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Measurement of glycosylated ferritin with Concanavalin A: Assay design, optimization and validation

Alexandre Raynor [a,*], Katell Peoc'h [a,b,c], Anne Boutten [a]

[a] Département de Biochimie, Hôpitaux Universitaires Paris Nord Val de Seine – site Bichat, Assistance Publique Hôpitaux de Paris, Paris, France
[b] Université de Paris, Centre de Recherche sur l'Inflammation, INSERM UMRs 1149, CNRS, ERL8252, UFR de Médecine Bichat, 75018 Paris, France
[c] Assistance Publique des Hôpitaux de Paris, APHP Nord, Laboratory of Excellence GR-Ex, France

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ABSTRACT

Introduction: Ferritin is the major iron-storage glycoprotein found in all tissues. Ferritin glycosylation can be assessed by the differential affinities of ferritin glycoforms for Concanavalin A (ConA), a lectin. The fraction of serum ferritin bound to ConA is called "glycosylated ferritin" (GF). Low GF reflects macrophagic activation and is an essential biomarker used in adult-onset Still's disease (AOSD), macrophage activation syndrome (MAS) and Gaucher disease diagnosis and therapeutic management. To date, no complete assay description and method validation according to the ISO 15189 standard has been published. This study aimed to describe and validate our method used for GF measurement and describe GF values observed in patients.
Materials and methods: Ferritin glycoforms were separated based on their affinities for ConA using commercially available TRIS-barbital buffer, Sepharose and ConA/Sepharose 4B gels. Ferritin concentrations were measured on the Siemens Dimension Vista 1500®. We analysed 16,843 GF values obtained between 2000 and 2021 from our database of patients.
Results: Optimal separation of ferritin glycoforms was obtained by 15-min incubation of serum with ConA/Sepharose at pH 8. The optimized volume were 0.4 mL for total serum ferritin (TSF) 30–1000 μg/L and 0.5 mL for TSF 1000–2500 μg/L. Serum with higher TSF should be pre-diluted in the TRIS-barbital buffer. Reproducibility of ferritin measurement in the TRIS-barbital buffer matrix was excellent (intra-assay CV < 1%; inter-assay CV < 4%). Reproducibility of GF assay was good (intra-assay CV < 10% for low and high ferritin samples, respectively; and inter-assay CV < 10%). Inter-operator variability was 21.6% for GF < 20%. Ferritin was stable for up to 3 days in the TRIS-barbital buffer. An inter-laboratory exchange program conducted with another French hospital showed good agreement between results. In our database, <20% GF levels were scarce, compatible with the low prevalence of Still's disease, MAS, and Gaucher disease. The 95% confidence interval for GF was [26–58]%, lower than values described in the literature for healthy individuals.
Conclusion: Thanks to good performances, this technique can become readily available for laboratories servicing patients with AOSD, MAS (including severe COVID-19 patients) and Gaucher disease patients.

1. Introduction

Ferritin is the major iron-storage glycoprotein. It is ubiquitous, although predominantly located in reticuloendothelial cells [1]. This 24-subunit heteropolymeric protein shell is made of two subunits: H (heavy, 21 kDa) and L (light, 19 kDa). Ferritin molecules contain various proportions of the two subunits depending on the tissue and physiological status: mainly L in the liver and the spleen and mostly H in the heart and the kidney [1]. While intracellular ferritin is not glycosylated, carbohydrate residues are added to L-ferritin in the cytosol when secreted into plasma [2]. Circulating ferritin mainly consists of L subunits with rare H subunits [3]. Ferritin glycosylation can be assessed by the differential affinity of its glycoforms for Concanavalin A (ConA). ConA is a lectin binding specifically accessible mannosyl residues on glycoproteins [4]. In healthy patients, a high percentage of circulating ferritin binds ConA and is thus called "glycosylated ferritin" (GF). GF is N-glycosylated [5

Abbreviations: AOSD, Adult-onset Still's disease; BCH, Bichat hospital; ConA, Concanavalin A; COVID-19, Coronavirus disease 2019; GF, Glycosylated ferritin (or GF percentage); HCL, Hospices Civils de Lyon hospital; MAS, Macrophage activation syndrome; QC, Quality control; Seph, Sepharose; TSF, Total serum ferritin.

* Corresponding author at: Service de Biochimie Hôpital Bichat, 46 rue Henri Huchard, 75018 Paris, France.
E-mail address: alexandre.raynor@aphp.fr (A. Raynor).

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6]. In healthy adults, GF is comprised between 50 and 80%. In patients with acute and chronic inflammatory syndromes, GF is decreased and ranges from 20 to 40% [5]. GF is a marker of active adult-onset Still's disease (AOSD), reflecting the intensity of inflammation and macrophagic activation [7]. GF is one of the major criteria of the Fautrel classification used to diagnose AOSD, with a threshold set at <20% [8]. The Fautrel classification has demonstrated, in an independently selected cohort, good sensitivity and specificity (87.0% and 97.8%). Positive and negative predictive values were also good (88.7% and 97.5%) [9]. GF is also used to monitor treatment in AOSD [7]. Low GF is also a good marker of macrophage activation syndrome (MAS) [10,11] and Gaucher disease [12], both for diagnosis and therapeutic management. Low GF is associated with hyperferritinemia in acute hepatitis or cancer. Recently, low GF has been reported as a sensitive biomarker of severe COVID-19 [13]. This observation could be related to macrophagic activation, one of the immune pathways involved in the infection (A Boutten and K Peoc'h, personal communication). Currently, a French clinical trial is retrospectively assessing the prognostic values of ferritin and GF in SARS-CoV-2 hospitalized patients [14]. Thus, GF determination, currently performed in a few specialized laboratories, is becoming a scorching topic from an analytical point of view.

