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Analytical Interference of Exemestane with Androstenedione Immunoassays

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Background: Exemestane, an aromatase inhibitor commonly used for breast cancer treatment, shares structural similarities with sex steroids analyzed in clinical laboratories. We aimed to investigate the influence of exemestane cross-reactivity in the measurement of sex steroids across various immunoassays.

Methods: We conducted a multicenter study involving measurements of androstenedione, testosterone, estradiol, progesterone, and 17-hydroxyprogesterone in serum samples from women undergoing exemestane therapy (N = 15; 25 mg/day). Measurements were performed using liquid chromatography-mass spectrometry (LC-MS) and various commercially available chemiluminescence immunoassays, ELISA, and radioimmunoassay. *In-vitro* cross-reactivity was assessed by adding exemestane and 17-hydroexemestane to serum samples.

Results: Patients undergoing exemestane therapy had markedly falsely elevated androstenedione results in all immunoassays evaluated (N = 4), which correlated with serum exemestane levels. *In-vitro* experiments confirmed this interference to be caused by cross-reactivity with exemestane. Additionally, one immunoassay yielded falsely elevated estradiol results in 20% of patients. However, *in-vitro* experiments did not confirm this to be caused by cross-reactivity with exemestane or 17-hydroexemestane.

Conclusions: Exemestane cross-reacts with androstenedione immunoassays, causing falsely elevated results in treated patients. This analytical interference may raise unnecessary concerns, leading to expensive diagnostic workups.

Key Words: Cross-reactivity, Exemestane, Hyperandrogenism, Immunoassay, Immunochemiluminescence, Liquid chromatography-mass spectrometry

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INTRODUCTION

Breast cancer is the most prevalent cancer in women. Patients with hormone receptor-positive breast tumors are commonly submitted to endocrine therapy. In this context, exemestane is a widely used aromatase inhibitor that disrupts estrogen production by irreversibly binding to aromatase, the key enzyme converting androgen to estrogen [1].

Current immunoassays for sex steroids lack accuracy because of cross-reactivity between the antibody used in the assays and endogenous or exogenous steroids other than the analyte [2]. Although liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays are recommended because of their higher specificity and sensitivity [3–5], automated immunoassays remain widely used in clinical laboratories. Using an immunoassay, we detected markedly elevated androstenedione levels in a 58-yr-old woman reporting to the Reproductive Endocrinology Clinic of Hospital Universitario Ramón y Cajal, who was being treated with exemestane for breast cancer. Following extensive clinical, imaging, and laboratory investigations, including LC-MS/MS measurements, interference of exemestane with the androstenedione immunoassay was deemed the most likely scenario. Building upon this clinical observation, we investigated the interference of exemestane with androstenedione and other current sex steroid immunoassays, both *in vitro* and *in vivo*, in women undergoing exemestane therapy for breast cancer.

MATERIALS AND METHODS

This multicenter study consisted of two parts: a patient-based study involving patients treated with exemestane and an *in-vitro* study using spiked human serum samples. The study was performed in compliance with the Declaration of Helsinki and was approved by the Ethics and Clinical Research Committee of Vall d'Hebron University Hospital (approval No. EOM: (AG) 060/2023 (6209)). Informed consent was obtained from all patients involved in the study.

In-vivo study of patients treated with exemestane

The study group consisted of 15 women with a mean age of 49 yrs (range, 30–76 yrs) who were being treated with a standard exemestane daily dose of 25 mg for advanced hormone receptor-positive luminal breast cancer. Eleven of the 15 patients were pre-menopausal and also received gonadotropin-releasing hormone analogs (goserelin or triptorelin) as adjuvant therapy. The control group consisted of eight healthy women, four of whom were pre-menopausal. Key exclusion criteria were a history or presence of any major or minor disease related to the hypothalamic–pituitary–gonadal axis and current use of hormone therapy. The mean age of the healthy women was 48 yrs (range 30–72 yrs). All subjects were recruited between April and June 2023 at the Clinical Laboratory of the Vall d'Hebron University Hospital (Barcelona, Spain). Blood samples were collected into gold-top serum separator tubes (Vacutainer), allowed to stand for 30 mins, and centrifuged at 25°C at 3,500×*g* for 10 mins. The serum was aliquoted and immediately frozen at −20°C until analyses, which were conducted within 3 months.

