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Serum prolactin revisited: parametric reference intervals and cross platform evaluation of polyethylene glycol precipitation-based methods for discrimination between hyperprolactinemia and macroprolactinemia

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Abstract

Background: Hyperprolactinemia diagnosis and treatment is often compromised by the presence of biologically inactive and clinically irrelevant higher-molecular-weight complexes of prolactin, macroprolactin. The objective of this study was to evaluate the performance of two macroprolactin screening regimes across commonly used automated immunoassay platforms.
Methods: Parametric total and monomeric gender-specific reference intervals were determined for six immunoassay methods using female (n = 96) and male sera (n = 127) from healthy donors. The reference intervals were validated using 27 hyperprolactinemic and macroprolactinemic sera, whose presence of monomeric and macroforms of prolactin were determined using gel filtration chromatography (GFC).
Results: Normative data for six prolactin assays included the range of values (2.5th–97.5th percentiles). Validation sera (hyperprolactinemic and macroprolactinemic; n = 27) showed higher discordant classification [mean = 2.8; 95% confidence interval (CI) 1.2–4.4] for the monomer reference interval method compared to the post-polyethylene glycol (PEG) recovery cutoff method (mean = 1.8; 95% CI 0.8–2.8). The two monomer/macroprolactin discrimination methods did not differ significantly (p = 0.089). Among macroprolactinemic sera evaluated by both discrimination methods, the Cobas and Architect/Kryptor prolactin assays showed the lowest and the highest number of misclassifications, respectively.
Conclusions: Current automated immunoassays for prolactin testing require macroprolactin screening methods based on PEG precipitation in order to discriminate truly from falsely elevated serum prolactin. While the recovery cutoff and monomeric reference interval macroprolactin screening methods demonstrate similar discriminative ability, the latter method also provides the clinician with an easy interpretable monomeric prolactin concentration along with a monomeric reference interval.

Keywords: assay interference; hyperprolactinemia; immunoassay; macroprolactinemia; polyethylene glycol precipitation; reference interval.

Introduction

Prolactin is a glycoprotein hormone secreted from the adenohypophysis whose principal function is exerted in the mammary glands by stimulating and sustaining lactation postpartum, as reviewed by Bernard et al. [1]. Elevated blood levels of prolactin (hyperprolactinemia) may have multiple causes including disorders of the hypothalamic-pituitary axis such as prolactin-secreting pituitary adenomas. Also, other conditions such as chronic renal failure, pregnancy, polycystic ovarian syndrome and primary hypothyroidism may cause hyperprolactinemia [2]. Medication such as dopamine antagonists may also lead to elevated prolactin because secretion in the pituitary is normally suppressed by dopamine. Symptoms related to hypersecretion of prolactin are gender specific and include galactorrhea, hypogonadism, infertility, and neurological symptoms such as visual field defects and headache.

Circulating prolactin predominantly exists as a bioactive monomer of 23 kDa that is recognized by the ubiquitously expressed prolactin receptor. Varying degrees of higher molecular weight complexes of prolactin are common in

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human sera and include dimeric prolactin (big prolactin) and prolactin•IgG complexes (big-big or macroprolactin) [3–6] as well as non-immunoglobulin (IgG)-type macroprolactin [7]. The biological activity of these forms, however, is minimal, and clinically, most patients with anti-prolactin autoantibodies lack the symptoms of hyperprolactinemia [1, 8, 9]. Thus, macroprolactin is considered clinically irrelevant and constitutes a challenging analytical problem in laboratory medicine as recently reviewed by the American Association of Clinical Endocrinologists and the American College of Endocrinology [10]. The significance of this challenge is underscored by the fact that the incidence of macroprolatinemia range between 4% and 46% among hyperprolactinemic patients depending on the referral population [11, 12].

While the most effective method (gold standard) in separating macroprolactin from monomeric prolactin is gel filtration chromatography (GFC), this method is not compatible with routine screening of a large number of samples. To this end, the use of polyethylene glycol (PEG)-mediated precipitation has been widely adopted as cost and time effective alternative approach. Currently, the presence of macroprolactin is evaluated by two different screening approaches based on either post-PEG recovery level of monomeric prolactin [12–14] or the use of post-PEG monomeric prolactin reference intervals [15, 16]. It is well known that immunoassay methods display different reactivity toward macroprolactin and that PEG treatment of sera results in co-precipitation of up to 20% of the monomeric prolactin present. Thus post-PEG recovery levels or post-PEG reference intervals (hereinafter called monomeric reference intervals) need to be determined for the specific assay method employed and using a representative healthy population. As manufacturers of prolactin assays constantly develop and improve their products there is a constant need for laboratory (re)evaluation of these tests given the clinical consequences such as imaging and pharmacologic or surgical treatment due to falsely elevated serum prolactin.