Our laboratory participated in the development of GF as a biomarker [5], particularly in AOSD [8,9] and Gaucher disease [12]. Our laboratory has recently been recognized as a reference laboratory for GF as a biological marker of AOSD, MAS and Gaucher disease. According to French regulations, our laboratory had to be accredited according to the ISO 15189 for this analysis. Although performed in our laboratory for >30 years, our GF assay method had not been published nor accredited. Furthermore, while methods derived from Worwood's are still widely employed today to measure GF [11,13,14], no complete assay method description and validation was reported in the literature to date.

The present study aimed to, following the ISO 15189 standard, i) describe the method we used to measure GF, adapted from Worwood *et al.* [2] and optimized for use in clinical laboratory environment, ii) evaluate the performances of ferritin measurement in TRIS-barbital buffer and iii) evaluate the performances of the GF assay. We also aimed to describe the frequent values observed in patients.

2. Materials and methods

2.1. Samples and regents

Serum samples were collected by venipuncture on separating tubes with gel (BD Vacutainer), centrifuged for 15 min at 2000 g at room temperature.

Reagents were prepared according to the manufacturer's instructions. TRIS-barbital buffer was prepared from a commercially available TRIS-barbital concentrated buffer (Sebia). Seventy five mL concentrated buffer (0.8 mmol/L barbital) were diluted with 925 mL distilled water (final concentration of 0.06 mmol/L). The buffer was adjusted to pH = 8 with hydrochloric acid (Carlo Erba). Sepharose (Seph) or ConA/Seph 4B gels (GE Healthcare Bio-Sciences) were washed and suspended in TRIS-barbital buffer (2 volumes Seph or ConA/Seph and 1 vol buffer) to obtain either Seph or ConA/Seph TRIS-barbital suspensions. The reagents were thoroughly mixed on a roller mixer before use. A low (69 μg/L) ferritin internal quality control (QC, Lyphocheck Assayed Clinical Chemistry Control, Biorad) was assayed with each batch of reagents.

2.2. Binding procedure

Total serum ferritin (TSF) was measured using a Vista® immunoassay on the Siemens Dimension Vista 1500® analyser (Siemens Healthineers®). Ferritin glycoforms were separated according to their differential affinities for ConA, based on a method previously described by Worwood *et al.* [2], with minor modifications. Briefly, serum was incubated either with ConA/Seph or with Seph (as a control) in a final incubation volume of 1 mL TRIS-barbital buffer for two hours at room temperature on a roller mixer at 5000 rpm. Then, samples were centrifuged at 3000 g at 20 °C for 10 min. Volumes of serum and reagents used in the assay depend on TSF and are detailed later. Unbound ferritin was recovered in the supernatants of the Seph and ConA/Seph samples and quantified by the Vista® immunoassay. GF percentage was calculated as follows: GF = 100 * (1 – (unbound ferritin in the ConA/Seph sample) / (unbound ferritin in the Seph sample)).

2.3. Binding conditions

2.3.1. Volumes of Sepharose and Concanavalin A/Sepharose

To determine the optimal volumes of Seph and ConA/Seph to be used, which are the volumes required to bind all GF, five patient samples with TSF 67 to 2257 μg/L and GF 10 to 50% were incubated with increasing ConA/Seph or Seph volumes (from 0.1 to 0.8 mL) in a final incubation volume of 1 mL TRIS-barbital buffer.

2.3.2. Optimal pH

To determine the optimal pH for maximal ConA binding, ConA/Seph and Seph reagents were prepared with TRIS-barbital buffer adjusted to pH 7.5, 8.0, and 8.5 and incubated with low and high (450 μg/L) ferritin QC (Lyphocheck Tumor Marker Plus Control, Biorad). GF were determined twice. Recoveries were calculated compared to GF value measured at pH 8.0, the original condition characterized by Worwood *et al.* [2].

2.4. Performances of ferritin measurement in TRIS-barbital buffer

The performance of the ferritin assay in the TRIS-barbital matrix was evaluated according to the ISO 15189 standard (criteria listed below).

2.4.1. Reproducibility

Intra-assay reproducibility was evaluated by a 10-fold measurement of patient samples with TSF 111, 1155, and 36900 μg/L, respectively. Inter-assay reproducibility was assessed over two years on low ferritin QC (Biorad).

2.4.2. Linearity

Linearity was evaluated by serial dilution in pH 8 TRIS-barbital buffer of a patient sample with TSF 407.0 μg/L at 87.5%, 75%, 62.5%, 50%, 37.5%, 25%, 12.5%, 6.25%, 3.13%, 1.56% and 0.78%. Recoveries were calculated. Measured and theoretical ferritin were compared using linear regression analysis.

2.4.3. Limit of detection

The limit of detection of the Vista® ferritin assay in the pH 8 TRIS-barbital matrix was calculated from the signal values of 30 measurements of a pH 8 TRIS-barbital buffer blank.

2.4.4. Stability of ferritin in TRIS-barbital buffer

ConA/Seph supernatants from patient samples with TSF 36 and 369 μg/L were centrifuged and stored at +4 °C. Ferritin was measured on days 0, 1, 2, and 3. Recoveries were calculated as compared to GF measured on Day 0.