In-vitro study using spiked human serum samples

Exemestane and 17-hydroexemestane were obtained from Merck (Darmstadt, Germany) and Toronto Research Chemicals (North York, ON, Canada), respectively. To investigate the extent of cross-reactivity of exemestane and/or 17-hydroexemestane in sex steroid immunoassays, a set of serum samples was spiked with exemestane or 17-hydroexemestane at the concentrations expected in serum following a 25-mg oral dose of exemestane [6, 7], i.e., at final concentrations of 0.0, 0.4, 4.0, and 40 ng/mL. Specifically, pooled serum was obtained from serum samples of postmenopausal women who were not taking exemestane by combining a fixed volume (0.5 mL) of each serum sample. After spiking of exemestane or 17-hydroexemestane at the indicated concentrations, the serum samples were aliquoted and immediately frozen at −20°C until analyses, which were conducted within 3 months. The concentration of androstenedione in the serum pool was 2.1 nmol/L, as measured using LC-MS/MS.

Hormone assays

Serum levels of androstenedione and four other steroids (testosterone, 17-hydroxyprogesterone, progesterone, and estradiol) were measured using various commercially available immunoassays in both the *in-vitro* and *in-vivo* studies. The immunoassays included Liaison XL from Diasorin (Saluggia, Italy; androstenedione, progesterone), Cobas e801 from Roche Diagnostics (Indianapolis, IN, USA; androstenedione, estradiol, testosterone, progesterone), Maglumi 800 from Snibe (Shenzhen, China; androstenedione, 17-hydroxyprogesterone), Atellica IM1600 from Siemens Healthineers (Tarrytown, NY, USA; estradiol, testosterone, progesterone), Architect i2000 from Abbott (Abbott Park, IL, USA; estradiol, testosterone, progesterone), DRG ELISA (DRG Instruments, Marburg, Germany; 17-hydroxyprogesterone), TECAN IBL ELISA (Tecan IBL GmbH, Hamburg, Germany; 17-hydoxyprogesterone), and DiaSource radioimmunoassay (RIA; Louvain-la-

Table 1. Characteristics of the steroid assays tested as provided by the manufacturers

Steroid	Assay	Manufacturer	Type of immunoassay	Analytical measuring interval	Traceability
Estradiol	Atellica IM 1600 (CLIA)	Siemens Healthineers	Competitive	70–11,010 pmol/L	ID-GCMS Internal reference standard
	Architect i2000 (CLIA)	Abbott	Competitive	92–3,670 pmol/L	ID-GCMS Internal reference standard
	Cobas e801 (ECLIA)	Roche	Competitive	92–11,010 pmol/L	ID-GCMS Certified Reference Material 6004a
	LC-MS/MS	Chromsystems	-	92–36,713 pmol/L	ID-LCMS Certified reference material (uncertainty 1.000 ± 0.005 mg/mL)
Testosterone	Atellica IM 1600 (CLIA)	Siemens Healthineers	Competitive	0.24–52 nmol/L	ID-LCMS Reference material NMIA M914
	Architect i2000 (CLIA)	Abbott	Competitive	0.15–30 nmol/L	GC-MS USP reference standard
	Cobas e801 (ECLIA)	Roche	Competitive	0.42–52 nmol/L	ID-GCMS Reference Measurement Procedure (JCTLM DB Identifier NRMeth 7)
	LC-MS/MS	Chromsystems	-	0.02–83.2 nmol/L	ID-LCMS NIST SRM971 (uncertainty 0.271 ± 0.006 ng/g)
Progesterone	Atellica IM 1600 (CLIA)	Siemens Healthineers	Competitive	0.67–190.8 nmol/L	GCMS Internal reference standard
	Architect i2000 (CLIA)	Abbott	Competitive	0.32*–127.2 nmol/L	Immunoassay Internal reference standard
	Cobas e411 (ECLIA)	Roche	Competitive	0.64–190.8 nmol/L	ID-GCMS Certified reference material BCR-348R and ERM-DA347
	Liaison XL (CLIA)	DiaSorin	Competitive	0.54–190.8 nmol/L	UV-spectrophotometry Internal reference standard (uncertainty 6.94%)
	LC-MS/MS	Chromsystems	-	0.10–79.5 nmol/L	ID-LCMS NIST SRM971 (uncertainty 1.903 ± 0.068 ng/g)
Androstenedione	Cobas e 411 (ECLIA)	Roche	Competitive	1.05–34.9 nmol/L	ID-LCMS Certified reference material NMIA M955
	Liaison XL (CLIA)	DiaSorin	Competitive	1.05–34.9 nmol/L	UV-spectrophotometry Internal reference standard (uncertainty 3.80%)
	Maglumi 800 (CLIA)	Snibe Diagnostics	Competitive	0.2*–34.9 nmol/L	Internal reference standard
	RIA	DiaSource	Competitive	0.1*–38.4 nmol/L	Immunoassay
	LC-MS/MS	Chromsystems		0.1–63 nmol/L	ID-LCMS Certified reference material (uncertainty 1.000 ± 0.005 mg/mL)
17-hydroxyprogesterone	ELISA	DRG solution	Competitive	0.47–60.6 nmol/L	Certified reference material Cerilliant H-085
	ELISA	TECAN IBL International	Competitive	0.09*–60.6 nmol/L	Immunoassay Internal reference standard (Sigma H5752; uncertainty 3.2%)
	RIA	DiaSource	Competitive	0.30–47.2 nmol/L	Certified reference material Cerilliant H-085
	Maglumi 800 (CLIA)	Snibe Diagnostics	Competitive	0.39–90.8 nmol/L	Internal reference standard
	LC-MS/MS	Chromsystems	-		ID-LCMS Certified reference material (uncertainty 1.000 ± 0.005 mg/mL)