The aim of this study was to evaluate the performance of monomeric prolactin reference interval- and monomeric prolactin recovery methods for hyper- and macroprolactinemia discrimination based on PEG precipitation using a panel of contemporary immunoassay methods including one, claimed to possess low sensitivity toward macroprolactin.

Materials and methods

Reference subjects and samples

Blood samples for determination of prolactin reference intervals were collected between 10:00 and 12:00 AM from healthy female (n = 96) and male (n = 128) donors. Only female donors in the fertile age and with regular menstruation were included. Participants who had night work within 7 days from blood sampling were excluded as well as participants (females) who used estrogen-containing contraceptives or were in hormone treatment for menopause. Blood samples were collected into serum gel tubes (Vacutainer 8.5 mL; BD) and left to clot at room temperature for at least 30 min, separated by centrifugation at 200 × *g* for 10 min within 2–3 h, and stored at 4 °C. The following morning each serum samples were divided into aliquots and one half stored untreated and one half subjected to PEG precipitation according to the macroprolactin screening protocol described below. All samples were stored frozen at – 8 °C until assay-specific prolactin measurements were carried out.

Hyperprolactinemic and macroprolactinemic serum samples for validation were obtained from routine analysis and stored at – 80 °C.

All participants provided informed consent and the study was conducted under the guidance of the Ethics Committee for Region of Southern Denmark (S–20162000–76).

Immunoassay methods

Total and post-PEG serum prolactin concentrations were determined in 2013/2014 using Architect prolactin 7K76 recalibrated version (Abbott), AutoDelfia prolactin; ref. B018–301 (PerkinElmer), Cobas e 411 prolactin II; ref. 03303093 190 (Roche), Dimension Vista prolactin; ref. K6462 (Siemens). Immulite 2000 prolactin; ref. L2KPR (Siemens), Kryptor BRAHMS prolactin; ref. 824.050 (Thermo Scientific). All immunoassays were calibrated against the WHO Third International Standard for prolactin (84/500). Prolactin units in mIU/L can be converted to ng/mL or µg/L by dividing by 21.2 for Cobas, Dimension Vista, and Immulite assays; by 21.0 for Architect and Kryptor assays; and by 36 for the AutoDelfia assay.

Interassay CVs for all automated analyzers were determined using Seronorm Immunoassay Liq-1/2 (Sero) and ranged from 2.2% to 7.8% (QC level 1; 148 mIU/L) and from 2.1% to 3.9% (QC level 2; 586 mIU/L).

Macroprolactin screening protocol

Our standard procedure to determine macroprolactin in patient samples is based on post-PEG prolactin (monomer) recovery < 50% analyzed using the AutoDelfia assay [17, 18]. Only samples above the upper limit of the reference interval are considered for macroprolactin testing. Samples with post-PEG prolactin recovery less than 50% are denoted "macroprolactin" and the recovered prolactin concentrations are provided to the clinicians. In short, precipitation using PEG is carried out by mixing 300 µL serum with an equal volume of 25% (wt/vol) PEG 6000 (Merck, product 8.04191.100) for 10 min followed by centrifugation for 30 min at 3000 rpm (1810 × *g*). The supernatant is transferred to a new tube and analyzed along with a non-precipitated but similarly diluted (deionized water) aliquot of the same serum sample.

For macroprolactin classification and validation in this study, sera were classified macroprolactinemic if PEG recovery was < 50% as determined by the AutoDelfia method and prolactin concentration was contained in the reference interval after correction for prolactin monomer content (Table 3 and Supplemental Data, Table S2) as determined by GFC/AutoDelfia analysis.

Gel filtration chromatography

GFC analysis was carried out by equilibrating a Superdex 75 10/300 GL gel filtration column (GE Healthcare) with PBS (137 mmol/L sodium chloride, 2.7 mmol/L KCl, 10 mmol/L sodium phosphate, 2 mM potassium dihydrogen phosphate, pH 7.4) using an ÄKTA Purifier FPLC system (GE Healthcare). Each serum sample was cleared by centrifugation for 5 min at 20.000 × *g* and 100 µL was injected and loaded onto the column and fractionated at a flow rate of 0.5 mL/min. A protein elution profile was obtained at a wavelength at 280 nm and fractions of 0.5 mL were collected and subjected to analysis for prolactin using the AutoDelfia assay. The molecular weight marker proteins (thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12, MW 1350–670,000) used as gel filtration standards (Bio-Rad Laboratories) were analyzed in triplicate to obtain a calibration curve for molecular weight determinations.

Statistical analysis

Data handling and statistical analysis was carried out using Microsoft Excel 2010 and Analyse it for Microsoft Excel 3.90.1 (Microsoft). Data outliers were detected using Dixon's Q-test for outlier detection, as implemented in the RefVal program [19].

