Practical Laboratory Medicine 17 (2019) e00134

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Effects of different tube types on patient classification using current diabetes decision limits

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ARTICLE INFO

Keywords:
Glucose
Lactate
Bias
Glycolysis
Preanalytics

ABSTRACT

Management of diabetes is a challenge starting in the pre-analytical phase with selecting the most appropriate glycolysis inhibitor. Study goal was to calculate the impact of tubes with different glycolysis inhibitors on the classification of the glycemic control of 157,415 consecutive hospital patients according to current WHO diabetes criteria.

Methods: Glucose and lactate were measured in parallel in samples from 68 healthy subjects collected and stored in different sample tubes from Sarstedt and Greiner. Bias to baseline conditions (fluoride heparin (FH) tubes, centrifugation within 1 h) was determined.

Results: In baseline samples, glucose concentration in fluoride/EDTA/citrate (FC) plasma was ~13% higher and lactate concentration ~20% lower compared to FH, fluoride oxalate, and fluoride EDTA plasma, and in serum. Glucose recovery after storage up to 48 h was 99–101% in the different tubes, but the effectiveness of glycolysis inhibition by FC was inconsistent. Based on the observed mean bias of 12% when FC tubes are used, we estimate an increase of 48.4–55.8% in the frequency of patients with impaired glucose levels using current WHO criteria.

Conclusion: Using current established decision limits, the number of patients with impaired glucose levels in the hospital would increase substantially with a strong impact on patient treatment and consumption of resources. The unpredictable failure of glycolysis inhibition in FC tubes does not allow to adjust the decision limits by a fixed factor. In the absence of prospective outcome studies with FC tubes, we recommend to measure glucose in samples containing FH.

1. Introduction

The increasing diabetes prevalence is a global health problem. More than 415 million people are living with the disease currently and a doubling is expected by 2030 [1]. In the absence of more specific biological markers to define diabetes, plasma and blood glucose testing with common accepted decision limits remains one of the basic diagnostic criteria. For diagnosing diabetes, fasting plasma glucose concentrations ≥7.00 mmol/L (126 mg/dL) or 2 h postprandial plasma glucose concentrations ≥ 11.10 mmol/L (200 mg/dL) have been established by the WHO in 1999 as decision limits [2] and are commonly accepted [3]. The range from 5.5 to 7.00 mmol/L is called “impaired fasting glucose” by the ADA [4].

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https://doi.org/10.1016/j.plabm.2019.e00134
Received 10 December 2018; Received in revised form 19 July 2019; Accepted 17 September 2019
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Tight plasma glucose monitoring is performed in many hospital patients: based on observational studies, inpatients with hyperglycemia with or without a prior diagnosis of diabetes have shown an increased risk of complications and mortality, a longer hospital stay, and a higher admission rate to the intensive care unit [5].

Preanalytical factors for glucose measurement are challenging and both the patient preparation (fasting, physical activity, smoking, and emotional stress) as well as the sample stability after blood draw before and after centrifugation can impact plasma glucose results *in-vitro* and therefore the patient classification based on glucose testing results [4].

A 5–7% decrease per hour of glucose concentration occurs in whole blood without stabilizers [6]. In 2002 the National Academy of Clinical Biochemistry (NACB) recommended to use sodium fluoride to stabilize glucose and to inhibit glycolysis [7]. Fluoride, however, only inhibits enolase, one of last enzymes involved in the complex glycolytic pathway, so inhibition of glycolysis will only be effective after approximately 2 h [8]. In 1988, Uchida proposed the acidification by citrate as an effective and fast glycolysis inhibitor acting on enzymes involved early in the glycolytic pathway [9]. NACB and WHO recommend to place blood tubes immediately after drawing into ice slurry and to separate plasma from cells within 30 min to prevent glycolysis [2,4], however this is difficult to perform under routine laboratory testing conditions and therefore not generally used [10].

Many studies have been published on the use of citrate as additive for the prevention of glycolysis. In a recent comprehensive review, the mean bias between natrium fluoride alone as glycolysis inhibitor versus additional citrate for effective rapid inhibition was ranging between 5.5 and 10.7% [10].