The analytical measuring interval corresponds to the range from the lower limit of quantification to the highest measurable value without dilution.
*The lower boundary is the lower limit of detection.
Abbreviations: CLIA, chemiluminescence immunoassay; ECLIA, enzyme-linked chemiluminescence immunoassay; ID-GCMS, isotope dilution gas chromatography-mass spectrometry; ID-LCMS, isotope dilution liquid chromatography-mass spectrometry; JCTLM, Joint Committee for Traceability in Laboratory Medicine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; UV, ultraviolet.

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Neuve, Belgium; androstenedione and 17-hydroxyprogesterone). The characteristics of the immunoassays are described in Table 1. In addition, the five steroids were measured using a CE-IVD-validated LC-MS/MS assay (MassChrom Steroids in Serum/Plasma with Sample Clean Up Columns - LC-MS/MS, Reference 7072/C; Chromsystems, Munich, Germany) using a Dionex UltiMate 3000 RSLCnano System coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Orbitrap Exploris 120; Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. LC-MS/MS was also used to quantify exemestane in serum samples. Standards and QCs were prepared using blank human serum spiked with exemestane. The method was linear from 0.5 to 50 ng/mL of exemestane, and the intra- and inter-assay CV were < 15%. Androstenedione-$^{13}C_3$ served as an internal standard. Fig. 1 illustrates the structural resemblances of the analytes, exemestane and 17-hydroexemestane.

Statistical analysis

Deming regression and Bland–Altman analysis were used to compare the concentrations of all steroids measured with the different immunoassays with those obtained using LC-MS/MS. Linear correlations between serum exemestane levels and bias in the immunoassay measurements were determined using Pearson's correlation coefficient (r). The extent of interference was evaluated using both dose-response experiments *in vitro* and patient specimens, following the CLSI EP07 guidelines [8]. Acceptance criteria for interference testing were based on the biological variation of the analytes [8], with estimates obtained from the European Federation of Clinical Chemistry and Laboratory Medicine biological variation database [9]. The exemestane concentration at which androstenedione interference occurred was estimated using interpolation from the dose-response graph. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Prism Software Inc., San Diego, CA, USA), and the Laboratory Evaluation and Accreditation Process checklist was used wherever applicable [10].

RESULTS

In-vivo study in patients treated with exemestane

We compared the androstenedione concentrations measured in serum samples of patients receiving exemestane or not using LC-MS/MS and four different immunoassays (Liaison XL, Cobas, Maglumi, and DiaSource RIA); the results are shown in Fig. 2A and 2B. Samples from patients treated with exemestane exhibited bias compared to LC-MS/MS that ranged from 22-fold to 309-fold for Liaison XL, from 18-fold to 197-fold for Cobas, and from 23 to 183-fold for Maglumi. In contrast, the bias of these immunoassays for control samples from women not treated with exemestane ranged from 0.8-fold to 2.9-fold. Serum exemestane concentrations significantly correlated with the bias observed in the androstenedione measurements with these immunoassays ($P < 0.05$) (Fig. 2C). When measuring androstenedione using the DiaSource RIA, the bias observed in serum samples of patients treated with exemestane ranged from 1.4-fold to 10.7-fold. This bias was significantly higher than that for non-treated patients only in serum samples with concentrations < 1.5 nmol/L, as determined using LC-MS/MS. In these samples, the bias ranged from 4.5-fold to 10.7-fold and was inversely proportional to the LC-MS/MS-based androstenedione levels (Fig. 2).

