Comparisons of Immunoassay and Mass Spectrometry Measurements of Serum Estradiol Levels and Their Influence on Clinical Association Studies in Men.

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Comparisons of Immunoassay and Mass Spectrometry Measurements of Serum Estradiol Levels and Their Influence on Clinical Association Studies in Men

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Context: Immunoassay-based techniques, routinely used to measure serum estradiol (E2), are known to have reduced specificity, especially at lower concentrations, when compared with the gold standard technique of mass spectrometry (MS). Different measurement techniques may be responsible for the conflicting results of associations between serum E2 and clinical phenotypes in men.

Objective: Our objective was to compare immunoassay and MS measurements of E2 levels in men and evaluate associations with clinical phenotypes.

Design and Setting: Middle-aged and older male subjects participating in the population-based Osteoporotic Fractures in Men (MrOS) Sweden study (n = 2599), MrOS US (n = 688), and the European Male Aging Study (n = 2908) were included.

Main Outcome Measures: Immunoassay and MS measurements of serum E2 were compared and related to bone mineral density (BMD; measured by dual energy x-ray absorptiometry) and ankle-brachial index.

Results: Within each cohort, serum E2 levels obtained by immunoassay and MS correlated moderately (Spearman rank correlation coefficient r_S 0.53–0.76). Serum C-reactive protein (CRP) levels associated significantly (albeit to a low extent, r_S = 0.29) with immunoassay E2 but not with MS E2 levels. Similar associations of immunoassay E2 and MS E2 were seen with lumbar spine and total hip BMD, independent of serum CRP. However, immunoassay E2, but not MS E2, associated inversely with ankle-brachial index, and this correlation was lost after adjustment for CRP.

Conclusions: Our findings suggest interference in the immunoassay E2 analyses, possibly by CRP or a CRP-associated factor. Although associations with BMD remain unaffected, this might imply for a reevaluation of previous association studies between immunoassay E2 levels and inflammation-related outcomes. (**J Clin Endocrinol Metab 98: E1097–E1102, 2013**)

Immunoassay-based techniques are routinely used in clinical and research settings for the measurement of serum estradiol (E2) levels. They have, however, a questionable specificity, especially at lower E2 concentrations, making this method unreliable in postmenopausal women and men (1–3). Assays based on mass spectrometry (MS) represent the gold standard method for the quantification of E2 in serum samples (4).

E2 has a pivotal role for bone mineral density (BMD) in men, with both cross-sectional and prospective studies describing associations between serum E2, mostly measured by immunoassay-based techniques, and musculoskeletal outcomes (for review see Reference 5). However, the lack of precise MS-based E2 assays may have contributed to the conflicting results regarding the association between E2 and other, nonmusculoskeletal clinical outcomes in men, eg, mortality and cardiovascular disease (CVD) (6–11).

The aim of the present study was to compare serum E2 levels assessed by both immunoassay-based techniques and MS technology in a large number of middle-aged and older men. We hypothesized that measurement of E2 concentrations by MS will be the more informative and reliable method and therefore should be used when investigating putative associations between serum E2 and clinical variables in men.

Materials and Methods

Study sample

The correlations between immunoassay and MS measurements of serum E2 were evaluated in the Osteoporotic Fractures in Men (MrOS) Sweden (n = 2599), MrOS US (n = 688), and European Male Aging Study (EMAS; n = 2908) cohorts. Details of the study sample are given in the Supplemental Material and Supplemental Tables 1–3 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Serum analyses and clinical phenotypes

Serum E2 levels were measured by both immunoassay (RIA or electrochemiluminescence) and MS in each study subject for all 3 cohorts. Details are given in the Supplemental Material and Supplemental Tables 4 and 5. Serum levels of high-sensitivity C-reactive protein (CRP) in MrOS Sweden and EMAS were measured by an immunoturbidimetric and a chemiluminescent immunometric assay, respectively, as described in the Supplemental Material.

Areal BMD of the lumbar spine and the proximal femur was assessed using dual-energy X-ray absorptiometry in MrOS Sweden and EMAS. Ankle-brachial index (ABI) in MrOS Sweden

was calculated for each leg by dividing the posterior tibial systolic pressure by the upper extremity pressure, and the lowest ABI was used. Details of these measurements are given in the Supplemental Material.

