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Corrected Fructosamine improves both correlation with HbA_{1C} and diagnostic performance

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ABSTRACT

Aims: There is increasing interest in using fructosamine measurements in screening for or managing diabetes, yet uncertainty remains as to whether these measurements should be corrected for variation in serum protein concentrations.

Methods: We considered all sets of simultaneous measurements of fructosamine, albumin, total serum protein (TP), fasting plasma glucose (FPG) and HbA_{1C} recorded in our laboratory over 10 years. The relationships between fructosamine and other variables were studied by multivariate linear regression and other analyses, and receiver operating curves (ROCs) were analysed to compare the diabetes screening performance of uncorrected fructosamine to those of albumin-corrected fructosamine (FA_{Alb}) and TP-corrected fructosamine (FA_{TP}).

Results: 40,938 sets of measurements were collected from 20,114 patients. Though correlation between fructosamine and serum proteins was strongest among patients with HbA_{1C} <6.5% (48 mmol/mol), it was also significant in the whole sample ($r = 0.193$ for albumin, $r = 0.213$ for TP). With diabetes defined by HbA_{1C} ≥6.5% (48 mmol/mol), the areas under the ROCs of FA_{Alb} (0.905) and FA_{TP} (0.895) were both significantly greater ($P < 0.001$) than that of uncorrected fructosamine (0.878). Correction of fructosamine for albumin or TP slightly improved its correlation with HbA_{1C}. There was no correlation of protein (albumin or TP) with log(fructosamine/protein).

Conclusions: Fructosamine concentration correlates significantly with albumin and total protein concentrations throughout their ranges. Correction of fructosamine improves its correlation with HbA_{1C} and its performance in detecting diabetes.

1. Introduction

In the management of established diabetes, glycated hemoglobin (HbA_{1C}) is the reference biomarker of control of glycemia. Recent advances in measurement standardization have led to its also being able to play a diagnostic role [1]. It nevertheless has its limitations. To begin with, HbA_{1C} levels are affected by a variety of genetic, hematological and disease-related factors: low levels are typical of later-stage chronic kidney disease, certain hemoglobinopathies (e.g. sickle cell disease and thalassemia), and conditions that shorten the lifespan of the erythrocyte (e.g. hemolytic anemia and spherocytosis) [2–4]; and high levels have been associated with iron deficiency and other conditions with decreased erythrocyte turnover [5]). HbA_{1C} levels are also difficult to interpret in the context of gestational diabetes. More generally, the fact that HbA_{1C} integrates glycemia over the lifespan of the erythrocyte means that it is relatively insensitive to shorter-term changes such as may be of interest for monitoring the effects of changes in therapy [6–7].

The above limitations of HbA_{1C} as a measure of glycemic control imply that there are niches for other biomarkers that could be used when HbA_{1C} cannot, because they do not suffer the corresponding limitation. Particular interest has focused on fructosamine (total glycated serum protein) and glycated serum albumin (GA), which reflect glycemia in the past 2–4 weeks and do not depend on hemoglobin metabolism. Recently, a cross-sectional analysis found both these analytes to be at least as strongly associated with microvascular complications of diabetes as HbA_{1C} is [9]; and a study of 2314 type 2 diabetes patients followed up for an average 6.5 years found the risk of progression of

Abbreviations: AUC, Area under the curve; CGM, continuous glucose monitoring; DCCT, Diabetes Control and Complications Trial; FA, uncorrected serum fructosamine; FA_{Alb}, albumin corrected fructosamine; FA_{TP}, total protein corrected fructosamine; FPG, fasting plasma glucose; GA, glycated serum albumin; JDS, Japanese Diabetes Society; JSCC, Japanese Society for Clinical Chemistry; NBT, nitroblue tetrazolium; NGSP, National Glycohaemoglobin Standardization Program; ROCs, Receiver operating characteristic curves.

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nephropathy to increase with the updated mean fructosamine level, with a hazard ratio slightly greater than that of updated mean HbA_{1C} [10].