The biases for testosterone and 17-hydroxyprogesterone in im-

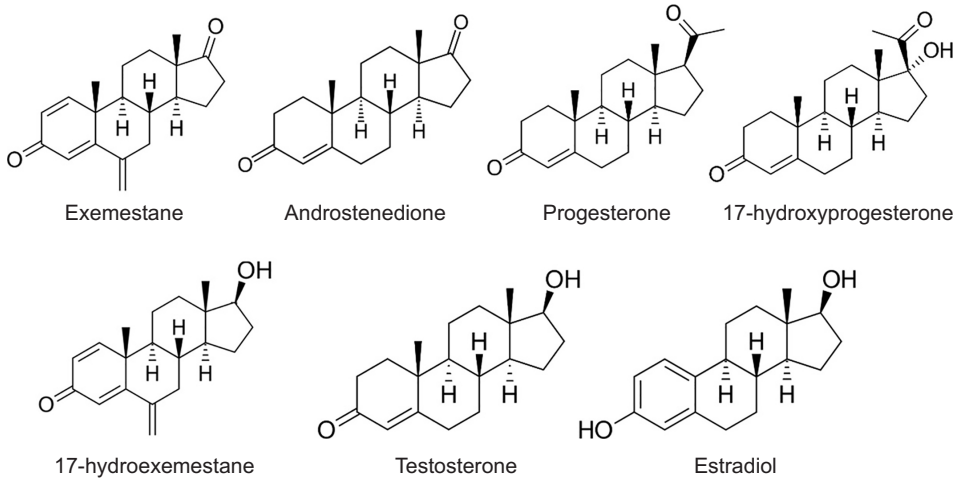

Fig. 1. Molecular structures of exemestane, androstenedione, progesterone, 17-hydroxyprogestrone, 17-hydroexemestane, testosterone, and estradiol.

