencing (NGS) technology, definition of copy number variations (CNVs) in human genetics were mainly rare changes in the quantity and structure of chromosomes. These included aneuploidies and rearrangements (1, 2, 3). Subsequently, with the advent of molecular technology, smaller and more abundant alterations were observed, including, various repetitive elements that involve short DNA sequences (micro and mini-satellites), insertions, deletions and duplications (4).

Targeted next-generation sequencing (NGS) is an established, but not the only, method for the detection of germline variants in cancer predisposition genes. While variants involving a few nucleotides, i.e., single-nucleotide variants (SNVs) and short insertion/deletion events (indels), can be detected accurately, the identification of larger genomic rearrangements (copy number variations (CNVs)) remains a challenge.

At present time, CNVs is considered a segment of DNA that is present at a variable copy number in comparison with a reference genome. They can derive from duplications, deletions, insertions and even translocations, and can vary in length, may be short or include thousands of bases (5, 6, 7, 8); for this research, it could be more adequate an average size of ~100 bp MLPA resolution level, as a parameter for defining CNV length. Several *in silico* tools have been developed to predict CNVs using targeted NGS data. However, several studies suggested that existing tools for CNV detection using targeted NGS data show limited accuracy and robustness (9, 10, 11, 12).

We investigated the performances of *in silico* CNV commercial prediction tool Celemics CNV Analysis Algorithm® in 13 cancer predisposition genes: *APC* (NM_000038.6), *ATM* (NM_000051.4), *BRCA1* (NM_007294.4), *BRCA2* (NM_000059.4), *CDH1* (NM_004360.5), *CHEK2* (NM_007194.4), *EPCAM* (NM_002354.3), *MLH1* (NM_000249.4), *MSH2* (NM_002878.4), *MSH6* (NM_000179.3), *MUTYH* (NM_001048174.2), *PALB2* (NM_024675.4), and *STK11* (NM_000546.5), evaluated in 80 patients with hereditary cancer syndrome, for those of who had the results by multiplex ligation-dependent probe amplification (MLPA) as the assay for the variation in copy number.

In this analysis, the algorithm predicted 8 CNVs, of which 1 (12.5%) it was a real CNV (exons 1 to 7 in *MSH2* gene). The remaining 7 (87.5%) were false positive (were not detected by MLPA). False positive predictions affected target genes: *APC* (figure 1)*, BRCA1, BRCA2, CDH1, MSH2* and *PALB2* without a clear predisposition for a gene o region.

The overall real CNV prevalence was 6.25% (5/80) (*MSH2* (n=3), *APC* (n=1) and *EPCAM* (n=1). Of these, 4 true positive CNVs were none predicted by CNV analysis algorithm.

As other *in silico* CNV prediction tools, the Celemics CNV algorithm uses read depth-based approaches. CNV is based on the hypothesis that a CNV determines the relative read depth per target region. Thus, low or high fluctuating read depths of a target region will likely affect accurate CNV prediction.