Results

Baseline characteristics of healthy female and male serum prolactin and parametric reference intervals for multiple commercial immunoassay methods

Female and male total and monomeric serum prolactin concentrations were determined using six different commercially available automated immunoassay methods. The female data consisted of samples from 96 individuals (age 19–66 years, mean 43 years) and the male data consisted of samples from 128 individuals (age 17–66 years, mean 40 years). The exclusion/inclusion criteria used for enrolling participants in this study were based on common biological causes affecting prolactin levels as described (Materials and methods). One sample was removed from the male panel as an outlier according to Dixon's Q-test. Female and male total and monomeric prolactin data appeared nongaussian distributed according to histograms and Anderson-Darling coefficients (1.93–4.78) as well as differences between mean and median values (Table 1). By log transformation, all data approached a normal distribution and Anderson-Darling coefficients declined (0.23–0.99). The transformed data were hence used to calculate parametric 95% reference intervals for each method investigated as presented in Table 2 and Supplemental Data, Table S1 along with manufacturer provided reference intervals. The upper 97.5th percentile reference limits ranged from 294 mIU/L (Kryptor) to 505 mIU/L (Architect) in females and from 291 mIU/L (Kryptor) to 398 mIU/L (AutoDelfia) in males. Monomeric prolactin reference intervals in females and males, respectively, ranged from 209 mIU/L (Dimension Vista) to 331 mIU/L (Kryptor) and from 194 mIU/L (Dimension Vista) to 303 mIU/L (Kryptor). The mean post-PEG recovery percentage in females and males, respectively, ranged from 61% (AutoDelfia) to 122% (Kryptor) and from 65% (Architect) to 120% (Kryptor).

Validation of monomeric prolactin reference intervals and comparison of macroprolactin screening methods

To evaluate the classification of hyper- and macroprolactinemia using monomeric reference intervals, we collected sera from patients with hyperprolactinemia and macroprolactinemia (n = 27) according to our existing screening regime (Materials and methods). Each sample was subjected to GFC separation, and the resulting fractions were analyzed using the AutoDelfia prolactin method. The resulting chromatogram of a representative hyperprolactinemic sample (S8) shows a major peak at 12 mL and a minor peak at 10 mL of elution volume (Figure 1A). From the molecular weight standard curve (Figure 1A, inset) the calculated native molecular size of the base peak corresponded to monomer prolactin (23–29 kDa) and the minor peak corresponded to dimeric prolactin (53–57 kDa) also referred to as big prolactin. For comparison, a macroprolactinemic sample (S25) yielded an additional prolactin peak at 8 mL which corresponded to macroprolactin (135–171 kDa) also referred to as big-big prolactin (Figure 1B). Using this GFC approach the monomer prolactin percentage was calculated for all 27 validation samples and used to correct total prolactin concentrations for monomeric prolactin content, as determined on each of five immunoassay platforms (Table 3). These data were used to obtain a robust assignment of the samples as true hyper- or macroprolactinemic (Materials and methods and Supplemental Data, Table S2). Sera that were reclassified with respect to the reference classification ranged from 4% (Cobas) to 15% (Architect and Kryptor) when monomeric reference intervals (Table 2) were applied to the validation set (Tables 3 and 4). For comparison to the widely used post-PEG recovery cutoff method where samples are classified

Table 1: Descriptive statistics for total and post-PEG prolactin (mIU/L) in serum samples from healthy male and female donors for the immunoassay platforms indicated.

Method	Minimum	Maximum	Mean	Median	SD	Normality[a]	Donor age[b]
Total prolactin							
Males (n = 127)							40 (17–66)
Architect	56	418	169	150	70	3.03 (0.56)	
AutoDelfia	51	490	185	168	80	2.25 (0.23)	
Cobas	56	460	179	156	76	3.21 (0.71)	
Dimension vista	43	379	146	126	64	3.51 (0.68)	
Immulite	40	392	146	124	67	3.85 (0.72)	
Kryptor	48	336	143	127	59	3.36 (0.65)	
Females (n = 96)							43 (19–66)
Architect	66	512	201	177	87	3.23 (0.54)	
AutoDelfia	75	781	231	200	122	4.78 (0.78)	
Cobas	74	524	208	189	85	2.52 (0.39)	
Dimension vista[c]	57	452	173	153	77	2.91 (0.47)	
Immulite	51	502	169	148	84	4.35 (0.99)	
Kryptor	54	414	154	141	57	1.93 (0.42)	
Post-PEG (monomeric) prolactin							
Males (n = 127)							40 (17–66)
Architect	39	249	108	97	40	2.70 (0.50)	
AutoDelfia	37	273	117	104	46	2.76 (0.60)	
Cobas	39	279	117	104	46	2.60 (0.39)	
Dimension vista	33	228	96	84	39	2.67 (0.44)	
Immulite	52	269	128	116	45	2.37 (0.49)	
Kryptor	64	367	165	149	58	2.53 (0.53)	
Females (n = 96)							43 (19–66)
Architect	51	292	121	111	43	2.73 (0.51)	
AutoDelfia	54	323	132	121	50	2.30 (0.38)	
Cobas	49	316	130	119	49	2.45 (0.43)	
Dimension vista[c]	41	271	107	97	42	2.57 (0.42)	
Immulite	68	354	144	130	51	2.74 (0.63)	
Kryptor	84	428	182	169	63	2.09 (0.33)	