To circumvent the influence of variation in serum albumin levels, GA is used clinically as the ratio of glycated to total serum albumin [8–9,11]. Also, the standardised reference method for measuring HbA1c promotes a standardized unit, millimoles of HbA1c per mole of Hb, which avoid fluctuation in blood hemoglobin levels [12]. By contrast, best practice for fructosamine in this respect is uncertain, controversy over whether measurements should be corrected for total albumin, total protein, or neither never having been resolved [13]. The study described here was undertaken to clarify whether fructosamine measurements obtained by a high-specificity enzymatic method should be corrected or not, and if so, with respect to albumin or total protein.

2. Materials and methods

2.1. Data

A profile was defined as comprising measurements of fructosamine, fasting plasma glucose (FPG), HbA_{1C}, albumin and total serum protein in blood samples from a single patient obtained at a single visit. Included in the study were all the complete profiles from diabetic and nondiabetic outpatients that were obtained and recorded in the clinical biochemistry laboratory of the University Hospital Complex, Santiago de Compostela (Spain) between March 2004 and March 2014. These profiles were divided in three groups according to whether the samples of origin were from a) patients with established type 1 or b) type 2 diabetes; c) patients with prediabetes, i.e. with FPG levels of 100–125 mg/dL (5.6–6.9 mmol/L) and/or HbA_{1C} levels of 5.7–6.4% (39–46 mmol/mol) [1]; or d) normoglycemic patients with FPG levels below 100 mg/dL (5.6 mmol/L) and HbA_{1C} levels <5.7% (<39 mmol/mol).

The study was conducted in accordance with the Declaration of Helsinki and data management was approved by the hospital's Ethics Committee. Informed consent was obtained from every patient prior to their inclusion in this or previous studies.

2.2. Analytical methods

Whole blood for determination of HbA_{1C} was collected in EDTA-containing tubes. HbA_{1C}, FPG and serum fructosamine, albumin and total protein were all determined on the day of collection.

HbA_{1C} was determined by high-performance liquid chromatography as implemented by Menarini Diagnostics HA-8121 and HA-8140 analyzers. The interassay coefficient of variation (CV) was 1.6% at an HbA_{1C} level of 5.9% (41 mmol/mol), and 0.9% at an HbA_{1C} level of 11% (97 mmol/mol). For this study, all HbA_{1C} values were converted from JDS/JSCC-referenced values (JDS = Japanese Diabetes Society; JSCC = Japanese Society for Clinical Chemistry) to DCCT-aligned units [14].

Fructosamine was determined by the GlyPro enzymatic method (Genzyme, Kent, UK) on a Cobas Mira analyzer (Roche). This assay uses proteinase K to digest serum proteins into small fragments, and ketoamine oxidase to catalyse the specific oxidation of the ketoamine bond of glycated fragments; the release of hydrogen peroxide, measured colorimetrically at 550 nm, is proportional to the concentration of fructosamine. The interassay CV was 1.8% at fructosamine = 175 μmol/L and 0.91% at 640 μmol/L. Fructosamine values were used either uncorrected (FA), or corrected for albumin or total protein concentration (respectively FA_{Alb} and FA_{TP}) as per Lin et al. [15]:

$$FA_{Alb}(\mu mol/L) = 42 \times FA/albumin, \quad (1)$$

$$FA_{TP}(\mu mol/L) = 70 \times FA/(total\ protein), \quad (2)$$

The mean serum concentrations of albumin and total protein in the whole data set being 42 and 70 g/L, respectively.

Glucose, albumin and total protein concentrations were determined on Advia Analyzer using kits from Siemens Healthcare Diagnostics. Globulin was calculated as total protein minus albumin.

2.3. Statistical analysis

The statistical significance of differences between groups was estimated by Student's *t*-test if the corresponding distributions were normal or by Wilcoxon's test otherwise. Correlations were evaluated as Pearson's correlation coefficient in the case of bivariate normal distributions or as Spearman's correlation coefficient otherwise. Normality was assessed by Kolmogorov–Smirnov tests.

Within each of four HbA_{1C} ranges (<5.7% (<39 mmol/mol), 5.7–7.0% (39–53 mmol/mol), 7.1–9.0% (54–75 mmol/mol), and >9.0% (>75 mmol/mol)), mean FA values in albumin interquintile intervals were compared by ANOVA followed by *post hoc* Bonferroni corrections for multiple comparisons if the groups were normally distributed, or by Kruskal-Wallis tests otherwise; and a parallel analysis was performed for FA_{Alb}. The relative impacts of albumin, globulins and FPG on FA were evaluated by multivariate linear regression as their standardized regression coefficients (*z* values, i.e. the regression coefficients divided by their SEs).