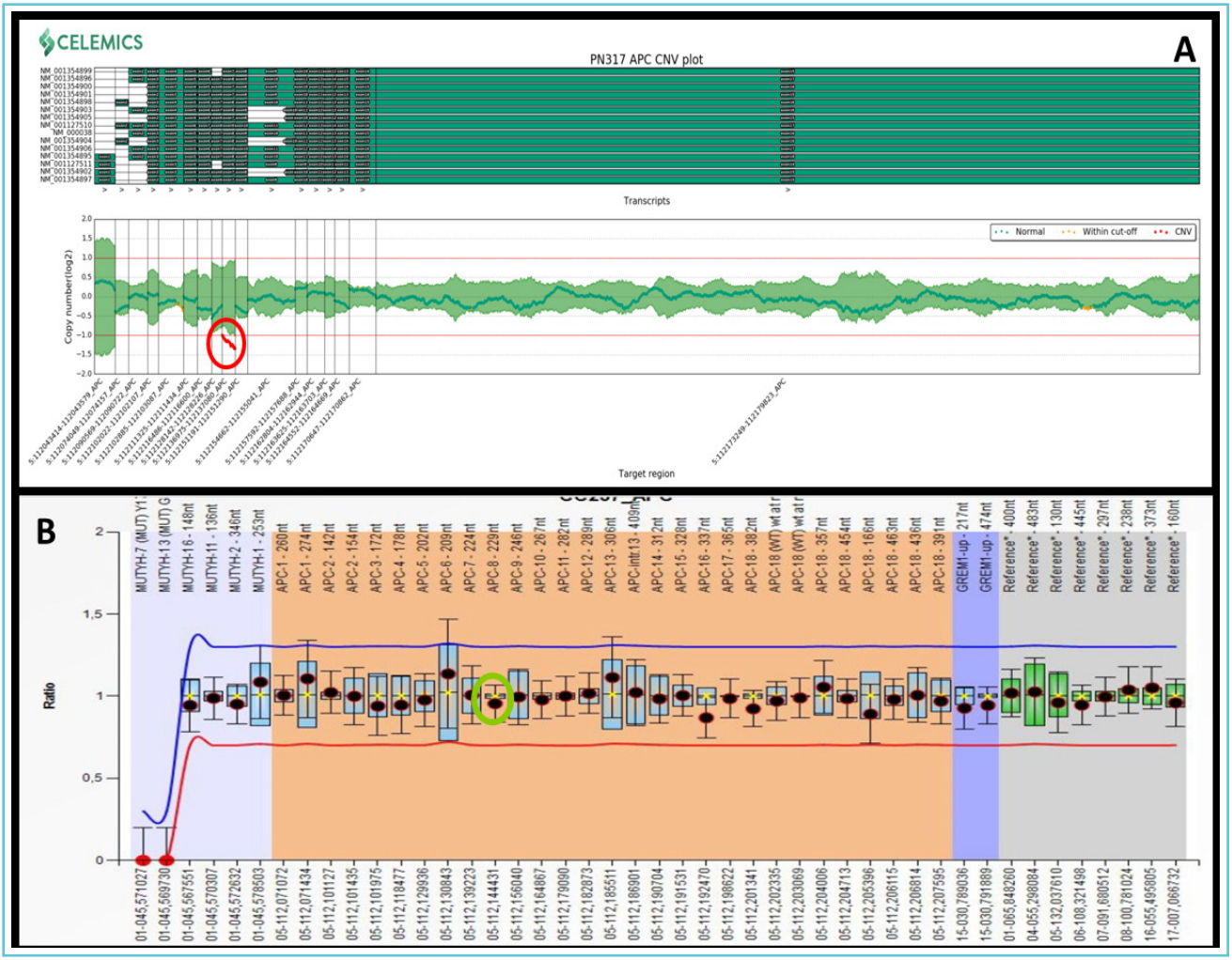
In this scenario, the probability of CNV analysis algorithm prediction representing a true positive CNV, its positive predictive value (PPV), was 12.5% (1/8). Although the series analysed is small, this value represents an important limitation to use the bioinformatics' estimation of CNV as the only analysis tool.

Comparing our data with those by Lepkes et al (N= 4208), we found that, the PPV values can vary greatly on the basis of different calculation algorithms. In their analysis, Lepkes et al. compared four bioinformatics calculation algorithms (the commercial tool incorporated in the CE-IVD-marked Sophia Genetics DDM pipeline®, and three publicly available tools, ExomeDepth, GATK gCNV and panelcn.MOPS) and established that the PPV values of these bioinformatics tools can vary between 7% to 68%, showing that there may be a great difference between the values of CNVs predicted by algorithms and their real existence (13).