[a]Normality was tested using the Anderson-Darling method. Numbers are given as the A^2 statistic and numbers in parentheses are A^2 statistics based on logarithm transformed data. [b]Donor age is represented as mean and range in parenthesis. [c]Dimension Vista females data (n = 95).

as macroprolactinemic when recovery is below a predetermined recovery cutoff, we calculated the percentage of the samples which would change classification at recovery cutoffs between 40% and 100% (Table 4). Accordingly, the monomeric reference interval method showed elevated discordant classification [mean 2.8; 95% confidence interval (CI) 1.2–4.4] compared to the post-PEG recovery cutoff method (mean 1.8; 95% CI 0.8–2.8). The two monomer/macroprolactin discrimination methods did not differ significantly (p = 0.089). Among macroprolactinemic sera evaluated by both discrimination methods, the Cobas and the Architect/Kryptor prolactin assays showed the lowest and the highest number of misclassifications, respectively. The post-PEG recovery cutoff method improved classification for Architect (40% cutoff), Kryptor (80% cutoff) and Immulite (40% and 50% cutoffs) compared to the monomeric reference interval method.

Comparison of Kryptor and AutoDelfia assay sensitivity to macroprolactinemia

Among the immunoassay methods tested, only prolactin measurements with the Kryptor method is claimed not to be interfered by macroprolactin and hence can be used without performing PEG precipitation of macroforms.

To explore the difference in macroprolactin sensitivity between our current AutoDelfia immunoassay and the Kryptor method, we performed Passing-Bablok regression for total and post-PEG hyperprolactinemic and macroprolactinemic samples (Figure 2A). For the hyperprolactinemic samples, we obtained a high level of agreement between the methods for both total and post-PEG prolactin measurements (Kryptor = $-182 + 0.78 \times$ AutoDelfia, r = 1.00 and Kryptor = $451 + 0.86 \times$ AutoDelfia, r = 1.00). On the contrary, the macroprolactinemic samples showed reduced

Table 2: Parametric reference intervals (95%) for total- and post-PEG prolactin (mIU/L) in serum samples from females and males for each immunoassay platform indicated.

Method	2.5th Lower percentile, mIU/L	97.5th Upper percentile, mIU/L	Manufacturer reported RI, mIU/L	Mean Post-PEG recovery, %
Total prolactin				
Females (n = 96)				
Architect	84 (75–94)	409 (365–458)	109–557	N/A
AutoDelfia	85 (75–97)	505 (445–574)	111–590	N/A
Cobas	89 (79–99)	418 (374–468)	102–496	N/A
Dimension Vista[b]	69 (61–78)	363 (322–409)	47–642	N/A
Immulite	64 (56–72)	367 (323–416)	40–530	N/A
Kryptor	71 (64–78)	294 (265–326)	80–435[a]	N/A
Males (n = 127)				
Architect	71 (64–78)	347 (314–383)	73–407	N/A
AutoDelfia	72 (65–80)	398 (357–443)	73–474	N/A
Cobas	73 (66–81)	372 (335–411)	86–324	N/A
Dimension vista	57 (51–64)	311 (280–346)	53–369	N/A
Immulite	56 (50–62)	317 (284–353)	53–360	N/A
Kryptor	59 (54–66)	291 (264–322)	59–254[a]	N/A
Post-PEG (monomeric) prolactin				
Females (n = 96)				
Architect	58 (53–64)	224 (203–247)	ND	63 (21–81)
AutoDelfia	60 (54–67)	254 (229–282)	ND	61 (15–87)
Cobas	58 (49–70)	257 (222–316)	ND	64 (38–84)
Dimension Vista[b]	48 (43–53)	209 (188–233)	ND	64 (26–80)
Immulite	71 (64–78)	262 (239–288)	ND	91 (34–135)
Kryptor	90 (82–99)	331 (301–363)	ND	122 (65–261)
Males (n = 127)				
Architect	50 (45–54)	208 (190–228)	ND	65 (25–81)
AutoDelfia	51 (46–56)	233 (212–257)	ND	65 (23–91)
Cobas	51 (46–56)	232 (211–255)	ND	66 (25–80)
Dimension vista	41 (37–45)	194 (176–214)	ND	67 (25–91)
Immulite	61 (56–72)	237 (218–258)	ND	88 (34–172)
Kryptor	80 (73–87)	303 (279–330)	ND	120 (45–218)