Optimal FA, FA_{Alb} and FA_{TP} thresholds for detection of diabetes (defined for this purpose by $HbA_{1C} \geq 6.5\%$ (48 mmol/mol)) were obtained from receiver operating characteristics (ROCs), the corresponding diagnostic sensitivities and specificities were calculated, and a parallel analysis was performed for diabetes defined by FPG ≥126 mg/dL (7.0 mmol/L). The statistical significance of differences between areas under ROCs (AUCs) was estimated by the test of DeLong et al. [16]. *P* values <0.05 were considered statistically significant. All calculations were performed using SPSS 17.0 or MedCalc 11.1.

To verify if there is any relationship of dependence between FA and albumin, we used the specific glycation index suggested by Schleicher er al. (17), This index is given as log (FA/serum Albumine) and they demostrated it should be indirectaly proportional to serum albumin concentration itself.

3. Results

The data set comprised 40,938 sets of measurements from 20,114 patients (Table 1). Consideration of FA levels in patient groups defined by the quintiles of the distribution of albumin concentrations (Fig. 1) might suggest, in keeping with early studies [18], that FA only depends on albumin at below-normal albumin levels: mean FA in the group with lowest albumin levels, 226 μmol/L, was significantly less ($P < 0.001$) than in the other groups (276–289 μmol/L), which did not differ significantly from each other in mean FA. However, this analysis ignores the dependence of FA on glycemia, variation of which among the albumin interquintile groups is shown by the existence of significant differences among their mean FPG and HbA_{1C} values ($P < 0.001$). The mean (SD) of FPG values in each interquintile of albumin were 147 (75); 147 (66); 156 (68); 148 (66) and 135 (55), with all of them expressed in mg/dL, while for HbA1c were 7.0 (2.2); 7.0 (1.8); 7.3 (1.8); 6.9 (1.7) and 6.4 (1.4) with all values expressed as percentages. FA in fact exhibited significant albeit weak correlation with albumin in the whole sample ($r = 0.193$), although correlation was much stronger among patients who were nondiabetic ($r = 0.452$) or pre-diabetic ($r = 0.433$) than among those with diabetes ($r = 0.177$–0.196) (Table 2).

A similar pattern was shown by correlation between FA and total protein ($r = 0.213$ in the whole sample), although the influence of globulins on FA ($r = 0.067$–0.123) was between 3 and 16 times smaller than that of albumin in the various patient subgroups. Contrariwise, correlation between FA and other measures of glycemia (FPG and HbA_{1C}) was much stronger among patients with diabetes than among those without (Table 2). Thus although variation in FA was determined predominantly by variation in glycemia in the diabetic range, and by variation in

Table 1
Characteristics of the study group and subgroups.[a]

Characteristic	Group				
	All	Type 1	Type 2	Prediabetes	No diabetes
Patients, n	20,114	324	7608	7181	5001
Visits, n	40,938	1862	22,476	10,177	6423
Male sex, %	54.6	58.9	54.5	56.9	50.2
Age, years	60.9 (17.2)	34.6 (14.0)	63.4 (14.0)	65.8 (14.2)	51.9 (21.8)
Hb A_{1c}, %	6.9 (1.8)	8.5 (1.7)	7.9 (1.7)	5.7 (0.4)	5.1 (0.4)
FPG, mg/dL	147 (66)	199 (96)	173 (67)	115 (25)	87 (9)
FA, μmol/L	272 (118)	421 (135)	319 (116)	200 (49)	183 (43)
Albumin, g/L	42 (4)	42 (3)	42 (4)	41 (5)	41 (4)
Total protein, g/L	69 (6)	69 (5)	69 (6)	69 (7)	68 (6)

[a] Data are mean (SD); FPG = fasting plasma glucose; FA = uncorrected fructosamine.