2.5. Performances of the GF assay

The performance of the assay was evaluated according to the ISO 15189 standard (criteria listed below). Samples were processed as described above.

2.5.1. Reproducibility

Intra-assay reproducibility was evaluated by a 10-fold measurement of low ferritin QC (Biorad) and a patient sample with TSF 5900 μg/L.

Inter-assay reproducibility was assessed over two years on the low ferritin QC (Biorad).

Since GF measurement is a manual method, we also studied inter-operator variability. Five different operators processed six patient samples covering normal and pathological GF values as previously described five times each.

2.5.2. Stability of reagents

GF was determined three-fold using the same reagents on days 0, 7, 14, 21, and 28 in low and high ferritin QC. Mean GF and recoveries as compared to GF on day 0 were calculated.

2.5.3. Inter-laboratory comparison

An inter-laboratory exchange program was organized in 2019 between *Bichat Hospital* (BCH) and the Biochemistry Laboratory of *Hospices Civil de Lyon* (HCL, Lyon, France), which used the same assay [2]. Successively, GF were measured in four and seven samples in each laboratory and reported with a clinical interpretation of data. Samples were categorized into those below and over the 20% GF threshold [8]. Inter-assay variability between BCH and HCL was determined for each sample, and mean variabilities in the two groups were calculated.

2.6. Glycosylated ferritin levels in patients

GF results were extracted from our laboratory's database. It contained 16,843 patients, mainly hospitalized, from 2000 to 2021. These patients were not selected. A cumulative frequencies plot was drawn in Microsoft Excel 2019. The linear range of GF was determined upon visual examination, and the 95% confidence interval for this range was calculated.

2.7. Statistics

Unless specified otherwise, results were interpreted using means, standard deviations (SD), and coefficients of variation (CV).

3. Results

3.1. Binding conditions

3.1.1. Volumes of Sepharose and Concanavalin-A/Sepharose

As shown in Fig. 1, for TSF 67 µg/L, complete binding of GF to ConA was achieved by adding 0.4 mL ConA/Seph. Adding extra ConA/Seph did not lead to a significant change in GF. For TSF 281, 530, 943 and 2257 µg/L, adding 0.4, 0.4, 0.4 and 0.5 mL ConA/Seph, respectively achieved complete binding of GF. Thus, when TSF is in the [30–1000] µg/L range, 0.4 mL ConA/Seph ensures complete GF separation. When TSF ranges between 1000 and 2500 µg/L, 0.5 mL ConA/Seph should be used. Samples with TSF > 2500 µg/L should be pre-diluted in TRIS-barbital buffer before measurement.

3.2. pH of reagents

For the low ferritin QC, GF measured at pH 7.5, pH 8.0, and pH 8.5 were 47.1, 54.1 and 55.3%, respectively. Recoveries were 87.0% at pH 7.5 and 102.2% at pH 8.5. For the high ferritin QC, GF measured at pH 7.5, pH 8.0, and pH 8.5 were 7.7, 10.6 and 10.0%, respectively. Recoveries were 72.8% at pH 7.5 and 94.0% at pH 8.5. pH 7.5 did not allow optimal binding of GF to ConA. Binding was similar at pH 8 and 8.5.

3.3. Incubation time

To shorten the 2 h incubation proposed by Worwood [2], samples with TSF 641 (GF: 70.6%) and 1536 µg/L (42.6%) were incubated for 15, 30, 45, 60, 120, and 180 min with the optimal ConA/Seph volumes. Complete binding of GF to ConA was achieved after a 15 min incubation. Further incubation did not lead to a significant change in GF (Fig. 2).