ND, not determined. [a]5th–95th percentile. [b]Female reference intervals using healthy donors (n = 95) subjected to exclusion criteria as described in Materials and methods (i.e. regular menstrual cycle, no hormonal therapy and no night shift). Numbers in parenthesis are 90% confidence intervals. Post-PEG recovery percentage is given as mean value and range in parenthesis.

agreement ($\text{Kryptor} = 321 + 0.06 \times \text{AutoDelfia}$, $r = 0.49$) in the absence of PEG precipitations and improved agreement ($\text{Kryptor} = -239 + 3.17 \times \text{AutoDelfia}$, $r = 0.71$) after PEG precipitation. Taken together, these results indicate that the two methods possess different sensitivity to macroforms of prolactin.

To further investigate this discrepancy, we compared the immunoreactivity of the two methods toward the different prolactin forms in GFC fractioned serum. The Kryptor method exhibited significantly lower reactivity against macroprolactin compared with the AutoDelfia method using a hyperprolactinemic sample with a significant content of macroprolactin (Figure 2B, Table 3, S12). When analyzing a macroprolactinemic sample with a predominant content of macroprolactin (Figure 2C, Table 3, S20) likewise less macro reactivity was obtained using the Kryptor method but still the vast portion of the total reactivity was achieved for the macro form.

Finally, we addressed the utility of all assay methods in classifying macroprolactinemic sera in the absence of PEG precipitation. We found that all macro sera (12/12) for all assay methods (except for serum S19, Kryptor, Table 3) whose initially elevated prolactin levels were, due to macroforms, classified as hyperprolactinemic along with their corresponding total prolactin reference intervals obtained in this study.

Discussion

In the present study, we have provided gender-specific total and monomeric prolactin reference intervals for six

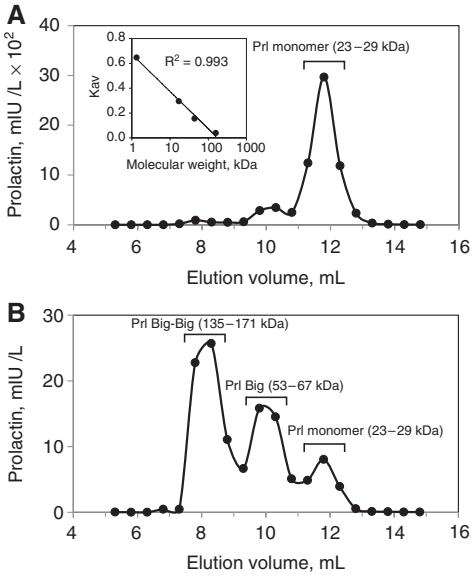

Figure 1: Analysis of prolactin monomer and macro species in serum. Serum (0.1 mL) was subjected to GFC and prolactin immunoreactivity was analyzed in the resulting fractions using the AutoDelfia method. The interpolated molecular weight (kDa) range of each peak fraction is indicated. (A) Serum from a patient (S8, Table 3) with hyperprolactineamia. Inset: GFC standard proteins; Kav plotted against molecular weight (kDa). (B) Serum from a patient with macroprolactinemia (S25; Table 3) causing discordant classification using post-PEG reference intervals. Prl monomer 23–29 kDa, Prl Big 53–67 kDa, Prl Big-Big 135–171 kDa.

commonly used immunoassay platforms in laboratory medicine. Except for male reference intervals for Cobas and Kryptor, the upper limits determined in this study (Table 2) were lower (range 12%–43%) than that reported by the manufacturers. This trend was also reported in an earlier study of multiple prolactin methods, despite the male reference intervals were derived from a smaller sample size [15]. In this study we excluded female donors due to confounding such as irregular menstruations, use of estrogen containing contraceptives or hormone treatment for menopause. Likewise donors who had recently been working at night were excluded (Materials and methods). Total serum prolactin is higher in females than males; accordingly, the female reference interval provided in this study was on average 15% higher than the male reference interval for all methods. Previously published reference intervals for three of the six methods studied here showed a 45% difference between females and males [15] and the manufacturer intervals showed a mean difference of 46%. None of the studies provided any details regarding exclusion criteria for the female participants, indicating that those reference intervals are applicable to a wider range of females.