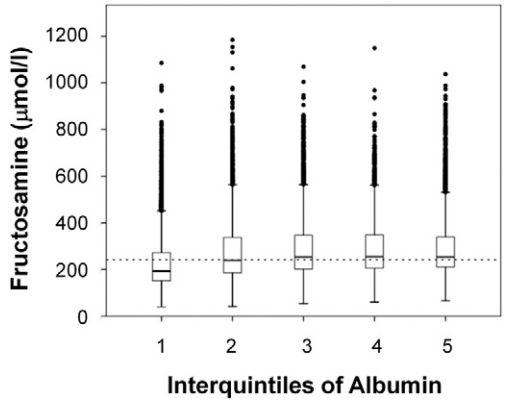

Fig. 1. Boxplots of fructosamine levels in albumin interquintile groups (1, 13–38 g/L, n = 7973; 2, 39–41 g/L, n = 8751; 3, 42–43 g/L, n = 8196; 4, 44 g/L, n = 6384; 5, 45–55 g/L, n = 9634). The dotted line reflects fructosamine median in all patients (241 μmol/L).

albumin among nondiabetic patients, it was never totally independent of either albumin or glycemia.

The universal influence of albumin is further supported by the data of Fig. 2a, which shows values of FA for albumin interquintiles within each of four HbA_{1C} ranges. For all HbA_{1C} ranges <9% (75 mmol/mol), FA increased significantly ($P < 0.001$) between successive albumin interquintiles. That among patients with HbA_{1C} >9% the increases in mean FA between the second and third albumin interquintiles, and between the third and fourth, were not statistically significant, is attributable to the wide variation of HbA_{1C} in this group, 9–18% (71–173 mmol/mol); the overall increasing trend was significant ($P < 0.001$), as in the other HbA_{1C} groups. Similar results were obtained when, within these same HbA_{1C} ranges, the trend in FA among groups defined by total protein quintiles was compared with the trend in FA_{TP}, the main difference being that FA_{TP} showed a greater tendency than FA_{Alb} to peak in the central interquintiles (results not shown).

Pursuing further the same reasoning as prompted the analysis of Fig. 2, Table 3 lists the results of multivariate regression of FA on albumin, globulins and FPG in each of three different HbA_{1C} ranges. The ratio between the influence of FPG on FA and that of albumin is seen to be about 52% among nondiabetic patients (HbA_{1C} <5.7%; 39 mmol/mol), about 73% among prediabetic patients (HbA_{1C} 5.7–6.4%; 39–46 mmol/mol), and about 209% among patients with diabetes (HbA_{1C} ≥6.5%; 48 mmol/mol).

In all except the highest of the HbA_{1C} ranges considered in Fig. 2, emendment of FA as FA_{Alb} successfully eliminated positive correlation with albumin (Fig. 2b). In the exceptional range HbA_{1C} >9% 75 mmol/mol, emendment introduced negative correlation of borderline significance, probably in great measure because in this less homogeneous group (*vide supra)* the subgroup of patients with albumin levels below the first quintile had a higher mean HbA_{1C} than the other albumin interquintile groups.

In the whole sample, emendment as FA_{Alb} improved correlation with both HbA_{1C} (r increasing from 0.779 to 0.807; Fig. 3a and b) and FPG (r increasing from 0.604 to 0.618), while correction as FA_{TP} achieved similar but smaller improvements ($r = 0.799$ for correlation with HbA_{1C} and $r = 0.610$ for FPG).

To investigate the extent to which correction for albumin or total protein might improve the performance of fructosamine in screening for diabetes, the set of first profiles obtained from the 20,114 patients was used to construct ROC curves for detection, by FA, FA_{Alb} and FA_{TP}, of diabetes defined by HbA_{1C} ≥6.5%; 48 mmol/mol (Fig. 4a) or by FPG ≥126 mg/dL; 7.0 mmol/L (Fig. 4b). With diabetes defined by HbA_{1C}, the various measures of fructosamine were ranked by their AUCs in the order FA_{Alb} (AUC = 0.905)>FA_{TP} (0.895)>FA (0.878) ($P < 0.001$ for comparison of FA with either of the others). The optimal diagnostic thresholds were FA_{Alb} = 241 μmol/L (diagnostic sensitivity 80.7%, diagnostic specificity 89.7%), FA_{TP} = 247 μmol/L (sensitivity 77.6%, specificity 89.3%) and FA = 246 μmol/L (sensitivity 74.8%, specificity 89.2%). With diabetes defined by FPG, the AUC-based ranking was the same - FA_{Alb} (0.834)>FA_{TP} (0.826)>FA (0.816) ($P < 0.001$ for both FA *vs.* FA_{Alb} and FA *vs.* FA_{TP}) - and the optimal diagnostic thresholds were FA_{Alb} = 234 μmol/L (sensitivity 72.8%, specificity 82.2%), FA_{TP} = 237 μmol/L (sensitivity 71.3%, specificity 82.3%) and FA = 233 μmol/L (sensitivity 71.4%, specificity 81.3%).