A summary of the optimal analytical conditions used to perform the

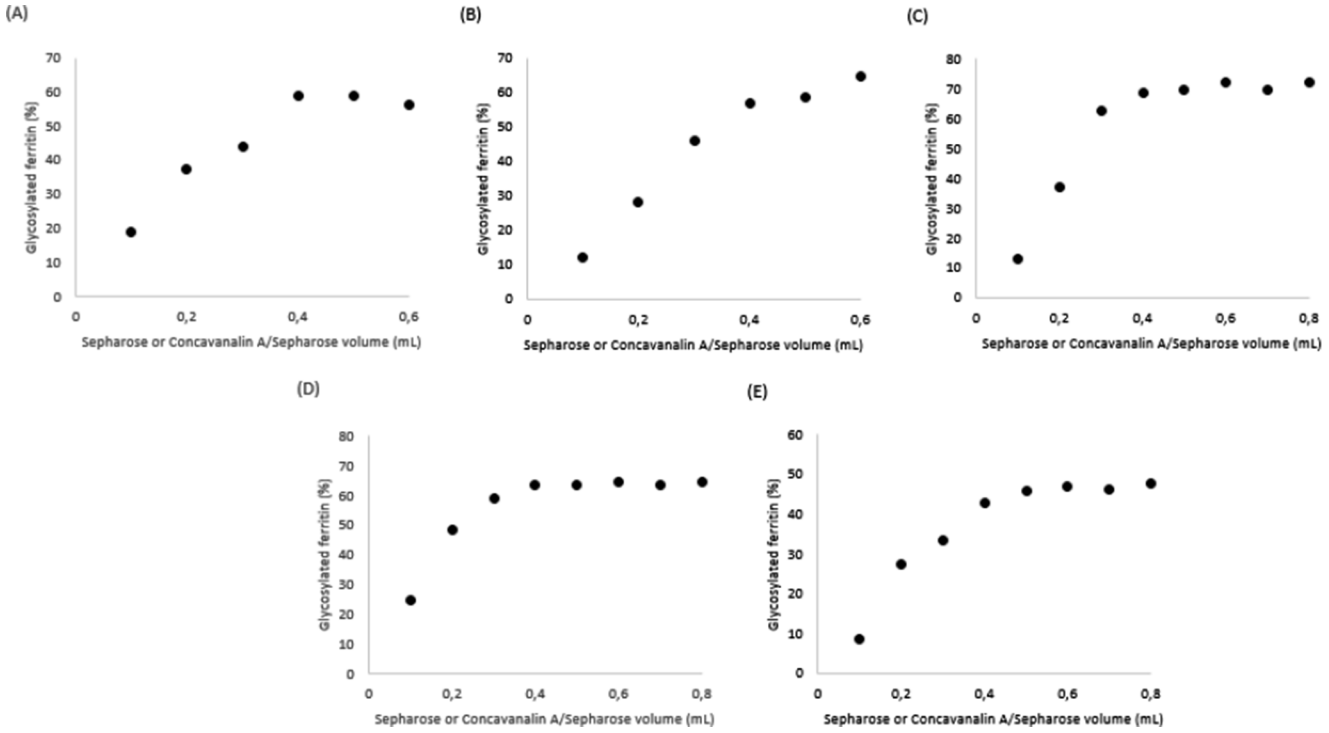

Fig. 1. Glycosylated ferritin (GF) according to Concanavalin A/Sepharose (ConA/Seph) volume. To determine the optimal volumes to be used in the assay according to total serum ferritin (TSF), five patient samples with TSF **(A)** 67, **(B)** 281, **(C)** 530, **(D)** 943, **(E)** 2257 µg/L were incubated with increasing ConA/Seph or Seph volumes in a final incubation volume of 1 mL TRIS-barbital buffer. For TSF 67-281-530-943 µg/L, 0.4 mL ConA/Seph allows binding of all GF. For TSF 2257 µg/L, 0.5 mL ConA/Seph is necessary to bind GF fully.

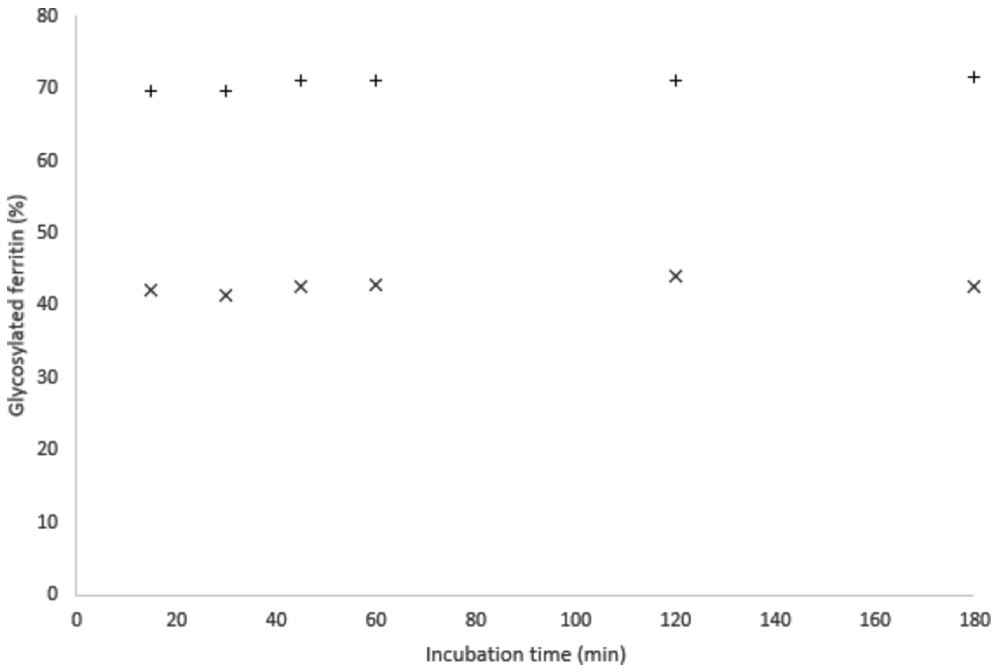

Fig. 2. Glycosylated ferritin (GF) according to incubation time and total serum ferritin. Two samples with GF 70.6% (represented as "+") and 42.6% (represented as "x") were incubated for 15, 30, 45, 60, 120, and 180 min with the optimal ConA/Seph. A 15-min incubation was sufficient to bind all GF in both samples since a longer incubation did not lead to a significant change in measured GF.

GF assay as determined by the previous experiments is provided in Table 1.

3.4. Performances of ferritin measurement in TRIS-barbital buffer

3.4.1. Reproducibility

Intra-assay CV were 0.0%, 0.7% and 0.5% for TSF 111, 1115 and 36900 µg/L respectively (n = 10). Inter-assay CV was 3.2% for the ferritin QC (n = 240).

3.4.2. Linearity

Recovery of ferritin in TRIS-barbital buffer ranged from 78.8% to 115.5% for ferritin concentrations between 3.2 and 355 µg/L. The fitting linear regression curve between theoretical and measured ferritin is represented in Fig. 3 ($R^2 = 0.9992$).

3.4.3. Limit of detection

The mean signal value was 3.160, and the signal SD was 0.149. The limit of detection was 0.073 µg/L.

3.4.4. Stability of ferritin in TRIS-barbital buffer

Recoveries were 100.0% on days 1, 2, or 3 compared to day 0 for both samples.