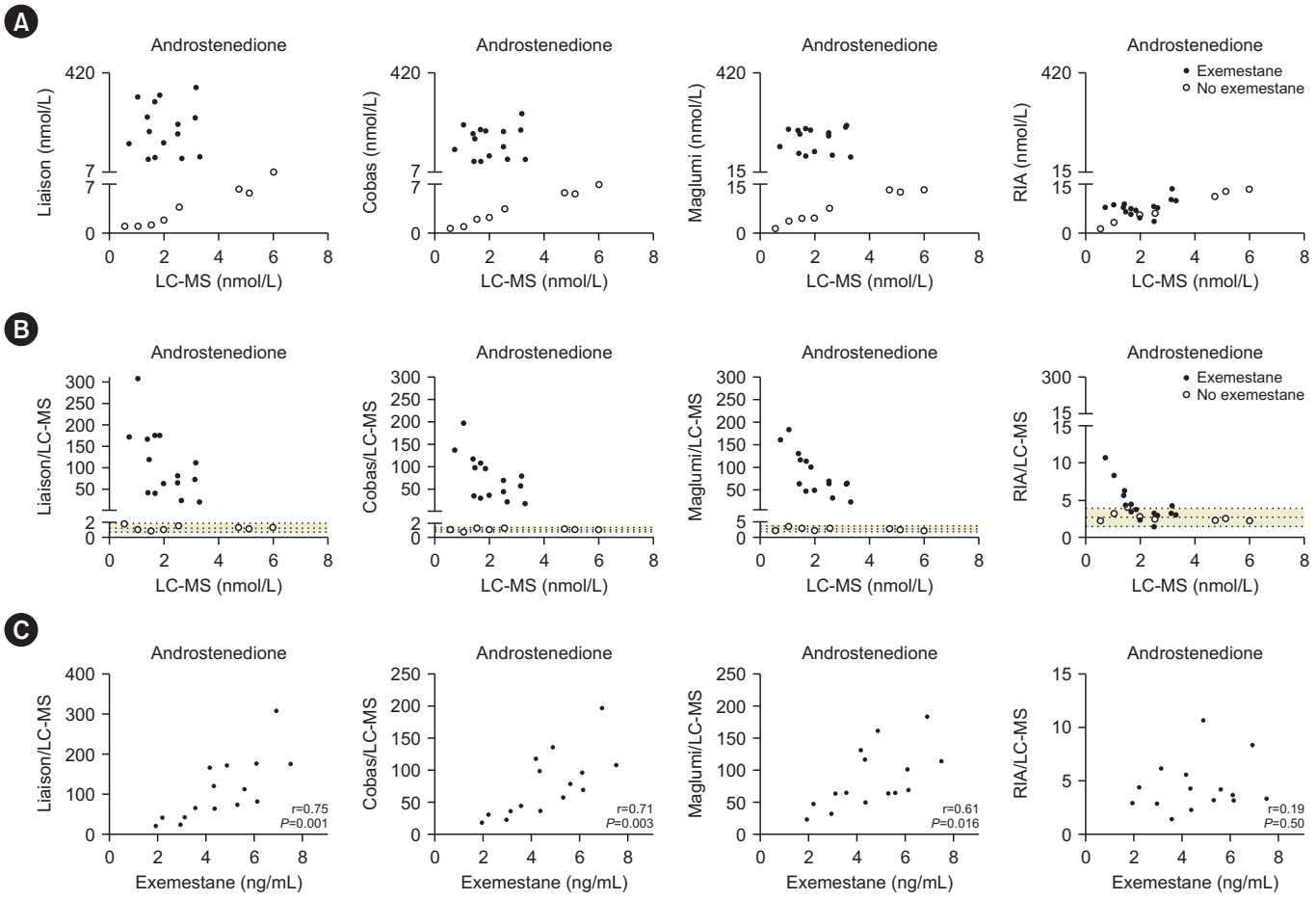

Fig. 2. Correlation (A) and Bland–Altman bias (B) plots of serum androstenedione concentrations measured using Liaison, Cobas, Maglumi, and DiaSource RIA versus LC-MS/MS. Because of the very large differences between the immunoassay and LC-MS/MS results, the ratio versus LC-MS/MS is plotted. (C) Correlation plots of serum exemestane concentrations in treated patients with the bias of androstenedione results for Liaison, Cobas, Maglumi, and DiaSource RIA.
Abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; RIA, radioimmunoassay.

munoassays compared to LC-MS/MS in serum samples from patients treated with exemestane did not differ from those for non-treated control samples (Fig. 3A–3D). Progesterone and estradiol levels were below or close to the detection limit of LC-MS/MS in all serum samples, except for two serum samples from pre-menopausal women not receiving exemestane. This was paralleled by different results for both progesterone and estradiol immunoassays, except for estradiol concentrations obtained with the Architect assay in three patients treated with exemestane, which notably exceeded the limit of detection of the assay (Fig. 3 E and 3F).

In-vitro study

In agreement with the results obtained for samples from patients treated with exemestane, the *in-vitro* spiking of exemestane in serum resulted in a large, concentration-dependent increase in androstenedione results as measured with the Liaison XL Cobas and Maglumi immunoassays. A similar but less pronounced pattern was observed for the DiaSource RIA (Fig. 4). The exemestane concentrations that produced significant interference in androstenedione measurements were estimated to be 0.072 ng/mL (Liaison), 0.068 ng/mL (Cobas), 0.40 ng/mL (Maglumi), and 1.60 ng/mL (DiaSource RIA) (Supplemental Data Fig. S1). Additionally, 17-hydroexemestane had a lower interference effect that was observed only with the Liaison XL and Cobas assays in samples spiked at high concentrations. No significant cross-reactivity was observed for the other steroids in any of the immunoassays evaluated, except for an increase in testosterone results measured with testosterone immunoassays when 17-hydroexemestane was spiked at high concentrations (Fig. 4).