Goal of our study was to estimate the impact of different tube types with different glycolysis inhibitors on the classification of the glycemic control in our hospital patients. Using a sensitive approach to compare the effectiveness of glycolysis inhibition in tubes with different stabilizers, both glucose and lactate were measured in parallel at all time points from all tubes. Lactate concentrations are low in normal subjects and the *in-vitro* glycolysis of 1 mmol/L glucose will be paralleled by a 2 mmol/L increase of lactate. Any increase in lactate would indicate an insufficient inhibition of glycolysis and any decrease of glucose without increase of lactate would indicate an accumulation of intermediate products of glycolysis [11,12].

2. Material and methods

The study was performed with healthy volunteers participating in a regular health check-up at the Marienhospital in Stuttgart, Department of Occupational Health. The study protocol was approved by the Ethics Committee of the Physicians Chamber of Baden-Württemberg (F-2016-087) and written informed consent was obtained from all participants.

Venous blood samples were drawn in 3 sets of tubes from each subject. Blood from 34 volunteers were collected in Sarstedt tubes (total of 12 tubes for each volunteer for glucose and lactate testing) and samples from another 34 healthy individuals were collected in Greiner tubes (total of 15 tubes). For the individual study participants, only tubes from only one manufacturer were used.

Sarstedt tubes included in the study were S-Monovette® Clotting Activator/Serum 04.1904.100 (“serum”), S-Monovette® Fluoride Heparin 05.1076 (“FH”), S-Monovette® Fluoride EDTA 04.1918.001 “(FE”), and S-Monovette® GlucoEXACT Citrate Fluoride 05.1074.001 (“FC”). The corresponding Greiner tubes were Serum separator tube VACUETTE® TUBE 454028 (“serum”), Sodium Fluoride/Sodium Heparin VACUETTE® 454218 (“FH”), Sodium Fluoride/K3E K3EDTA VACUETTE® 454091 (“FE”), Sodium Fluoride/Potassium Oxalate VACUETTE® 45406 (“FOX”) and Fluoride/EDTA/Citrate VACUETTE® GLUCOMEDICS 454347 (“FC”). FOX tubes are not available from Sarstedt.

Citrate additives in the FC tubes tested are liquid, so all results in the FC tubes were multiplied with a factor of 1.16, as recommended by the tube manufacturers.

The tubes of Set 1 and Set 2 were centrifuged within 60 min after collection (at 10 min, 2500×*g*, 10 °C). Set 1 samples were stored at 4 °C thereafter. The tubes were measured directly after centrifugation (i.e.1–2 h after blood drawing, “baseline”), 24 ± 1 h, and 48 ± 1 h after the blood drawing. Set 2 samples were centrifuged and stored at room temperature (“RT”) for the rest of the study period and measured after 24 and 48 h. Set 3 samples were kept at RT for 48 h and centrifuged immediately before testing (Table 1).

All glucose and lactate measurements were performed on Abbott ARCHITECT ci8200 systems using Abbott glucose (3L82), a hexokinase method traceable to NIST SRM 965 (IDMS) [28], and lactic Acid (9P18), a lactate colorimetric oxidase method, traceable to reagent grade lactate, reagents.

The imprecision of the glucose assay was 1.4%, the bias 2.0% and the total error 4.8% with an allowable total error in RiliBÄK ≤11.0% and ≤10.0% in CAP and RCPA, respectively. The imprecision of the lactate assay was 1.0% and the bias 2.4% and the total error 4.4% with an allowable total error in RiliBÄK ≤11.0% [13] respectively ≤10.0% in RCPA, as checked with Liquichek Assayed Chemistry Controls (Bio-Rad).

The possible impact of glycolysis inhibition by FC tubes on patient results were simulated using 157,415 consecutive patient results of the Marienhospital in Stuttgart with request for glucose testing in the central laboratory.

2.1. Statistical analysis

All statistical analysis was performed using Analyse-it version 4.8 and SAS JMP 12. For each sample tube, differences due to storage length and temperature were calculated for each study person with the respective baseline FH tube used as reference.

3. Results

Results for the different Greiner tubes and Sarstedt tubes were similar and therefore presented together.

Table 1
Setup of the study: Blood was drawn in 3 sets each of either Sarstedt or Greiner tubes: Serum, fluoride/heparin (FH), fluoride/EDTA (FE), fluoride oxalate (FOX – Greiner only), and fluoride/EDTA/citrate (FC). Testing points, time of centrifugation, and storage conditions are indicated for each of the 3 tube sets. Each type of tube was analyzed 6 times in total, from 3 separate tubes. Baseline testing was performed from set 1 tubes only.