3.5. Performances of the GF assay

3.5.1. Reproducibility

Intra-assay CV were 4.8% and 7.3% for the QC and patient sample (mean GF: 44.7% [41.2–50.0] and 19.9% [17.4–21.8], n = 10), respectively (Table 2). Inter-assay CV was 8.7% for the QC (mean GF: 43.1% [33.0–53.0], n = 240). Inter-operator variability CV (n = 5) were 21.6%, 8.0%, 13.0%, 6.2%, 6.9% and 19.5% for samples with mean GF 14.1% [12.0–18.2], 28.1% [26.6–31.9], 30.4% [24.3–34.7], 41.2% [38.8–45.3], 53.1 [48.1–57.1] and 62.8% [42.6–72.5], respectively.

3.5.2. Stability of reagents

Recoveries for the low ferritin QC (GF: 49.0%) were 95.8%, 100.0%, 69.3% and 74.4%, on days 7, 14, 21 and 28 respectively, as compared to day 0. At 21 and 28 days, the underestimation of GF was significant and could change the clinical interpretation. Recoveries for the high ferritin QC (GF 10.5%) were 97.0%, 97.0%, 63.7%, and 66.1%, respectively, with no change of the clinical interpretation. Considering the results for both QC, we applied a 14-day expiration for the reagents.

3.5.3. Inter-laboratory comparison

Results of the inter-laboratory comparison program are presented in Table 3. Mean GF for the GF < 20% group were 16.5%, 7.5%, 11.5%, 15.5%, and 21%, respectively, and inter-assay CV were 12.9%, 9.4%, 6.1%, 41.1%, and 20.2% respectively. Mean CV in this group was 17.9%. Mean GF for the GF > 20% group were 34%, 47.5%, 67.5%, 50.5% and 50.5%, respectively and inter-assay CV were 12.5%, 4.5%, 13.6%,

Table 1
Optimal analytical conditions to perform the glycosylated ferritin assay.

	Total serum ferritin (µg/L)					
	<30	30–300	300–500	500–1000	1000–2500	>2500
Serum (mL)	Test not performed	0.25	0.2	0.1	0.1	Pre-dilution necessary
ConA/Seph and Seph (mL)		0.4	0.4	0.4	0.5	
TRIS-barbital buffer pH 8 (mL)		0.35	0.4	0.5	0.4	
Final volume (mL)		1				
Incubation time (min)		15 min				

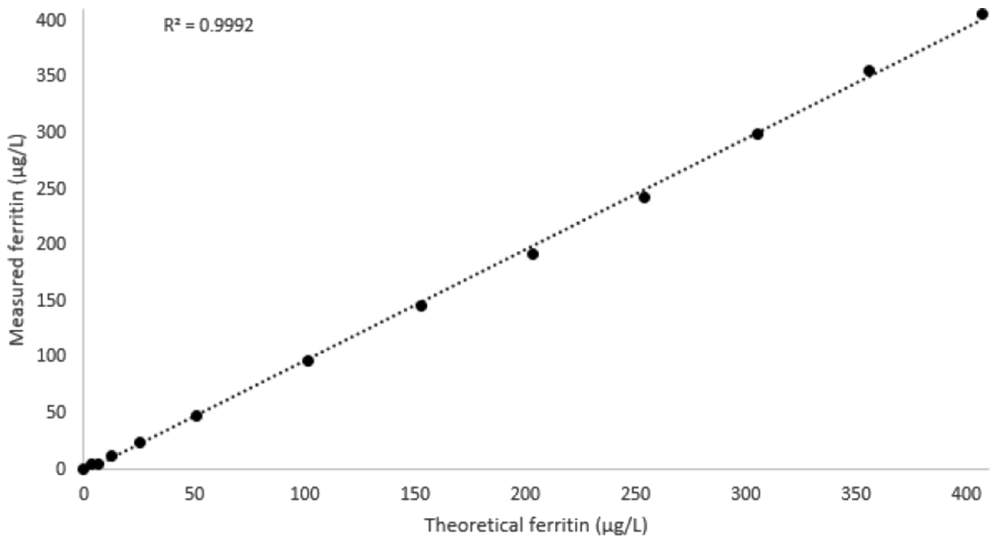

Fig. 3. Measured ferritin concentration according to theoretical ferritin in pH 8 TRIS-barbital buffer. Serial dilutions evaluated linearity in TRIS-barbital buffer of a patient sample with 407 µg/L total serum ferritin. The linear regression curve of measured ferritin according to theoretical ferritin, is shown as a dashed line. The calculated coefficient of determination is $R^2 = 0.9992$.

Table 2
Intra-assay and inter-assay reproducibility of the glycosylated ferritin assay.

	Intra-assay reproducibility		Inter-assay reproducibility
	Ferritin QC	Patient serum	Ferritin QC
n	10	10	240
TSF (µg/L)	72	5900	69
Mean GF (%)	44.7	19.9	43.1
SD	2.1	1.5	3.8
CV (%)	4.8	7.3	8.7
GF range	41.2–50.0	17.4–21.8	33.0–53.0

CV: coefficient of variation, n: number of samples, GF: glycosylated ferritin, SD: standard deviation, TSF: total serum ferritin, QC: quality control.

18.2%, and 9.8% respectively. Mean CV in this group was 11.7%. Only one sample (mean GF = 21%) had a discordant clinical interpretation between HCL and BCH according to the 20% decision level in AOSD.