The most relevant hypothesis at present explaining the great differences found between predicted and real CNVs strongly suggest that target region sequencing coverage along with target region characteristics, such as GC content, length, low sequencing coverage, determined the accumulation of false positive CNV predictions (13, 14, 15).

Future directions are strongly orientated to improve the use of CNVs NGS-derived information. However, verification of *in silico* predicted CNVs is required due to its high frequencies of false positive predictions.

Figure 2 **Graphic representations of the calculation of a deletion (*in silico*) and MLPA analysis (*in vitro*) for exon 8 in *APC* gene***



** A) Graphic representation of the calculation of a deletion (in silico predicted CNV, red circle) in exon 8 of the APC gene. B) Graphic representation of the exon analysis of the APC gene by MLPA (in vitro) showing the absence of a deletion in exon 8 (green circle).*

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Competing interests

Authors state no conflict of interest.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Dasatinib-induced chylothorax: a clinical laboratory's perspective

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ABSTRACT

Dasatinib is a tyrosine kinase inhibitor drug used for chronic myeloid leukaemia (CML) treatment. Chylothorax has been rarely reported as a secondary effect of dasatinib occurring especially in long-term treated patients, although its pathophysiology is not yet fully understood. Laboratory analysis of the pleural effusion is crucial for chylothorax diagnosis. We report a case of a 53-year-old male patient presenting a chylothorax after 14 years of dasatinib therapy where the clinical laboratory was key in the diagnosis.

INTRODUCTION

Dasatinib is a second-generation tyrosine kinase inhibitor drug used for chronic myeloid leukaemia (CML) treatment. It is commonly prescribed to adult patients with the following types of CML: newly diagnosed patients who are 'Philadelphia chromosome positive (Ph+)', 'accelerated' and 'blast' phases when other treatments do not achieve remission. In addition, it is prescribed in Ph+ acute lymphoblastic leukaemia (ALL) or 'lymphoid blast' crisis when patients do not tolerate previous treatments (1,2).

The most common side effects of dasatinib therapy are skin rash, dyspnoea, abdominal pain, pancytopenia, hypertension, bleeding events that may require dose interruption or transfusion, and liquid retention including pleural effusion (1).

Pleural effusion occurs in as many as 28-33% of long-term treatments (3). However, pleural effusion in the form of chylothorax has rarely been reported and its pathophysiology is not fully understood (4,5).

We describe a case of a patient who presented with chylothorax after being treated with dasatinib for 14 years.

CLINICAL-DIAGNOSTIC CASE

A 53-year-old male with a 16-year history of CML and ongoing dasatinib treatment for 14 years presented to the emergency department with symptoms of fever, dyspnea, and abdominal pain. Physical examination revealed a blood oxygen saturation level of 95% and chest radiography showed pleural effusion on the left lung (Figure 1). Consequently, a chest ultrasound-guided left thoracentesis was performed for evacuation and diagnosis. The extracted fluid exhibited a turbid and milky white appearance. Biochemical analysis using AU5800 (Beckman Coulter®) revealed a total protein concentration of 50 g/L (<30 g/L is suggestive of transudate), adenosine deaminase activity of 22 U/L (>45 U/L is suggestive of tuberculosis), cholesterol concentration of 1.29 mmol/L, and triglyceride concentration of 6.58 mmol/L (>1.25 mmol/L is suggestive of chylothorax) (Table 1).

Table 1 Results of biochemical analysis of pleural fluid by AU5800 and automatized cytological analysis by Sysmex XN-1000

Biochemistry		Cytology	
Glucose	6.72 mmol/L	Cells	3.45 x10^3 cell/L
Protein	50 g/L	Erythrocytes	2x10^5 cell/L
Lactate dehydrogenase	142 U/L	Lymphocytes	75 %
Cholesterol	1.29 mmol/L	Mesothelium cells	0 %
Triglycerides	6.58 mmol/L	Macrophages	21 %
Adenosine deaminase	22 U/L	Neutrophils	3 %

Automated cytological analysis using Sysmex XN-1000 revealed 3.45x103 cells/L and 2x105 erythrocytes/L. Cytocentrifugation, staining with May Grünwald-Giemsa and microscopic observation showed 75% mature lymphocytes, 21% macrophages and 3% neutrophils (Figure 2). Microbiological cultures, pathological anatomy, and immunophenotype studies yielded negative results.