	Set 1	Set 2	Set 3
Centrifugation	1 h after blood drawing	1 h after blood drawing	48 h after blood drawing
Baseline testing	Yes	No	n.a.
Storage	4 °C	RT	RT
24 h testing	Yes	Yes	n.a.
48 h testing	Yes	Yes	Yes

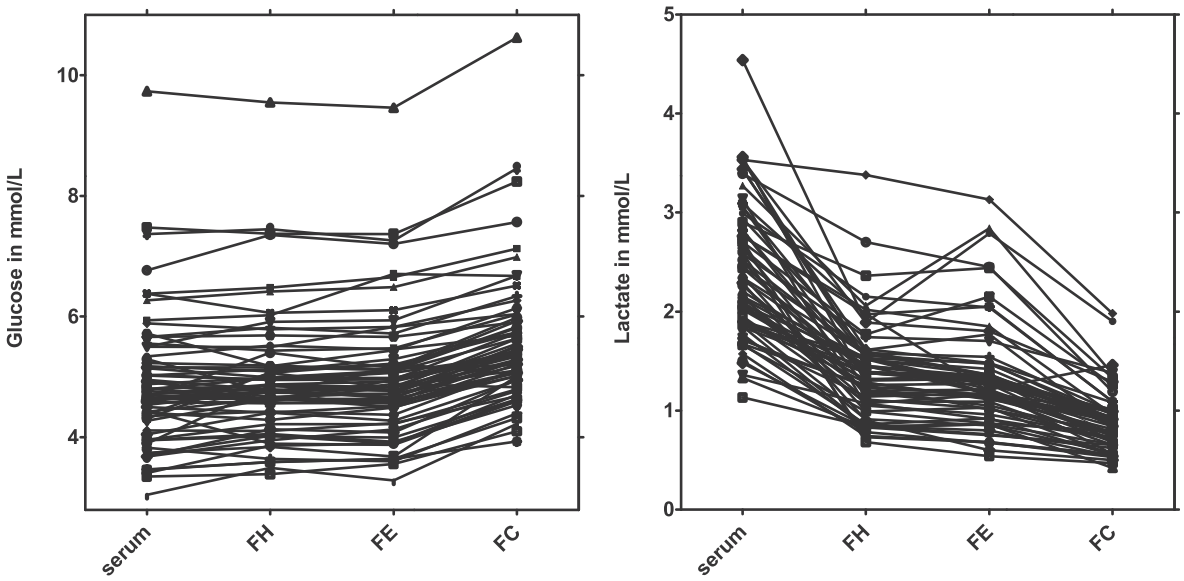

Fig. 1. Comparison of glucose and lactate concentrations at baseline in individual patients (n = 68) in tubes with different glycolysis inhibitors.

FH tubes centrifuged within 1 h and analyzed immediately thereafter were used as reference for the other tubes with different glycolysis inhibitors and different storage conditions ("baseline"). Fig. 1 summarizes the results for the different tube types at baseline: Glucose levels measured in FC tubes are highest in all subjects tested when compared to the FH reference, to FE, and to serum tubes. Lactate levels were lowest in FC tubes and highest in serum.

An overview of glucose and lactate concentrations ranges in the different tube types with different centrifugation and storage condition are presented in Fig. 2. The box-and-whisker plots show the median, interquartile ranges, and the 5th to 95th percentiles for the different tube types. Glucose median and interquartile range results in FE, FH, and FOX tubes are similar at different storage/centrifugation conditions. However, results for FC tubes are higher. Serum glucose results decrease from baseline to testing after 48 h significantly. At baseline, lactate results are lowest in FC tubes. In all tubes, increasing lactate concentrations are observed at later time points vs. Baseline values. This lactate increase is most pronounced in serum tubes followed by FC tubes.

Table 2 summarizes the mean glucose and lactate concentrations and the mean differences in mmol/L as well as percent deviation from the individual testing points to the concentrations measured at baseline in the FH tube. Highest glucose and lowest lactate means were seen in the FC tubes.

Mean glucose results at all testing points were within 0.2 mmol/L of the baseline value in the FC, FE, FOX, and FH tubes, independent of storage and centrifugation conditions. In serum, glucose levels decreased from baseline to 48 h by an average of 1.5 mmol (when samples are centrifuged and analyzed within 2 h after blood draw) and by 2.9 mmol/L (when centrifugation was delayed).

Compared to FH samples, the mean plasma glucose at baseline was 12.8% higher in FC tubes, 4.3% higher in FOX tubes and within 0.4% in the FE tubes. The mean serum values at baseline were 1.4% lower.