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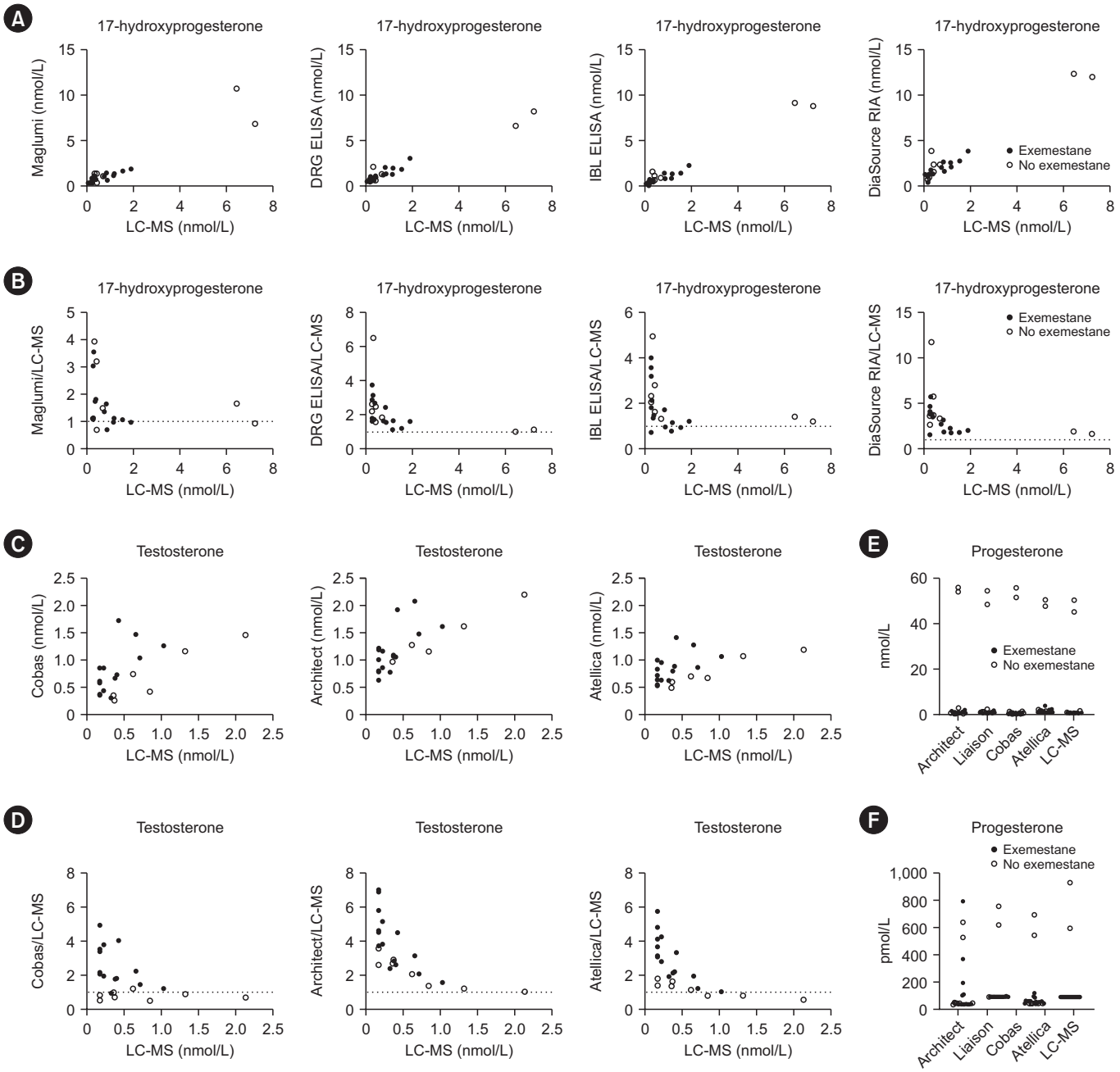

Fig. 3. Correlation (A) and Bland–Altman bias (B) plots of serum 17-hydroxyprogesterone concentrations measured using Maglumi, DRG ELISA, IBL ELISA, and DiaSource RIA versus LC-MS/MS. Correlation (C) and Bland–Altman bias (D) plots of serum testosterone concentrations measured using Cobas, Architect, and Atellica versus LC-MS/MS. Serum concentrations of progesterone (E) and estradiol (F) obtained with different commercial immunoassays versus LC-MS/MS.
Abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; RIA, radioimmunoassay.

DISCUSSION

The main finding of the current study is that exemestane cross-reacts with current serum androstenedione immunoassays. First, the possibility of cross-reactivity was initially raised by a clinical case in which other clinical, imaging, and biochemical evidence of severe hyperandrogenism did not correlate with the substantially high androstenedione levels measured in the patient. The suspicion of cross-reactivity was strongly supported by the finding of normal androstenedione concentrations in the