The assessment of the covariates in each cohort and of the parameters used for screening in MrOS Sweden is also described in the Supplemental Material.

Statistical analyses

Agreement of serum E2 assay techniques was evaluated by calculating Spearman rank correlation coefficients and visualized using Kernel density plots and Bland-Altman analyses. Associations among the study variables were examined with Spearman rank correlations. The independent associations between serum E2 levels, measured either by immunoassay or MS, and clinical phenotypes (lumbar spine and total hip BMD and ABI) were calculated using multiple linear regression models. Differences in the mean serum E2 levels, measured either by immunoassay or MS, according to quintiles of CRP were assessed using ANOVA followed by a Tukey's post hoc test. Values are given as means $\pm$ SD, unless otherwise indicated.

Results

Characteristics of the study subjects and assays

The baseline characteristics of the study subjects in the MrOS Sweden, MrOS US, and EMAS cohorts are shown in Supplemental Tables 1–3. The subjects from the MrOS Sweden and MrOS US cohorts are older men, whereas the participants in EMAS are middle-aged men. The characteristics of the immunoassays and MS techniques used to analyze serum E2 are presented in Supplemental Tables 4 and 5.

Assessment of agreement and bias between methods for the analysis of serum E2 in men

The MS techniques were cross-calibrated using 50 samples from the MrOS US study and displayed a strong correlation (Spearman rank correlation coefficient $r_S = 0.95$, $P < .001$) (Supplemental Figure 1) (12). However, caution must be taken when interpreting or comparing this concordance level for the 2 MS methods because it was ob-

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tained from a small number of selected samples. For more details, see the Supplemental Material.

When comparing the immunoassay and MS measurements of serum E2 in each of the 3 cohorts, r_S was 0.53 in MrOS US ($P < .001$), 0.64 in MrOS Sweden ($P < .001$), and 0.76 in EMAS ($P < .001$) (3), demonstrating a rather moderate correlation (Figure 1A and Supplemental Figure 2). The distribution of the immunoassay and MS E2 concentrations in the Kernel density plots is shown in Supplemental Figure 3. The Bland-Altman plots showed that in the MrOS Sweden cohort, immunoassay E2 levels were on average slightly higher than those obtained with MS (Supplemental Figure 4A), whereas in the MrOS US cohort, immunoassay E2 levels were somewhat lower than those obtained by MS (Supplemental Figure 4B).

Serum CRP levels associate with serum E2 levels measured by immunoassay

To investigate whether standard population characteristics associated differentially with both E2 assay techniques used, the MrOS Sweden cohort was used as a screening cohort to evaluate the associations between serum E2 levels obtained by either immunoassay or MS and general characteristics, metabolic parameters, CRP, and lifestyle factors (Supplemental Table 6). Importantly, specifically for serum CRP levels, the association differed substantially according to the E2 measurement technique: CRP levels associated significantly (albeit to a low extent) with immunoassay-based E2 levels ($r_S = 0.29$, $P < .001$) but not with the E2 values measured by MS ($r_S = -0.01$, $P = $ NS) in MrOS Sweden (Supplemental Table 6). The

fasting state did not affect this association (fasting serum samples, n = 1797, $r_S = 0.29$, $P < .001$ for the immunoassay method; $r_S = -0.02$, $P = $ NS for the MS method). A similar association between serum CRP levels and E2 levels, measured by immunoassay ($r_S = 0.11$, $P < .001$) but not by MS ($r_S = 0.03$, $P = $ NS) was also observed in the EMAS cohort. Analyzing serum E2, measured either by immunoassay or MS, according to quintiles of CRP, illustrated that with each quintile increment in serum CRP, there is also an increase in mean immunoassay E2 levels but not mean MS E2 values (Figure 1B).

Associations of immunoassay- and MS-based E2 levels with clinical phenotypes

Similar associations of immunoassay E2 and MS E2 were seen with lumbar spine and total hip BMD in both the MrOS Sweden and EMAS cohorts, independent of serum CRP levels (Table 1). As expected, ABI, which is lowered in atherosclerosis-based lower-extremity peripheral arterial disease (13), was negatively associated with serum CRP levels in the MrOS Sweden cohort. Importantly, serum E2 measured by immunoassay, but not by MS, was significantly inversely associated with ABI (Table 1). This association between immunoassay E2 and ABI was lost after adjustment for CRP levels.