At baseline, the lowest mean plasma lactate concentration was observed in FC tubes and lactate concentrations increased over time with a mean increase of 50% at 48 h.

Compared to mean plasma lactate results in FH tubes at baseline ("reference"), mean lactate results were 24% lower in FC tubes, 5% lower in FE tubes, but 10% higher in FOX tubes and 70% higher in serum tubes. The prolonged storage demonstrated increased lactate levels in all plasma tubes and higher results were seen in the samples stored at RT versus 4 °C. After 48 h, lactate concentrations in FC, in FE, and in FH were essentially identical. In FOX, higher mean lactate concentrations were measured.

The efficacy of glycolysis inhibition was tested by comparing samples at baseline with samples stored for 48 h at room temperature.

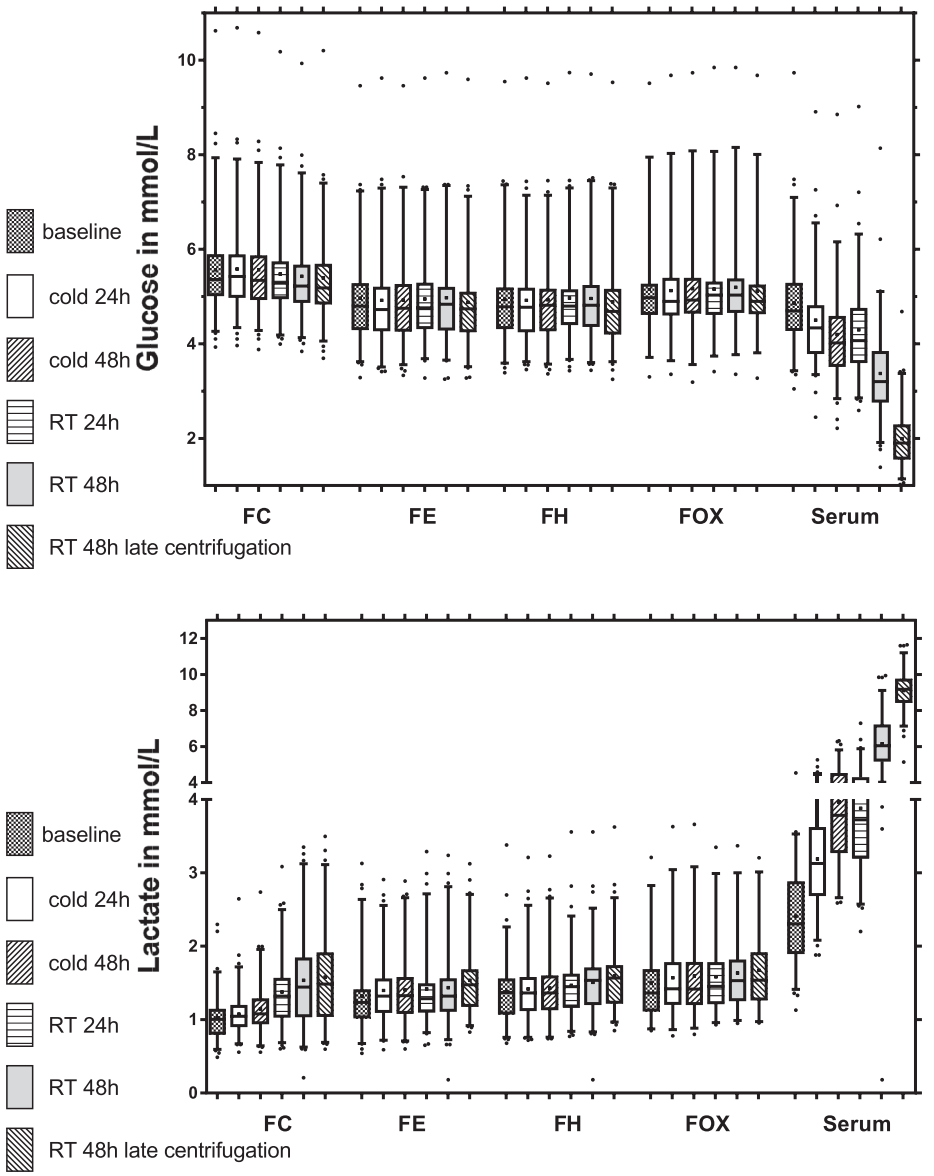

Fig. 2. Glucose (A) and lactate (B) concentrations at different time points in the different tube types under the different centrifugation and storage conditions. Box-and-whisker plots show the median plus interquartile ranges and the 5th to 95th percentiles. The mean is indicated by a +.