Finally, we investigated whether albumin correlated better with specific glycation, given as log(fructosamine/albumin) (= log FA_{Alb}–log 42) than with corrected or uncorrected fructosamine, as was reported by

Table 2
Correlation coefficients between glycemic markers and serum proteins in each group of patients[a].

Parameter	All		Type 1		Type 2		Prediabetes		No diabetes	
	FA	HbA_{1c}	FA	HbA_{1c}	FA	HbA_{1c}	FA	HbA_{1c}	FA	HbA_{1c}
Albumin	0.193	-0.058	0.196	−0.127	0.177	−0.131	0.433	−0.025*	0.452	0.007**
Total protein	0.213	0.001**	0.183	−0.064	0.193	−0.076	0.385	−0.029	0.394	0.011**
Globulins	0.116	0.064	0.067	0.037**	0.100	0.048	0.123	−0.017**	0.113	0.009**
FPG	0.604	0.681	0.275	0.321	0.484	0.559	0.180	0.115	0.109	0.175
HbA_{1c}	0.779	1.0	0.752	1.0	0.692	1.0	0.133	1.0	0.046	1.0

[a] $p < 0.01$ in all cases except those labelled with *($p = 0.014$) or with **(no significant correlation). FA = uncorrected fructosamine. FPG = fasting plasma glucose.

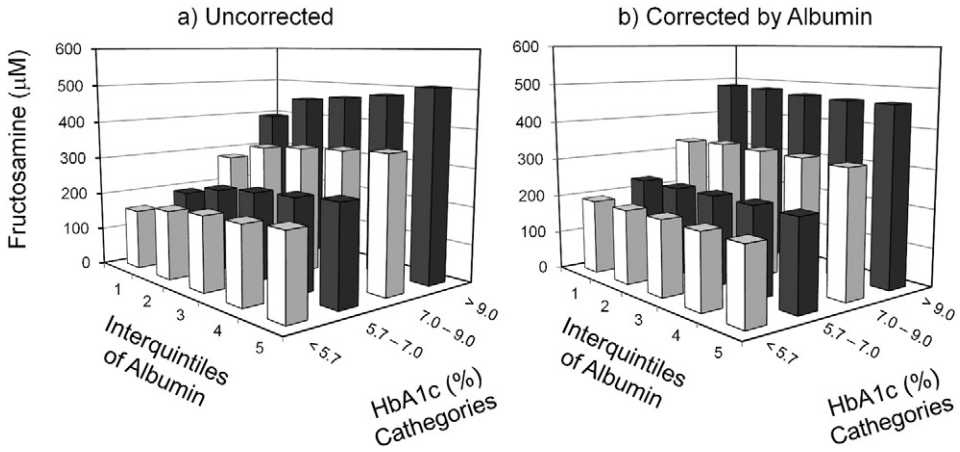

Fig. 2. Fructosamine values uncorrected (a) or Albumin-corrected (b) for albumin interquintiles within each of four HbA_{1C} ranges. For all HbA_{1C} ranges uncorrected FA increased significantly ($p < 0.001$) between successive albumin interquintiles. After correction the trend was not significant.

Schleicher et al. [17]. To minimize distortion due to the influence of glycemia on fructosamine levels, the whole population of participants with HbA_{1C} between 5 (31 mmol/mol) and 10% (86 mmol/mol) was divided in HbA_{1C} classes 0.5% wide (5.0–5.4%, 5.5–5.9%, etc.), but in none of these groups was there significant correlation between albumin and log(fructosamine/albumin), r ranging from −0.017 to 0.051. By contrast, in the whole sample log(fructosamine/albumin) was closely correlated with both fructosamine ($r = 0.939$) and HbA_{1C} ($r = 0.784$) ($P < 0.0001$ in both cases).