The elevated triglyceride concentration (Table 1) and mature lymphocyte predominance (Figure 2) suggested the presence of chylothorax, which may result from trauma, surgery, infection, or malignancy; however, these causes were not apparent in this case. Although rare, the treatment with kinase inhibitors is a possible cause of chylothorax. Therefore, a drug-related chylothorax was suspected, and the patient's dasatinib treatment was discontinued, resulting in clinical improvement. After draining the pleural effusion, treatment with octreotide, an analogue of somatostatin that inhibits digestive secretions and reduces lymphatic flow, was prescribed to prevent chylothorax.

DISCUSSION

Chylothorax is a rare condition that results from damage to the thoracic duct, leading to leakage of chyle from the lymphatic system into the pleural space (6). The diagnostic test for chylothorax involves the analysis of pleural fluid obtained by thoracentesis. Macroscopically, the fluid appears milky due to the high content of chylomicrons. However, this appearance is not specific to chylothorax, and a differential diagnosis is needed to rule out empyema and pseudochylothorax, which are cholesterol-rich pleural effusions commonly associated with chronic

Figure 1 Thorax radiography performed as the patient arrived at the emergency room. The patient showed pleural effusion on the left lung

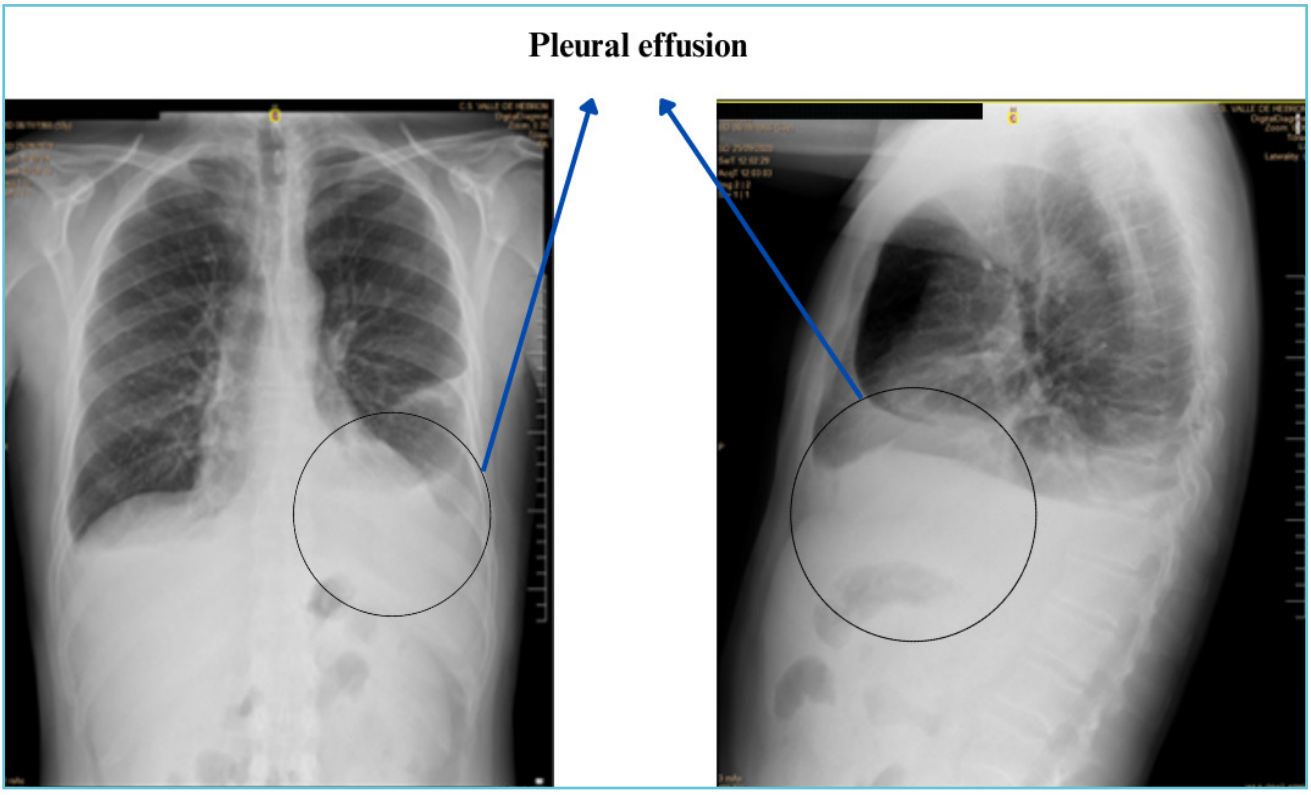
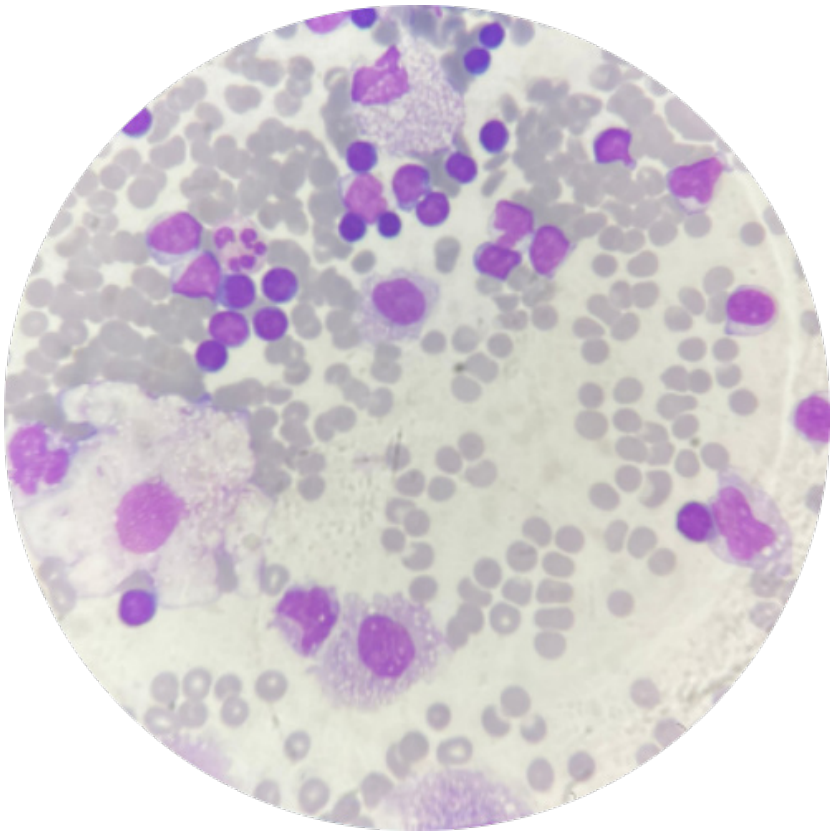


Figure 2 Microscopic image of a cytological extension of pleural effusions stained with May Grünwald-Giemsa (100x)



inflammatory disorders. The typical cytology observed in chylothorax is a predominance of mature lymphocytes. In 1980, Staats and collaborators introduced criteria for the biochemical diagnosis of chylothorax, which is defined by the presence of chylomicrons in pleural fluid and is strongly suggested by a triglyceride concentration >1.25 mmol/L. When triglyceride levels are between 0.57-1.25 mmol/L, an electrophoresis of pleural fluid lipoprotein should be performed to detect chylomicrons (8).