3.6. Glycosylated ferritin levels in patients

The cumulative frequencies plot of GF in patients from our database is presented in Fig. 4. The curve appears linear between 30 and 85% cumulative frequencies, corresponding to [25–60]% GF, with kinks below and above this range. The 95% confidence interval for these values was [26–58]% lower than values described in the literature for healthy individuals. Less than 20% GF levels were scarce.

4. Discussion

A process to measure GF was initially described by Worwood *et al.* in 1979 [2]. Low GF is a diagnostic marker of AOSD [8,9], also used to monitor therapy [7]. It is also currently a diagnostic marker of MAS and Gaucher disease [10–12]. Furthermore, current trials are undergoing to determine the value of low GF as a sensitive biomarker of severe COVID-19 for prognosis [13,14]. For these many reasons, GF is a hot topic from an analytical point of view in clinical laboratories.

Despite this, no complete assay method description and validation in a clinical environment meeting ISO 15189 demands was published since Worwood *et al.* Here, we described the optimal assay conditions we use, including incubation time, pH, and volumes of ConA/Seph to ensure maximal binding of GF to ConA. All samples with TSF > 30 µg/L could be measured. We decided not to process samples with TSF < 30 µg/L since low ferritin concentrations lead to a significant imprecision when calculating ratios. A pre-dilution is used to measure GF in samples with extreme TSF, frequent in Still's disease and MAS. Thus, unbound ferritin concentrations in the Seph and ConA/Seph samples remain in the Siemens Vista® ferritin assay analytical range (0.5–2000 µg/L in serum). A volume of 0.4 or 0.5 mL ConA/Seph in a final volume of 1 mL allowed complete binding of GF (Fig. 1) (Table 1). pH 8 allowed optimal ConA binding, and GF was underestimated with pH < 8. This observation was in agreement with the initial description of the assay by Worwood *et al.* [2]. Then, we evaluated the possibility of reducing the incubation time, particularly in samples with high TSF. A 15-min incubation was enough to bind all GF even in samples with high TSF, meaning that the assay is

Table 3
Results of the glycosylated ferritin inter-laboratory exchange program between *Bichat* and *Hospices Civils de Lyon* hospitals.

GF (%)	<20						>20				
n	**6**						5				
BCH GF (%)	7	11	11	12	15	18	31	44	49	54	61
HCL GF (%)	8	<14	20	11	18	24	37	57	46	47	74
Mean GF (%)	7.5	n/a	15.5	11.5	16.5	21	34	50.5	47.5	50.5	67.5
SD	0.7	n/a	6.4	0.7	2.1	4.2	4.2	9.2	2.1	4.9	9.2
CV (%)	9.4	n/a	41.1	6.1	12.9	20.2	12.5	18.2	4.5	9.8	13.6
Mean CV (%)	17.9						11.7				

BCH: *Bichat* hospital, HCL: *Hospices Civils de Lyon* hospital, n: number of samples.
Both laboratories determined glycosylated ferritin (GF). Mean, standard deviation (SD), and coefficient of variation (CV) were calculated for each measurement pair. Samples were divided into two groups: those with mean GF < 20% (diagnosis threshold in adult-onset Still's disease) and those >20%. For each group, the mean CV was calculated.

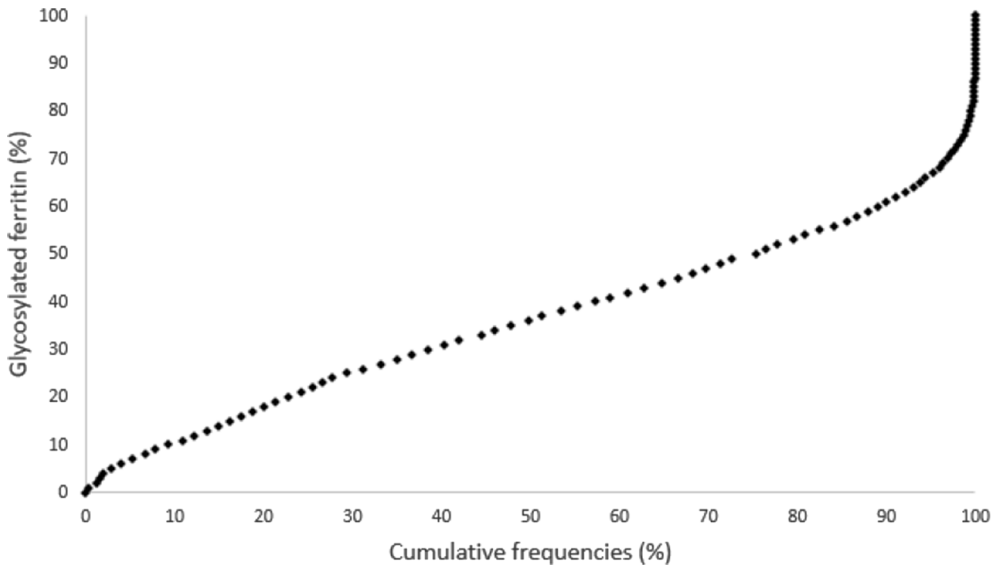

Fig. 4. Cumulative frequencies plot of glycosylated ferritin (GF) measurements collected in our database of 16,843 samples from hospitalized patients and outpatients. The curve appears linear in the [30–85]% range, corresponding to [25–60]% GF, with kinks below and above this range. The 95% confidence interval for these values was [26–58]%.

quick to perform. Finally, excessive ConA volumes or incubation time did not impact GF measure, arguing for the absence of non-specific binding of ferritin to ConA.