Glycolysis inhibition was essentially complete in FE tubes and FH tubes: when comparing the difference between baseline value to values seen after 48 h and room temperature storage, there was only minimal glucose decrease and lactate increase, with the expected 1:2 ratio. In FC tubes however, glycolysis inhibition was less effective in some samples while other samples showed excellent glycolysis inhibition. In all serum tubes, glucose decrease and parallel lactate increase was very high, as expected (Fig. 3).

Fig. 4 demonstrates the expected impact of switching to additional citrate glycolysis inhibition using FC tubes. This simulation was performed with 157,415 consecutive samples from the hospital with request for glucose testing in the central laboratory. 12%, the average bias of all samples excluding RT storage for 48 h, was chosen for this simulation.

This bias would increase the number of patients meeting the criteria for “impaired fasting glucose” from 34.3 to 52.4% (an increase of 52.8%), the criteria “diabetes (fasting conditions)” from 15.4% to 24.0% (an increase of 55.8%) and of the criteria “diabetes from random sample/2hrs after oral glucose load” from 3.1% to 4.6% (an increase of 48.4%).

4. Discussion

Glucose is one of the most frequently measured laboratory tests but pre-analytics in glucose testing remains still a challenge since incomplete inhibition of *in-vitro* glycolysis decreases glucose concentrations and increases lactate concentrations - in most patients in a

Table 2
A. Mean glucose and lactate concentrations measured in different tube types under different centrifugation and storage conditions. B. Delta glucose and lactate results (mmol/L): glucose and lactate results at different time points and different storage/centrifugation conditions versus baseline in FH tubes, C. Percent difference (%) between glucose and lactate results at baseline (FH tube) and testing results in the different tubes at different time points and different storage/centrifugation conditions
Baseline conditions are indicated by bold/underline.

	Mean glucose (mmol/L)					Mean lactate (mmol/L)				
A	FC	FE	FOX	FH	Serum	FC	FE	FOX	FH	Serum
Baseline	5.57	4.96	5.15	**4.94**	4.87	1.07	1.35	1.56	**1.42**	2.42
cold 24 h	5.58	4.92	5.13	4.92	4.50	1.13	1.43	1.63	1.45	3.17
cold 48 h	5.57	4.93	5.17	4.93	4.20	1.19	1.44	1.65	1.47	3.92
RT 24 h	5.48	4.95	5.16	4.97	4.30	1.42	1.45	1.65	1.50	3.86
RT 48 h	5.43	4.97	5.20	4.96	3.37	1.58	1.47	1.70	1.54	6.10
RT 48 h late c.	5.39	4.87	5.11	4.89	1.99	1.60	1.56	1.73	1.62	9.00
	Glucose: Mean delta to baseline results in FH tube (mmol/L)					Lactate: Mean delta to baseline results in FH tube (mmol/L)				
B	FC	FE	FOX	FH	Serum	FC	FE	FOX	FH	Serum
Baseline	0.62	0.02	0.20	**0.00**	−0.08	−0.35	−0.07	0.14	**0.00**	1.00
cold 24 h	0.64	−0.02	0.18	−0.03	−0.44	−0.29	0.01	0.21	0.03	1.75
cold 48 h	0.62	−0.02	0.22	−0.01	−0.75	−0.23	0.02	0.23	0.05	2.50
RT 24 h	0.53	−0.00	0.21	0.02	−0.65	−0.00	0.03	0.23	0.08	2.44
RT 48 h	0.48	0.03	0.25	0.02	−1.57	0.16	0.05	0.28	0.12	4.68
RT 48 h late c.	0.45	−0.07	0.17	−0.06	−2.95	0.18	0.14	0.31	0.20	7.58
	Glucose: Mean delta (%) to baseline results in FH tube					Lactate: Mean delta (%) to baseline results in FH tube				
C	FC	FE	FOX	FH	Serum	FC	FE	FOX	FH	Serum
Baseline	12,75%	0,40%	4,25%	**0,00%**	−1,42%	24%	5%	−10%	**0%**	−70%
cold 24 h	12,96%	−0,40%	3,85%	−0,40%	−8,91%	21%	−1%	−15%	−2%	−123%
cold 48 h	12,75%	−0,20%	4,66%	−0,20%	−14,98%	16%	−1%	−16%	−3%	−176%
RT 24 h	10,93%	0,20%	4,45%	0,61%	−12,96%	0%	−2%	−16%	−6%	−172%
RT 48 h	9,92%	0,61%	5,26%	0,40%	−31,78%	−11%	−3%	−20%	−8%	−329%
RT 48 h late c.	9,11%	−1,42%	3,44%	−1,01%	−59,72%	−13%	−10%	−22%	−14%	−534%