Dasatinib exerts its mechanism of action by inhibiting tyrosine kinases, particularly the ABL family, platelet-derived growth factor receptor beta (PDGFR-β), KIT and Src. Although the physiopathology of chylothorax induced by dasatinib remains unclear, one possible mechanism proposed by Gorham is related to the inhibition of PDGFR-β (9). This receptor plays a role in the regulation of lymphangiogenesis, and its inhibition leads to the formation of abnormal lymphatic vessels and leakage into the pleural space. Another proposed mechanism is linked to the inhibition of Src kinase, whose activity is involved in the regulation of vascular permeability and stability of the pleural epithelium (4, 10).

In conclusion, chylothorax is a rare adverse effect of long-term dasatinib treatment. Our case report highlight the decisive contribution of the biochemical analysis and cytological study of the pleural fluid for the diagnosis and treatment of chylothorax.

LEARNING POINTS

- Dasatinib, a drug used for CML treatment, can rarely cause chylothorax as secondary effect.
- Macroscopic, cytological and biochemical study of a pleural effusion is crucial for differential diagnosis of chylothorax, pseudochylothorax and empyema.
- High triglyceride concentration together with low cholesterol concentration and a high proportion of matures lymphocytes support the diagnosis of chylothorax.

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Scientific journals should encourage, not hinder, debates about their published papers

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LETTER TO THE EDITOR

The revolution in electronic publishing now allows for papers to be continuously critiqued through letters to the editor, online comments, tweets and other means. However, established top-ranked journals still pose serious barriers regarding cultivation, documentation and dissemination of post publication critiques (1). To improve on this situation, Hardwicke et al. published a set of rules, one being for journals to actively encourage and highlight post publication critique to their readership. In this commentary, I present a case whereby the editors of a top ranked journal hindered the discussion/debate between authors and expert readers. Highlighting and publishing such cases will likely put pressure on journals to modify their current policies and actively encourage post publication review. Like Hardwicke et al., we believe

that post publication review is a major vehicle for advancing and accelerating science, by encouraging debates, resolving disagreements and revealing flaws in already published (and in many cases seemingly high-impact) papers.

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INTRODUCTION

A recent paper (1) documented that only a small fraction of the 330 top-ranked scientific journals published critiques of their published papers. Undoubtedly, the minuscule number of post-publication critiques slows the advancement of science, by not actively engaging the readers and by not promoting healthy scientific debates. Those who want to raise concerns have very limited fora to express their opinions. Many journals publish "letters to the editor" to address reader's comments and to give the opportunity for authors to respond. But not all journals encourage such policies, many have numerous restrictions and a few of them are unwilling to publish critiques, presumably for non-scientific reasons (see below). In general (1), published critiques are a rare, with only 2% of published papers being linked to a comment, but admittedly, this percentage differs between disciplines.

I here describe a case, with the hope of helping to catalyze changes, and put some pressure on journals to follow the Hardwicke et.al. recommendations (1), which I wholeheartedly sponsor. My vision is that debates can contribute decisively to scientific progress and should be encouraged.

CASE REPORT

In 't Veld et al (2) presented in a top-ranked journal a method for cancer detection by using transcripts isolated from platelets exposed to cancer tissues. Since this paper was societally consequential, public media invited me, as an expert, to comment on the validity and applicability of these findings in clinical practice. In parallel, I prepared a critique, indicating that it is unlikely for the described method to have value for early cancer detection (3). I used the sensitivity and specificity of the test, as mentioned by the authors, to calculate the positive predictive value (PPV) (the positive predictive value represents the chances of someone having cancer if the test is positive). The pretest probability of somebody having cancer was about 1% (which is equal to the prevalence of cancer in the screened population). I calculated that if the test was positive, the PPV was only modestly increased to about 3%, making the test unsuitable for practical applications

The finding of low PPV in cancer and other disease screening is a common deficiency (3). I also remind that 7 years ago, I drew attention that the same technology, may not be promising for cancer detection, for similar reasons (4). The mere fact that this technology did not as yet advance to the clinic, after an almost a decade, confirms that likely, it has important limitations.