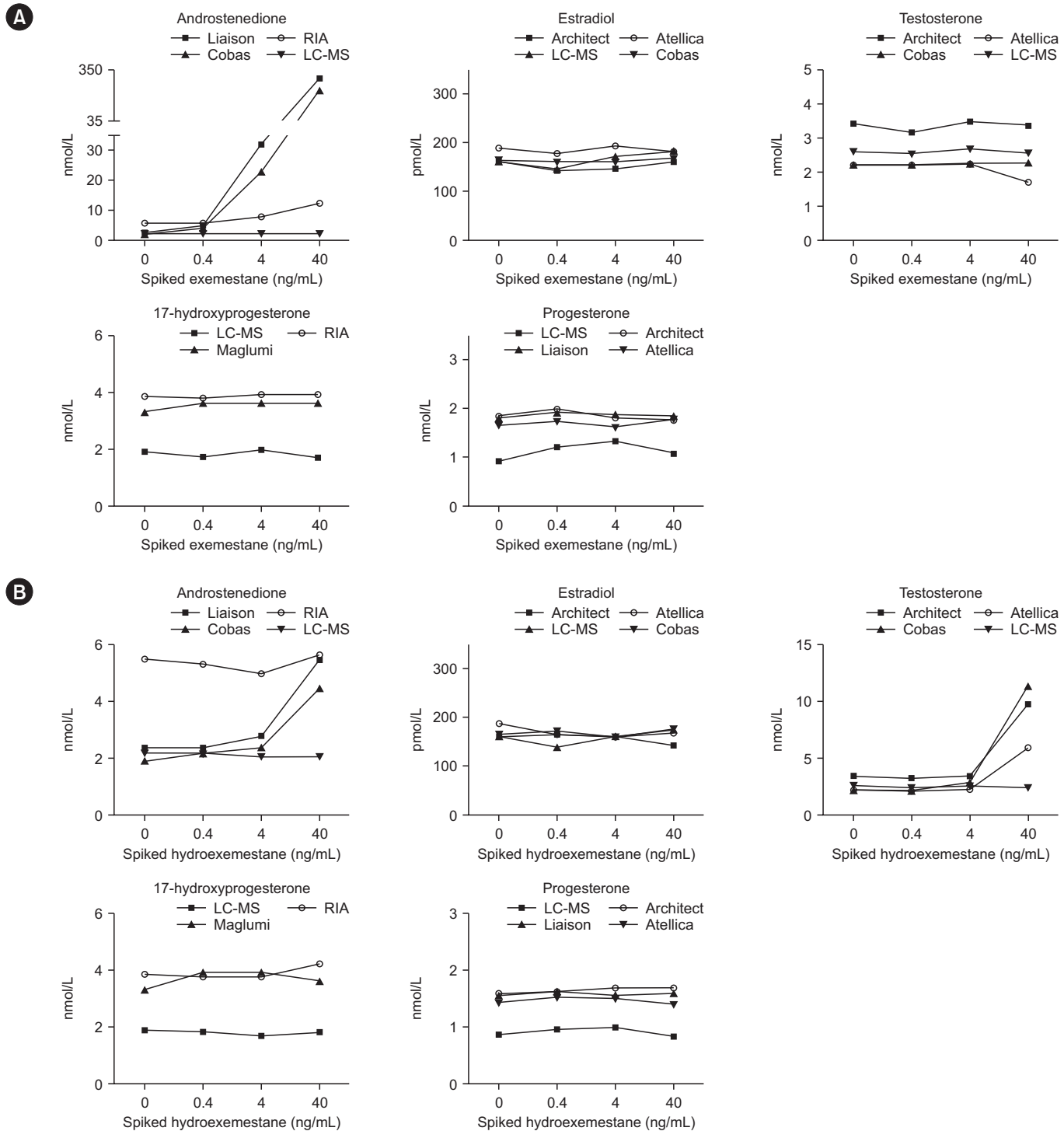

Fig. 4. Serum concentrations of androstenedione, estradiol, testosterone, 17-hydroxyprogesterone, and progesterone obtained with different commercial immunoassays versus LC-MS/MS for pooled sera spiked with exemestane (A) or 17-hydroexemestane (B) at 0, 0.4, 4, and 40 ng/mL.

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same samples when assayed with LC-MS/MS. Second, a very high bias in androstenedione measurements was observed when comparing results from direct chemiluminescence immunoassays with those of LC-MS/MS in serum samples obtained from 15 women undergoing exemestane therapy. Third, this bias positively correlated with the serum levels of exemestane. Fourth, spiking experiments using serum samples confirmed the high cross-reactivity caused by exemestane in immunoassays.

Compared with that for the chemiluminescence immunoassays, the magnitude of the interference in androstenedione measurements was considerably smaller for the DiaSource RIA, likely because of higher antibody specificity and/or reduced exemestane ligation with the RIA protocol. This agrees with the estimated exemestane levels that significantly affected androstenedione measurements in the *in-vitro* study, which were < 0.4 ng/mL for the chemiluminescence immunoassays and 1.60 ng/mL for the DiaSource RIA. However, the fact that the measured exemestane concentrations in the patients in our *in-vivo* study ranged from 1.8 to 7.5 ng/mL suggests that androstenedione measurements will be interfered with in most patients treated with exemestane at the standard dose of 25 mg.

These results support the use of LC-MS/MS for androstenedione measurements in patients receiving exemestane and indicate the need for manufacturers of androstenedione immunoassays to include information on exemestane cross-reactivity (%) in the inserts of the reagents, which is not available in any of the manufacturers' inserts (April 2024). Unawareness of this interference, in addition to unnecessary explorations that may be expensive and time-consuming, may inevitably increase anxiety in a patient who is already dealing with a serious condition such as breast cancer.