predictable manner. The objective of our study was to compare tubes with different glycolysis inhibitors stored under different conditions and processed at different time intervals on glucose results and we simulated its impact on the identification of hyperglycemia and diabetes in our hospitalized patients.

We evaluated the impact of glycolysis on glucose and lactate levels in a three-step approach:

In a first step, differences between tubes were studied at baseline (i.e. centrifuged within 1 h and analyzed immediately thereafter) and compared to the standard procedure in the laboratory (i.e. testing from FH tubes). The observed positive bias on glucose concentrations was 12.75% in FC tubes. The bias was smaller and very similar in FE (0.4%) and serum (−1.42%) and slightly larger in FOX tubes (4,25%).

In a second step, the influence of timing for centrifugation and storage for up to 48 h at 4 °C versus RT was assessed. Glucose and lactate concentrations in FE, FOX, and FH tubes were stable even after 48 h and even with storage at RT. Use of serum cannot be recommended due to massive glycolysis as reflected by decrease in glucose and parallel increase in lactate, as expected. Predictably, storage at shorter periods of time and at low temperature caused lower glycolysis than storage at room temperature without centrifugation for 48 h (Fig. 2) [14]. A promising alternative to glycolysis inhibitors might be the use of mechanical barriers in lithium heparin tubes [15] as well as the use of gel barrier tubes containing lithium heparin [14], when rapid centrifugation in swing-out rotors at the site of blood drawing is feasible.

Thirdly, the effectiveness of glycolysis inhibition was evaluated by checking the extent and the ratio between decrease in glucose and increase in lactate between baseline samples and the samples stored for 48 h at RT. In FH and FE tubes, the concentrations and the ratio between measured glucose and lactate levels at baseline and at 48 h were nearly identical, which indicates an essentially complete inhibition of glycolysis. The results in FC tubes, however, were heterogenous: In some samples, FC achieved an excellent inhibition of glycolysis. In other FC tubes respectively samples from other patients, however, marked decreases of glucose up to 1 mmol/L and lactate increases up to 2 mmol/L (in the expected 1:2 ratio for glucose decrease and lactate increase) were observed. For lactate testing, FE as well as FH indicate an excellent long-term stability, with a back draw of a slight positive bias. This bias, however, is very low in normoglycemic subjects (Fig. 1). Our data indicate that FC tubes show a time-dependent increase in mean lactate concentration when samples are stored at 4 °C and an even higher increase when samples are stored at RT.

Our data indicates, that on average, the glucose results are 13% higher with FC tubes compared to the commonly used FH tubes. Compared to our standard procedure for glucose measurement, the use of fluoride-citrate-EDTA tubes resulted in glucose results which were in average 13% higher.

When tubes with liquid or powder citrate additives became commercially available, different studies have also examined the effects of adding citrate on glucose test results. This more effective, early inhibition of glycolysis by liquid or powdered citrate additive has been reported to inhibit degradation by glycolysis at an earlier stage and resulting in 6–11% higher plasma glucose levels [16]. In our study