All monomeric upper reference limits were markedly lower than that obtained for total serum prolactin except for Kryptor which was also reflected in the mean post-PEG recovery which ranged from 65% for Architect and AutoDelfia to 120% for Kryptor. These recoveries reflect not only protein precipitation by PEG (i.e. macroprolactin, big prolactin, and some degree of monomeric prolactin) but also positive or negative PEG interference of the immunoassay per se. While common practice for macroprolactin screening among hyperprolactinemic sera has been reporting the presence of macroprolactin when post-PEG recovery was below a certain cutoff (typically below 40% or 50%) depending on the method used [20], some researchers have advocated for the use of post-PEG monomeric reference intervals [16]. To this end, we evaluated the ability of both approaches to correctly classify hyperprolactinemic and macroprolactinemic sera as determined using GFC analysis (Figure 1 and Tables 3 and 4). We found that while the monomeric reference interval approach had a marginally higher rate of discordant classification for the Architect, Immulite, and Kryptor methods, the AutoDelfia and Cobas methods reclassified equally at low levels for both approaches. Notably, the optimal recovery cutoff for three methods was 40%, whereas the optimal recovery cutoff for Immulite and Kryptor was found to be 50% and 80%, respectively. The latter result was expected due to a higher mean post-PEG recovery found for female and male sera using these methods as previously discussed.

From a clinician's point of view, laboratory reporting of a monomeric (post-PEG) prolactin concentration along with a monomeric reference interval may be more useful and easier to interpret than the reporting of macroprolactin along with a recovered prolactin concentration. Nevertheless, the recovery approach is more easily transferable to laboratories which are not establishing local reference intervals and where the population composition differ significantly from that of which published reference intervals where derived.

Clearly, macroprolactin screening of hyperprolactinemic sera requires more laborious preanalytical handling in the laboratory and thus, methods that are absolutely insensitive to macroprolactin should be preferred. Intriguingly, one of the assays (Kryptor) has been claimed not to detect macroprolactin and hence be used without any PEG precipitation step. Using the validation sera, the macroprolactinemic samples expectedly showed poor agreement in the absence of PEG precipitations and improved agreement after PEG precipitation (removing macroforms), indicating that the two methods possess different sensitivity to macroforms of prolactin. By evaluating two of the macroprolactinemic samples by GFC analysis, this

Table 3: Classification of macroprolactin containing serum using monomeric prolactin reference intervals.

Immunoassay platform			Architect		AutoDelfia		Cobas		Immulite		Kryptor	
ID	Serum classification and gender hyper (H); macro (M) prolactin[a]	GFC monomer prolactin, %[b]	Prolactin, mIU/L[c]									
			Total serum	Post-PEG monomeric	Total serum	Post-PEG monomeric	Total serum	Post-PEG monomeric	Total serum	Post-PEG monomeric	Total serum	Post-PEG monomeric
S1	H (♀)	92	2373	1890 (H)	2592	2016 (H)	2374	1950 (H)	1632	1760 (H)	2016	2457 (H)
S2	H (♀)	74	1008	714 (H)	1080	720 (H)	933	700 (H)	742	784 (H)	840	945 (H)
S3	H (♂)	95	1365	1092 (H)	1440	1080 (H)	1336	1102 (H)	954	1081 (H)	1218	1407 (H)
S4	H (♂)	83	2919	2268 (H)	3240	2376 (H)	2947	2205 (H)	2438	2353 (H)	2499	2751 (H)
S5	H (♀)	86	4452	3990 (H)	5040	3528 (H)	4749	4304 (H)	3880	3731 (H)	3675	4851 (H)
S6	H (♀)	90	6090	4431 (H)	6984	5472 (H)	5894	4982 (H)	4940	4113 (H)	5250	5775 (H)
S7	H (♀)	88	100,569	84,420 (H)	109,872	89,640 (H)	100,276	75,387 (H)	>71,550	64,872 (H)	82,194	70,938 (H)
S8	H (♀)	87	107,058	88,557 (H)	112,248	90,072 (H)	103,329	89,952 (H)	>71,550	63,536 (H)	81,564	74,151 (H)
S9	H (♀)	89	36,246	32,466 (H)	37,512	32,904 (H)	36,591	37,651 (H)	27,009	27,009 (H)	30,450	28,518 (H)
S10	H (♀)	90	23,142	21,567 (H)	24,192	20,304 (H)	23,362	24,316 (H)	16,790	16,133 (H)	19,467	17,976 (H)
S11	H (♀)	90	61,614	56,049 (H)	64,008	59,616 (H)	54,675	53,509 (H)	42,654	50,180 (H)	51,912	47,922 (H)
S12	H (♀)	36	1953	834 (H)	2088	936 (H)	1346	984 (H)	1297	984 (H)	1023	1252 (H)
S13	H (♂)	58	2902	1260 (H)	3456	1440 (H)	2328	1408 (H)	2417	1221 (H)	2071	1724 (H)
S14	H (♂)	41	1810	594 (H)	1944	576 (H)	1170	664 (H)	1297	664 (H)	937	844 (H)
S15	H (♀)	31	1943	273 (H)	2232	288 (H)	1149	293 (H)	1467	293 (H)	725	376 (H)
S16	M (♀)	27	1334	153 (M)	1368	144 (M)	640	134 (M)	1043	195 (M)	510	229 (M)
S17	M (♀)	20	1214	**229 (H)**	1368	**288 (H)**	742	250 (M)	840	**293 (H)**	489	**380 (H)**
S18	M (♀)	12	1401	126 (M)	1584	144 (M)	494	140 (M)	916	157 (M)	298	218 (M)
S19	M (♀)	9	1086	137 (M)	1152	144 (M)	430	153 (M)	598	195 (M)	290	216 (M)
S20	M (♀)	9	1943	141 (M)	2304	144 (M)	742	148 (M)	1272	201 (M)	466	218 (M)
S21	M (♀)	19	2297	**267 (H)**	1872	**288 (H)**	869	**301 (H)**	1645	**352 (H)**	685	**414 (H)**
S22	M (♀)	10	1649	174 (M)	2088	144 (M)	630	187 (M)	1018	225 (M)	384	**395 (H)**
S23	M (♀)	8	1823	143 (M)	2232	144 (M)	570	153 (M)	1085	174 (M)	397	212 (M)
S24	M (♀)	7	2545	**246 (H)**	3240	216 (M)	797	242 (M)	1459	244 (M)	540	317 (M)
S25	M (♀)	16	1252	**405 (H)**	1512	216 (M)	594	187 (M)	907	**452 (H)**	424	**470 (H)**
S26	M (♀)	7	3364	183 (M)	4320	144 (M)	1276	193 (M)	1908	214 (M)	592	242 (M)
S27	M (♂)	35	678	141 (M)	648	216 (M)	483	134 (M)	530	218 (M)	403	**302 (H)**