4. Discussion

In this study we measured fructosamine with a high-specificity enzymatic assay in large samples of diabetic, prediabetic and nondiabetic patients. The good correlation with HbA_{1C} suggests that in general the participants had stable glycemia when their analytical profiles were obtained. Our results clearly show that fructosamine concentrations are affected by differences in serum protein concentrations over the whole range of the latter and at all levels of glycemia. Although the effect is small, especially among patients with normal or high albumin levels, correcting fructosamine measurements for albumin or total protein improves both correlation with HbA_{1C} and diagnostic performance in the detection of diabetes defined by $HbA_{1C} \geq 6.5\%$ (48 mmol/mol) or FPG >126 mg/dL (7.0 mmol/L). The finding that the influence of serum albumin on fructosamine levels is four or five times that of globulin is in keeping with albumin being far more abundant (it makes up 50–60% of plasma protein in normal individuals [19], and representing more than 80% of the total molecules), and with the modest difference between correction for albumin and correction for total protein as regards correlation with HbA_{1C} and diagnostic performance.

It has been argued that fructosamine levels should not depend on serum protein concentration, because the latter (or more exactly, the concentration of lysine residues susceptible to glycation) is always vastly in excess of the reactive open-chain form of glucose [20]. The observation that fructosamine levels do in fact depend on serum protein may be due to the pool of open-chain glucose being constantly replenished by isomerization of the cyclic form [21]. It has also been argued that fructosamine levels should not depend on serum albumin concentration – or at least, should not be corrected for serum albumin concentration – because although a higher albumin concentration may increase the glycation rate it is also accompanied by a reduction in the half-life of albumin [17]. Schleicher et al. supported this contention with the observation of good negative correlation between albumin and log(fructosamine/albumin) among 63 nondiabetic subjects with serum albumin levels of 17–48 g/L [17]. In the present study log(fructosamine/albumin) exhibited no correlation with albumin at any HbA_{1C} level, and in the whole study group exhibited close correlation with fructosamine and HbA_{1C}. These latter correlations are in keeping with the analysis of Schleicher et al. – that they were not observed by Schleicher et al. themselves is attributable to their using a small group of patients with a limited range of non-diabetic HbA_{1C} values. The discrepancy concerning correlation between albumin and log(fructosamine/albumin) is more intriguing, but again seems likely to derive from the difference in study size.

While at least one previous study found no correlation at all between serum protein and fructosamine levels [22], in a number of others, as in ours, such correlation has been observed [23–26]. Although the influence of serum proteins was in all these cases greatest at sub-normal levels, it was not limited to this range as has been claimed by others [27–29]. The discrepancies among these and other results (most of them obtained 15 years or more ago) may be due to a number of factors. They include small study size [17,23,25,29,30] and differences in the composition of the study group, which has variously consisted of Maoris [31], normal children [29], individuals with diabetes [25], individuals without diabetes [17,23,29–31], or mixes with various proportions of participants with and without diabetes [22,30,32,33]. Additionally, all these earlier studies determined fructosamine concentrations by non-enzymatic methods – most of them by the nitroblue tetrazolium (NBT) assay, which suffers from interference (amounting to about half the fructosamine concentration in healthy persons) due to a nonspecific reducing activity of NBT in serum [18,34]. The present study, though limited geographically to the population served by a single tertiary hospital laboratory, included 40,938 analytical profiles from 20,114 participants, and employed a highly specific enzymatic method for the determination of fructosamine. We emphasize, moreover, that the study group was not only large, but was also not limited to diabetic patients or to diabetic patients plus an arbitrary number of nondiabetic individuals: it comprised all patients for whom the relevant profile had been requested in the past 10 years, and was therefore truly representative of the population

Table 3
Magnitudes of the effects of predictor variables on fructosamine in the multivariate linear regression analysis.

	HbA_{1c}					
	<5.7%		5.7–6.4%		>6.4%	
Variable	b (SE)	z[a]	B (SE)	z[a]	b (SE)	z[a]
Albumin	4.339 (0.088)	49.5	4.858 (0.124)	39.1	5.624 (0.185)	30.5
Globulins	0.972 (0.095)	10.2	1.078 (0.128)	8.4	1.632 (0.192)	8.5
FPG	0.043 (0.0017)	25.6	0.047 (0.0017)	28.6	0.069 (0.0011)	63.7

[a] Z value or standardized coefficient was calculated as the regression coefficient divided by its SE. FPG = fasting plasma glucose.