After establishing optimal analytical conditions to separate ferritin glycoforms, we evaluated the performances and limits of the assay. Since unbound ferritin is measured in a matrix not evaluated by Siemens Healthineers®, it was first necessary to evaluate the performances of ferritin measurement in the TRIS-barbital buffer. Intra-assay (CV < 1%) and inter-assay reproducibility (CV < 4%) were excellent. We confirmed the linearity of the assay from 3.2 to 407 µg/L ferritin with an absence of bias. The detection limit was 0.073 µg/L, lower than that established by Siemens Healthineers® (0.5 µg/L) and our previous study on serum [15]. Ferritin was stable for three days at 4 °C in TRIS-barbital buffer, when separating and storing supernatants immediately after separation, before GF measurement.

Finally, we validated the performance of the GF assay. Intra-assay and inter-assay reproducibility in patients and QC samples were excellent (CV < 10%), especially for a manual assay. Higher CV was found for inter-operator variability for GF < 20% (CV 21.6%). While this high variability was analytically significant, it was of lesser clinical significance. Indeed, all measurements remained below the 20% threshold used in AOSD diagnosis and thus did not impact the clinical interpretation of results [8]. Marinova et al. [16] reported a brief evaluation of the performances of a GF assay similar to ours. They reported a better inter-assay CV than ours for low GF (CV < 15%). This report was the only one in literature since Worwood *et al.* to describe a GF assay method and performances in a clinical laboratory, to the best of our knowledge. However, this evaluation only focused on 20 determinations and did not assess the effects of time, reagent lots, and operator variability. Thus, this study cannot be used as a basis for comparing performances in the context of ISO 15189 accreditation. Despite these good results, the authors recognized the complexity of the method and considered that it should be performed only in laboratories that have developed the necessary technical skills.

The inter-laboratory comparison program between HCL and BCH Biochemistry laboratories showed good agreement, with acceptable inter-laboratory reproducibility (mean CV 17.9% and 11.7% for samples with GF below and above the 20% threshold, respectively) and the absence of conflicting interpretation according to the 20% decision level but in one sample (mean GF = 21%) (Table 3). Because of this, and because of a noticeable inter-operator variability in samples with GF < 20%, we decided to confirm all measurements of GF < 25% by a second determination and to report the mean of both measurements to the physician. A confirmatory sample could also be requested, although, to date, the GF intra-individual variability remains unknown.

We showed that reconstituted assay reagents were stable for 14 days. ConA binding to GF was reduced when stored longer, resulting in GF underestimation with possible consequences on clinical interpretation. Of note, despite the assay evaluation criteria of ISO 15189, we decided not to evaluate interferences nor inter-sample contamination. Since we measured serum ferritin diluted in TRIS-barbital buffer, our matrix was less susceptible to interferences than serum, and therefore no additional investigations were performed. Regarding interferences, according to the manufacturer's notice, hemolysis, icterus and lipemia did not significantly affect the ferritin assay. Regarding inter-sample contamination, we only used single-use material. Siemens Healthineers®' experiments showed no inter-sample contamination risk in the Dimension Vista 1500® analyser, so we did not conduct additional investigations.

Finally, we aimed to evaluate the levels of GF in patients. On the largest sample published until now, including 16,843 measurements between 2000 and 2021, we found that the 95% confidence interval for GF was [26–58]%. This range was similar to those previously reported in the literature for healthy individuals [5], albeit lower. This can be explained by significant inflammation or hematological malignancies associated with decreased GF [5,17] which are frequent in hospitalized patients. We also receive many samples from patients with Still's disease, MAS, and Gaucher disease, with lower GF. We evidenced that <20% GF is exceptional, agreeing with the low prevalence of AOSD, MAS, and Gaucher disease. Of note, red blood cell transfusion has been shown to increase GF levels [18]. We did not study this phenomenon in this work as transfusions remain marginal in the studied population, so the resulting bias of GF towards higher values would probably be insignificant. We did not propose a reference interval since we did not select individuals based on their conditions or treatments.

Reporting total GF or non-GF in µg/L has been suggested in a few clinical studies [12]. In this work, we decided to study GF as a percentage of TSF exclusively, following standard clinical practices. The increasing relevance of GF and its use in diagnostic and therapeutic algorithms call for the standardization of the method and the development of quality programs. Indeed, if the number of laboratories performing GF assay grows, it could lead to greater variability in reported GF values and possibly erroneous interpretations considering decision thresholds.

In the past, this led to a critical re-evaluation of the analytics of other markers such as creatinine, cystatin, HbA1c, or INR.

In conclusion, our work demonstrated that serum GF could be measured performantly in a clinical laboratory environment thanks to ConA, and with good performances considering ISO 15189 criteria. Using only easy-to-procure, commercially available reagents, we showed that this assay was fast to perform, offered good reproducibility for most samples, and tolerable imprecision for samples with strongly pathological GF. Therefore, this technique may become more widely implemented in hospital laboratories servicing patients with AOSD, MAS (including severe COVID-19 patients), or Gaucher disease.

Authors' contributions

Katell Peoc'h (KP), Anne Boutten (AB), and Alexandre Raynor (AR) designed the research protocol; KP, AB, and AR were involved in treating and collecting data; KP, AB, and AR controlled the accuracy of collected data and conducted the statistical analyses; AR performed experiments; AB and AR wrote the draft of the manuscript, that was then corrected and approved by all authors.