In addition to confirming and characterizing the interference of exemestane in androstenedione immunoassays, we evaluated its influence on other sex steroid immunoassays in breast cancer patients undergoing exemestane therapy. In contrast to the falsely increased androstenedione results in patients undergoing exemestane therapy, we did not observe relevant effects on testosterone, 17-hydroxyprogesterone, and progesterone assay results. In line herewith, exemestane added *in vitro* did not show interference in any of the immunoassays evaluated other than androstenedione assays. Although the spiking of 17-hydroexemestane at 40 ng/mL increased testosterone results in all immunoassays, the C_{max} expected for this metabolite in the clinical setting is 10 times lower [7,11], which explains the lack of significant interference in the patient samples. Progesterone levels in all patients undergoing exemestane therapy were < 1.2 ng/mL (< 3.8 nmol/L) for the different immunoassays, and 90% of the results were < 0.5 ng/mL (< 1.6 nmol/L). Additionally, exemestane spiking did not significantly affect any of the immunoassays, including the Cobas assay, for which a two-dimensional molecular similarity prediction raised the possibility of cross-reactivity, potentially leading to apparent concentrations of approximately 0.5 ng/mL [12].

Given that estradiol measurements are used to monitor the efficacy of the pharmacological action, the potential interference of exemestane is of particular clinical relevance [13]. In our study, all treated patients presented estradiol levels below or close to the limit of detection, except for three patients who presented high levels with Architect (Abbott) (195, 371, and 793 pmol/L). In agreement with this finding, Mandic, *et al*. [14] have previously reported a case of a high estradiol level measured using Abbott in a patient undergoing exemestane therapy. In our study, this affected 20% of samples, and we expected this interference to be caused by exemestane or, more probably, 17-hydroexemestane, because of the higher structural similarity of the latter with estradiol (–OH at C_{17}) (Fig. 1). However, no cross-reactivity was observed in the spiking studies, raising the possibility that other minor metabolites may be the cause of interference. In the previous case report [14], a high estradiol level was also found using the Cobas assay. Notably, exemestane spiking experiments performed by Krasowski, *et al*. [12] did not reveal the Cobas assay to have cross-reactivity with exemestane, which is in agreement with our results after spiking up to 40 ng/mL of this drug. In addition, we did not observe cross-reactivity with 17-hydroexemestane. Given that none of the patients in our study presented cross-reactivity with the Cobas assay, the possibility of interference with this assay may be considered low at the standard 25-mg oral dose.

This study had some limitations. We evaluated only a selection of steroids, based on their close structural similarity to exemestane or 17-hydroexemestane and their frequent measurement in clinical laboratories, with several automated assays available. However, other steroids assayed in clinical practice may also pose a risk of cross-reactivity. Another limitation, of the *in-vitro* study, is that we did not evaluate the cross-reactivity of other less abundant metabolites derived from exemestane, such as 6-hydroxyexemestane and 4-hydroxyexemestane. Additionally, the *in-vivo* study was focused on breast cancer patients receiving the standard daily oral dose of 25 mg, as this has been established to be the minimum effective dose for maximum estrogen suppression [15]. Hence, our results may not be extrapolatable to other research or clinical scenarios that may use higher

doses. Notably, exemestane use has been observed in association with abuse of anabolic androgenic steroids [16].

In summary, among patients undergoing 25-mg oral exemestane therapy for breast cancer, androstenedione immunoassays yield falsely elevated concentrations due to cross-reactivity with exemestane. Moreover, some immunoassays may be associated with high estradiol results in a minority of patients, although the exact mechanism underlying this finding remains to be elucidated. Our findings emphasize the imperative need for a comprehensive screening of all sex steroid immunoassays for potential cross-reactivity with exemestane and its metabolites. Considering that such cross-reactivity can significantly influence clinical decisions in managing breast cancer, further investigation is warranted to determine the extent of this issue across various diagnostic assays. Moreover, we strongly advocate the use of LC-MS/MS assays for measuring steroid hormones in routine clinical settings to prevent inaccurate clinical decisions associated with the lesser accuracy of immunoassays. Cross-reactivity in immunoassays may result in undue patient concerns, unwarranted diagnostic techniques, or even inadequate therapeutic decisions for managing breast cancer and other hormone-sensitive malignancies.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2024.0362.

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AUTHOR CONTRIBUTIONS

Conceptualization: Giralt M, Ferrer R, Casals G, and Escobar-Morreale HF. Methodology: Giralt M, Ferrer R, Díaz-Troyano N, Vega B, Luque-Ramírez M, Martínez S, Fernández B, Martínez I, Fabregat A, Urgell E, Casals G, and Escobar-Morreale HF. Writing – original draft: Giralt M, Ferrer R, Díaz-Troyano N, Casals G, and Escobar-Morreale HF. Writing – review & editing: Giralt M, Ferrer R, Díaz-Troyano N, Vega B, Luque-Ramírez M, Martínez S, Fernández B, Martínez I, Fabregat A, Urgell E, Cardona I, Casals G, and Escobar-Morreale HF. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

None declared.

RESEARCH FUNDING

None declared.

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