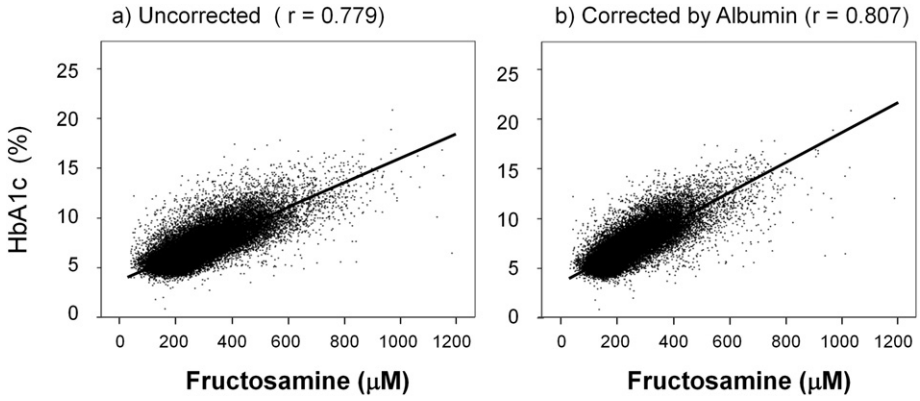

Fig. 3. Correlations between HbA1c and fructosamine (FA). (a) uncorrected FA or (b) albumin-corrected FA.

for whom the issue of whether or not to correct for serum protein is pertinent.

A limitation of this study is the absence of continuous glucose monitoring (CGM) data, which prevents evaluation of whether correction of fructosamine for albumin or total protein improves correlation with CGM-derived measures of glycemia as it does correlation with HbA_{1C} and FPG. Finally, it is interesting that the improvements in correlation with HbA_{1C} and in diagnostic performance upon correction for albumin were not only similar to those achieved by correcting for total protein – a similarity attributable to the much smaller influence of globulins on fructosamine (see Table 2) – but were in both cases greater than when FA_{TP} was used. This may perhaps be due to possible heterogeneity among globulins as regards susceptibility to glycation.

In conclusion, in this study we found that serum fructosamine concentration is significantly influenced by serum protein concentration, not only when serum concentration is low but throughout its entire range. Correcting fructosamine for albumin or total protein improves its correlation with HbA_{1C} and FPG, and slightly but significantly increases its ability to predict that HbA_{1C} exceeds 6.5% (48 mmol/mol) or FPG 126 mg/dL (7.0 mmol/L). Though the correction is slight for most patients, we recommend its application, which in the vast majority of cases will involve no extra cost, requests for fructosamine determination invariably being accompanied by a routine request for albumin determination and which is the best for patients care.

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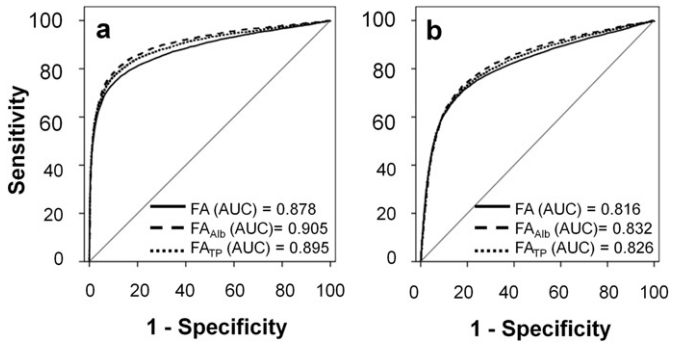

Fig. 4. ROCs for comparison of fructosamine (——), FA_{Alb} (– –) and FA_{TP} (▪▪▪▪▪▪▪▪) for detection of diabetes defined by HbA_{1C} ≥48 mmol/mol (6.5%) (a) or by FPG ≥7.0 mmol/L (126 mg/dL) (b).

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Acknowledgements

Author contribution: SRS designed the study, collected analysed and interpreted data, performed statistical analyses, and wrote the draft of the article. FC contributed to discussion and edition of the manuscript, he also wrote the final version of the manuscript and performed graphic artwork. JRG collected, analysed, interpreted data and contributed to discussion. All authors revised and approved the final version of the manuscript.

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