CRediT authorship contribution statement

Alexandre Raynor: Conceptualization, Methodology, Software, Writing – original draft, Writing – review & editing, Data curation, Investigation. **Katell Peoc'h:** Writing – review & editing, Resources. **Anne Boutten:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] F.M. Torti, S.V. Torti, Regulation of ferritin genes and protein, Blood 99 (10) (2002) 3505–3516.
[2] M. Worwood, S.J. Cragg, M. Wagstaff, A. Jacobs, Binding of Human Serum Ferritin to Concanavalin A, Clin. Sci. 56 (1) (1979) 83–87.
[3] M. Worwood, S. Dawkins, M. Wagstaff, A. Jacobs, The purification and properties of ferritin from human serum, Biochem. J. 157 (1) (1976) 97–103.
[4] M.F.A. Bierhuizen, M. Wit, C.A.R.L. Govers, W. Ferwerda, C. Koeleman, O. Pos, W. Dijk, Glycosylation of three molecular forms of human alpha 1-acid glycoprotein having different interactions with concanavalin A. Variations in the occurrence of di-, tri-, and tetraantennary glycans and the degree of sialylation, Eur. J. Biochem. 175 (2) (1988) 387–394.
[5] C. Van Reeth, G. Le Moel, Y. Lasne, M.C. Revenant, J. Agneray, M.F. Kahn, et al., Serum ferritin and isoferritins are tools for diagnosis of active adult Still's disease, J. Rheumatol. 21 (5) (1994) 890–895.
[6] M. Cazzola, G. Bergamaschi, L. Tonon, E. Arbustini, M. Grasso, E. Vercesi, G. Barosi, P.E. Bianchi, G. Cairo, P. Arosio, Hereditary hyperferritinemia-cataract syndrome: relationship between phenotypes and specific mutations in the iron-responsive element of ferritin light-chain mRNA, Blood 90 (2) (1997) 814–821.
[7] S. Vignes, Percentage of glycosylated serum ferritin remains low throughout the course of adult onset Still's disease, Ann. Rheum. Dis. 59 (5) (2000) 347–350.
[8] B. Fautrel, E. Zing, J.-L. Golmard, G. Le moel, A. Bissery, C. Rioux, S. Rozenberg, J.-C. Piette, P. Bourgeois, Proposal for a new set of classification criteria for adult-onset still disease: Proposal for a New Set of Classification Criteria for Adult-Onset Still Disease, Medicine (Baltimore) 81 (3) (2002) 194–200.
[9] D. Lebrun, S. Mestrallet, M. Dehoux, J.L. Golmard, B. Granger, S. Georgin-Lavialle, L. Arnaud, G. Grateau, J. Pouchot, B. Fautrel, Validation of the Fautrel classification criteria for adult-onset Still's disease, Semin. Arthritis Rheum. 47 (4) (2018) 578–585.
[10] L. Fardet, P. Coppo, A. Kettaneh, M. Dehoux, J. Cabane, O. Lambotte, Low glycosylated ferritin, a good marker for the diagnosis of hemophagocytic syndrome, Arthritis Rheum. 58 (5) (2008) 1521–1527.
[11] F. Debaugnies, B. Mahadeb, C. Nagant, N. Meuleman, D. De Bels, F. Wolff, P. Gottignies, A. Salaroli, P. Borde, M. Voué, F. Corazza, Biomarkers for Early Diagnosis of Hemophagocytic Lymphohistiocytosis in Critically Ill Patients, J. Clin. Immunol. 41 (3) (2021) 658–665.
[12] J. Stirnemann, A. Boutten, C. Vincent, A. Mekinian, D. Heraoui, B. Fantin, O. Fain, F. Mentré, N. Belmatoug, Impact of imiglucerase on the serum glycosylated-ferritin level in Gaucher disease, Blood Cells Mol. Dis. 46 (1) (2011) 34–38.
[13] M. Fauter, S. Viel, S. Zaepfel, P. Pradat, J. Fiscus, M. Villard, L. Garnier, T. Walzer, P. Sève, T. Henry, Y. Jamilloux, Low glycosylated ferritin is a sensitive biomarker of severe COVID-19, Cell. Mol. Immunol. 17 (11) (2020) 1183–1185.
[14] https://clinicaltrials.gov/ct2/show/NCT04469153 (accessed on Dec. 6th 2021).
[15] A. Bruneel, M. Dehoux, A. Barnier, A. Boutten, External Evaluation of the Dimension Vista 1500® Intelligent Lab System: Dimension Vista 1500® Evaluation, J. Clin. Lab. Anal. 26 (5) (2012) 384–397.
[16] M. Marinova, M. Nabergoj, C. Artusi, M.M. Zaninotto, M. Plebani, A Case of Atypical Urticaria, Clin. Chem. 62 (4) (2016) 558–560, https://doi.org/10.1373/clinchem.2015.243220.
[17] A.M. Konijn, R. Kaplan, R. Or, Y. Matzner, Glycosylated serum ferritin in patients with hematological malignancies before and after bone marrow transplantation, Leuk. Lymphoma 7 (1–2) (1992) 151–156, https://doi.org/10.3109/10428199209053616.
[18] A. Ishihara, T. Yamauchi, K. Ikeda, Y. Fukuyoshi, T. Yokoyama, Y. Yonemura, M. Uchiba, H. Matsui, Glycosylated ferritin as an improved marker for post-transfusion iron overload, Int. J. Hematol. 113 (4) (2021) 537–546, https://doi.org/10.1007/s12185-020-03056-9. Epub 2021 Jan 5.