[a]Serum samples are classified as hyperprolactinemic (H) when immunoassay concentrations corrected for prolactin monomer as determined by GFC/AutoDelfia analysis are higher than the platform specific reference interval. Macroprolactinemic (M) samples are classified as such if PEG recovery is <50% as determined by the AutoDelfia method and prolactin concentration is contained in the reference interval after correction for prolactin monomer content as determined by GFC/AutoDelfia analysis. Gender for macroprolactin cases are indicated in parenthesis (♂, male; ♀, female). [b]Monomer prolactin percentage was calculated based on GFC chromatogram as shown in Figure 1. [c]Method specific classification: Hyperprolactinemia, post-PEG monomeric concentration above upper limit of post-PEG monomeric RI; Macroprolactinemia the post-PEG monomeric prolactin concentration is contained by the method specific post-PEG monomeric reference interval. Discordant classification is indicated in bold and summarized in Table 4.

Table 4: Discordant classification of hyper- and macroprolactinemia as determined using the post-PEG reference interval and post-PEG recovery methods.

Immunoassay platform	Architect	AutoDelfia	Cobas	Immulite	Kryptor
					n (%)
Post-PEG RI method	4 (15)	2 (7)	1 (4)	3 (11)	4 (15)
Post-PEG recovery method					
Recovery cutoff, %					
40	2 (7)	2 (7)	1 (4)	3 (11)	12 (44)
50	4 (15)	4 (15)	1 (4)	1 (4)	9 (33)
60	4 (15)	4 (15)	2 (7)	3 (11)	8 (30)
80	7 (26)	10 (37)	7 (26)	4 (15)	3 (11)
100	16 (59)	15 (56)	13 (48)	10 (37)	9 (33)

difference in macroprolactin sensitivity was confirmed by higher reactivity toward monomeric prolactin by the Kryptor method (Figure 2B and C). However, the method also showed some reactivity toward macroprolactin and failed to classify the macroprolactinemic sera correctly in the absence of PEG precipitation. Thus, our data supports the continued use of PEG precipitation of hyperprolactinemic sera for macroprolactin screening for all of the methods tested in this study.

The incidence of macroprolatinemia in our hospital laboratory setting was estimated to be 10% (n = 924), using a PEG recovery cutoff of 50%. This number may be positively biased because our laboratory receives patient samples from other sites for macroprolactin screening. Other studies have reported very different macroprolatinemia incidences ranging from 4% to 46% [11, 12]. Despite these differences, interference from macroprolactin remains a clinically important challenge for laboratory specialist and endocrinologist and thus, requires the implementation of a validated screening strategy. According to clinical guidelines for the diagnosis and treatment of hyperprolactinemia, pituitary magnetic resonance imaging is recommended to establish the diagnosis and to evaluate the treatment [21]. Thus, patients with macroprolactinemia may be subjected to unnecessary and expensive diagnostic procedures.