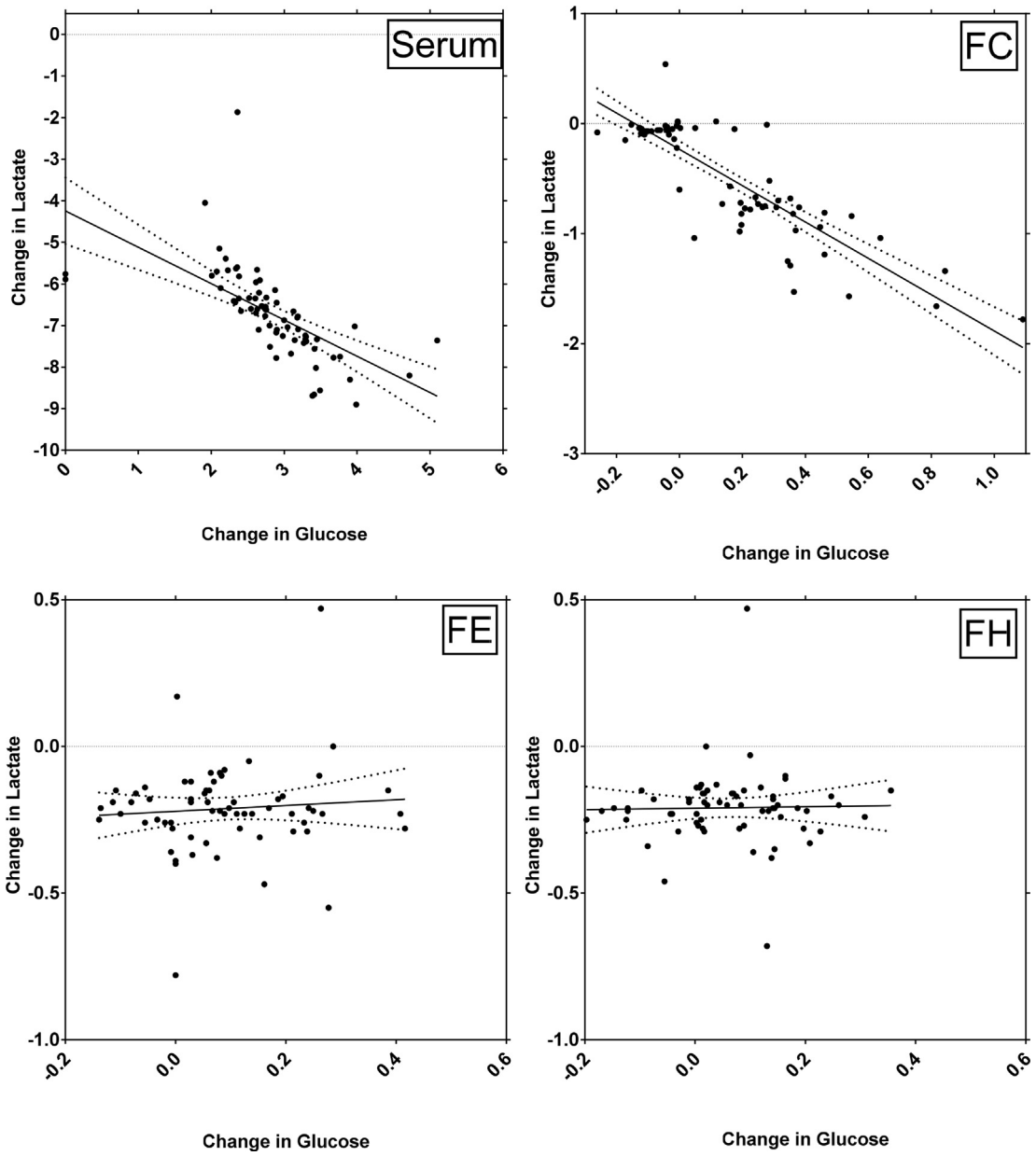

Fig. 3. Change in lactate in mmol/L versus change in glucose in mmol/L in different tubes stored at RT for 48 h, compared to samples analyzed immediately after the blood drawing (baseline). Please note different scales.

we saw in average 12.7% higher values for glucose, however, could confirm by simultaneous lactate measurement that use of citrate does not reliably inhibit glycolysis in all patient samples.

With hyperglycemia being defined as a fasting blood glucose greater than 7.0 mmol/L, 22%–46% of the hospitalized patients have been reported to fall into this category [17,18]. From our cohort of 157,415 hospitalized patients, we estimate an increase by 55.8% from 15.4% to 24.0%. These frequency data compare well with the increase of the frequency of diabetes from the results of oral glucose testing in pregnant women after switching to FC tubes [19].

In brief, switching to FC tubes would increase substantially the percentage of patients with diagnoses of impaired fasting glucose and diabetes when using the established decision limits (high false-positive rate). In some patients however, the long-term stability of glucose in FC tubes was poor which speaks against changing the decision limits, to avoid an increased rate of false-negative results in diabetes screening. Our data employing lactate testing indicate that even in FC samples with no failure of inhibition of glycolysis, lactate increases over time more in FC tubes than in all other plasma tubes tested (FH, FE, FOX).