I submitted my critique as a "letter to the editor", carefully avoiding offensive language. The Editor-in-Chief (EIC) indicated that they discussed my letter and decided not to publish it. The Editor did not mention any specific deficiencies of my letter and did not question my PPV calculations, which, as mentioned, were based on the author's data. I protested the decision and asked her to reconsider, or provide specifics as to why the letter was not acceptable (such as if it had calculation or other errors, offensive statements etc.). I also invited the EIC to review my letter externally, so that she formulates a better opinion. I did indicate that journals have an obligation to publish critiques of papers published, for the purpose of advancing science, finding the actual truth and informing the lay audience/non-experts, about

questionable "medical breakthroughs" (a major point made also by Hardwicke et. al.et al.) (1).

The EIC replied back with a negative answer. I was not surprised since in my 30-year career in publishing, I have never seen an editor changing a decision regarding rejection (although others may have different experiences).

In short, the editor asked me to take up the issues with the authors, in private, or in public, at scientific meetings that I may or may not attend. In essence, the editor shut the door for a debate. I believe that the action was inappropriate and hindered the advancement of science through a civilized scientific dialog.

In my deliberations with the Editor, I indicated that I have 30 years experience in cancer biomarkers and during my long career, like Ioannidis, I revealed many deficiencies of numerous technologies that have been touted as "revolutionary" in the past (5-8). These include the recent Theranos scandal (7). I thought that the EIC should have an excellent chance to initiate a debate as to the validity of the proposed test, between other experts, the authors and our group. Instead, the editor decided to shut down the discussion.

What other avenues do authors like us have, in order to challenge seemingly flawed papers and protect the integrity and avoid contamination of the scientific literature? One avenue would be to publish our critique elsewhere. In such occasions, where I tried to submit critiques related to papers published in other journals, I was justifiably told by the editors of these journals, that the best forum to publish our critiques are the journals that originally published the papers. In some cases, we did manage to get our opinions published in other journals including this incidence (3), with the hope that our opposition will be documented and be visible to interested audiences in the future, through PubMed searching.

Last but not least, it is worth examining why some top-ranked journals decide to block certain scientific debates related to papers that they publish. While some letters may be inappropriate for legitimate reasons, such as conflict of interest, this is an easily addressable concern since the editors have the opportunity to review the critiques externally and then decide. However, our belief is that editors of top-ranked journals, do not like to publish debates and possible flaws in papers that they thoroughly reviewed and finally decided to publish. They are likely concerned that their journals may lose some prestige if they are proven to occasionally publish flawed science that leads to misleading press releases or to retractions. But flawed papers, sooner or later, will prove to be wrong, even if some of them reach citation classic status. One paper we challenged in the past, received more than 3,000 citations (9) before it was shown by an independent validation by the Early Detection Research Network (EDRN) investigators to be flawed by bias (10) and after the authors received (undeserved) prestigious awards and lots of related grants.

We are well aware of many papers (maybe too many!) in the biomarker field which became citation classics and were considered valid for many years, before confirmatory experiments showed that they were flawed (6). Similar experience is shared by Ioannidis (8). In another communication we suggested, like Hardwicke et al., (1) that the outcomes of scientific debates should be published, in an effort to clean the literature from misleading information (5).

I congratulate the authors (1) for an insightful study on publication practices and debates in the scientific literature. I hope that my own commentary will help convince editors to encourage comments for their papers published, even if the comments are not congratulatory for their journals. In such case, the journals may seemingly lose some prestige but in essence,

they contribute to the advancement of science by finding the truth, in the long run.

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