The accuracy of the reference intervals provided in this study was limited by the sample size, especially for the female donors. Also, it is likely that the use of exclusion criteria for selecting healthy donors contributed to lower reference intervals and thus being less representative of a general population. In our hospital setting, we recommend the application of those criteria to avoid confounding due to biological cyclic variation. Validation of the monomeric reference intervals was limited by the number of sera selected due to the relatively high number of downstream analyses required for cross-validation on the five platforms including GFC/AutoDelfia. Unfortunately, lack of sufficient validation sample material prevented the evaluation of the Dimension Vista method in relation to macroprolactin screening. Another limitation of this study was the assignment of macroprolactinemia among the sera used for validation. We used sera whose concentrations initially were above the upper reference limit. Then total prolactin concentrations were corrected for actual monomeric prolactin content (as determined by GFC) to become normo-prolactinemic according to the total prolactin reference interval. This strategy could potentially lead to an overestimation of the number of samples classified as macroprolatinemic in our study because the use of a true monomeric reference interval based on GFC analysis of each sample expectedly would be lower due to the discrimination between prolactin isoforms. The use of a monomer-corrected reference interval according to a previous reported upper range of monomeric prolactin of 85% for 21 normal sera [15] did not change classification for any specimen (Supplemental Data, Table S2). Finally, we cannot exclude biased macroprolactin assignment due to flawed AutoDelfia assay recognition of macroforms in the GFC fractions. However, the degree of recovery correlated with the content of macroforms detected using GFC/AutoDelfia analysis (Table 3). Based on the monomeric reference intervals determined in this study, discordant classification ranged 4%–15% for all sera (Tables 3 and 4) and overall agreement of classification was 90%. Taken together, the specificity of the monomeric reference interval method is moderate to high but for sera with post-PEG prolactin concentrations being close to the upper limit, the post-PEG recovery percentage could additionally be used for estimating the magnitude of macroprolactin present in the patient sample.

Current macroprolactinemia screening methods, based on PEG precipitation, perform similarly regarding macroprolactin detection. For clinical use, however, the

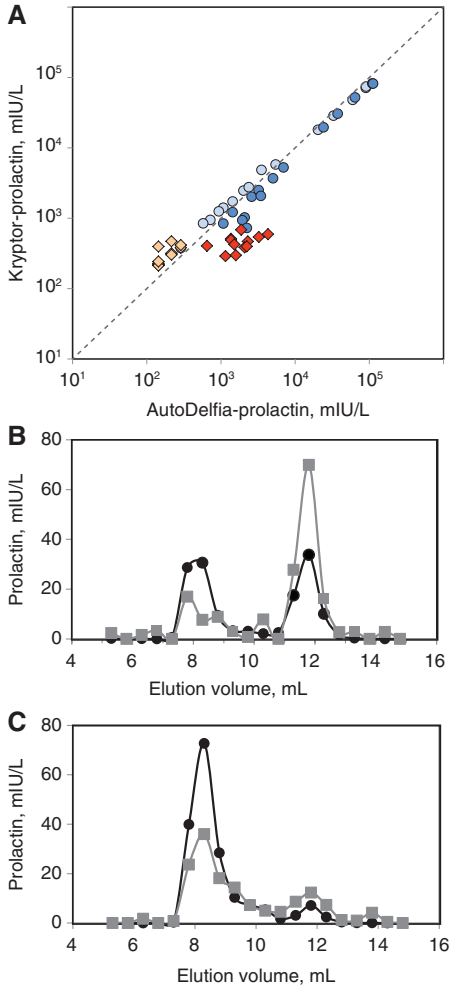

Figure 2: Assessment of macroprolactin sensitivity for Kryptor and AutoDelfia immunoassay methods.
(A) Passing-Bablok regression of prolactin analyzed using the methods indicated on total hyperprolactinemic sera (blue circles; r = 1.00), post-PEG hyperprolactinemic sera (light blue circles; r = 1.00), total macroprolactinemic sera (red squares; r = 0.46), post-PEG macroprolactinemic sera (orange squares; r = 0.64), (B) Prolactin immunoreactivity in GFC fractionated sera (AutoDelfia: grey squares; Kryptor: filled circles) using hyperprolactinemic serum, H12, Table 3 (B). Macroprolactinemic serum, S20; Table 3 (C).

monomeric reference interval method is simpler by providing an easy interpretable monomeric prolactin concentration along with a monomeric reference interval. Among the prolactin assays studied, no assay could accurately classify hyperprolactinemia in the presence of macroprolactin without use of PEG precipitation.

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