Decision limits for fasting blood glucose and for hyperglycemia were derived from studies before commercial tubes with additional inhibition of glycolysis using citrate additive were available [2]. Published guidelines propose in addition to immediate centrifugation

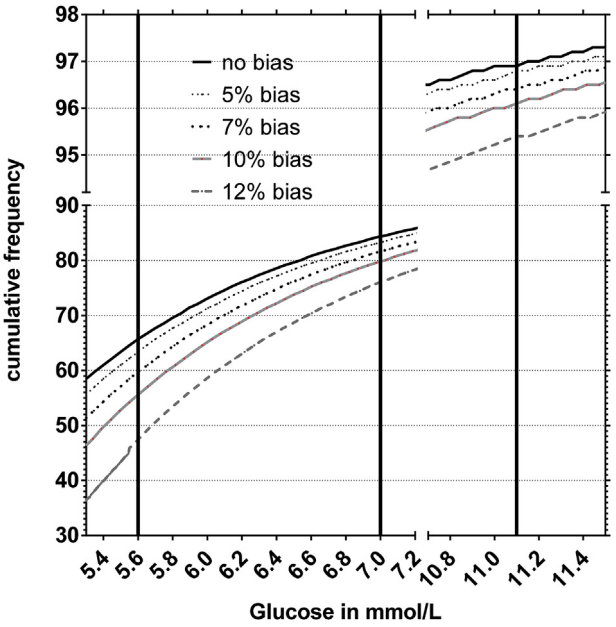

Fig. 4. Effects of different systematic biasses on the frequency of diagnosing impaired fasting glucose (5.6 mmol/L), of diagnosis of diabetes in fasting samples (7.0 mmol/L) and of diagnosis of diabetes by 2 h postprandial glucose testing. Frequency curves were obtained from 157,415 consecutive routine glucose tests from the Marienhospital Stuttgart.

an immediate storage of samples in an ice/water slurry for a maximum of 30 min to minimize glycolysis [4]. Although glycolysis is slower at cold temperature, it cannot be stopped completely, and inter-individual glycolytic rates vary [20–22]. Handling of samples in an ice/water slurry is not practical and is therefore not well accepted under routine conditions [23].

Our study revealed other shortcomings of citrate additive for glycolysis inhibition. An unresolved problem was the unpredictable failure of glycolysis inhibition in some samples, a problem which occurs more frequently with tubes with a certain galenic (i.e. containing granulated NaF/citrate) but was also present in tubes containing liquid additives such as those used in our study. No effective glycolysis inhibition for longer storage such as 48 h at RT was observed in some samples, in particular when glucose decrease and lactate increase were studied together. Our study proves that these differences are caused in fact by failure of glycolysis inhibition (i.e. bias) and not by random scatter of glucose testing (i.e. imprecision).

The strengths of our study are the standardized handling of samples and glucose as well as the parallel lactate measurements for all samples. A limitation of the study is that the true glucose concentration in these samples was not assessed by measuring the reference measurement procedure but instead the results were compared with results from FH samples. In addition, the study population comprised only of healthy individuals. Therefore, the range of glucose concentrations was limited to near reference intervals and decision limits, and it remains unknown if the results (in particular the mean bias of 12.7%) will be similar at other concentrations. Addition of lactate testing allowed a more sensitive analysis of glycolysis compared to the evaluation based on glucose levels alone. Potential limitations of our study are the time delay between phlebotomy, centrifugation, and measurement (~1 h), by which glycolysis using sodium fluoride alone was not inhibited effectively, and the use of sample tubes of one lot each only from both manufacturers tested.

In conclusion, compared to our routine glucose measurement using FH plasma, the use of the fluoride-citrate-EDTA mixture as glycolysis inhibitor increased glucose concentrations by ~13% in average due to effective inhibition of glycolysis in these samples. The simulation studies demonstrated that the use of FC tubes in place of FH tubes would increase the number of patients with impaired fasting glucose or hyperglycemia by more than 50% according to WHO criteria. Since the glycolysis inhibition is not uniform across all FC samples, we advise against using a factor to compensate for the observed difference between FH and FC tubes. Therefore, we recommend using sodium fluoride samples for measuring both glucose and lactate. The routine use of FC tubes should be discouraged until the decision limits for diagnosis of diabetes have been re-evaluated by outcome studies using citrate as the glycolysis inhibitor.

Conflict of interest

None of the authors have any conflict to report.

Acknowledgment

This work was supported in part by an unrestricted grant from Abbott GmbH & Co. KG, Wiesbaden, Germany.

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