Discussion

The 2 MS methods used to measure serum E2 levels in this study revealed a strong correlation, as was shown previ-
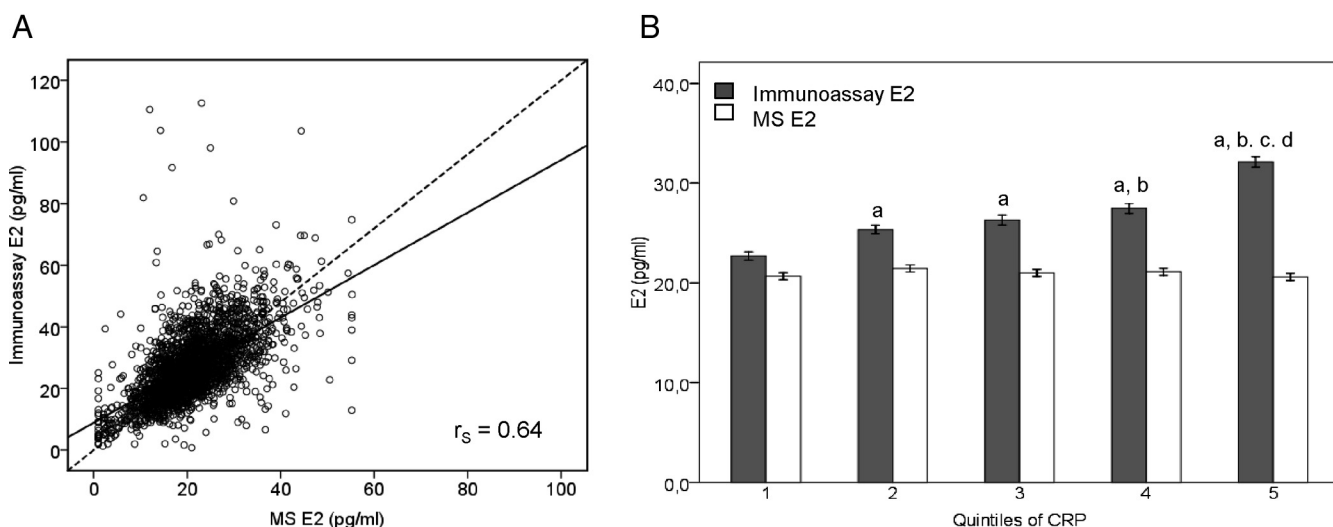
Figure 1. Correlation between immunoassay and MS E2 and relation with CRP levels. A, Scatter plot of the correlation between E2 measured by immunoassay and MS in MrOS Sweden (n = 2599). The Spearman rank correlation coefficient (r_S) as well as the linear regression line (solid line) and the line of full agreement (dashed line) is indicated. B, Mean serum E2 levels (±SE), measured either by immunoassay or MS, according to quintiles of serum CRP in MrOS Sweden. Limits of CRP levels were 1.56 or less for quintile 1, greater than 1.56 and 1.92 or less for quintile 2, greater than 1.92 and 2.44 or less for quintile 3, greater than 2.44 and 3.64 or less for quintile 4, and greater than 3.64 mg/L for quintile 5. a, $P < .05$ vs quintile 1; b, $P < .05$ vs quintile 2; c, $P < .05$ vs quintile 3; d, $P < .05$ vs quintile 4.

Table 1. Independent Associations Between Serum E2 Levels Measured Either by Immunoassay or MS and Sex Steroid-Related Phenotypes

	Model 1		
	Immunoassay E2	**MS E2**	**hsCRP**
Lumbar spine BMD, g/cm^2 per quintile			
MrOS Sweden	0.016 ± 0.003 (<0.001)	0.019 ± 0.003 (<0.001)	0.001 ± 0.003 (NS)
EMAS	0.014 ± 0.004 (<0.01)	0.012 ± 0.005 (<0.05)	−0.006 ± 0.005 (NS)
Total hip BMD, g/cm^2 per quintile			
MrOS Sweden	0.010 ± 0.002 (<0.001)	0.013 ± 0.002 (<0.001)	−0.004 ± 0.002 (<0.05)
EMAS	0.009 ± 0.003 (<0.05)	0.008 ± 0.004 (<0.05)	−0.005 ± 0.004 (NS)
ABI, ratio per quintile			
MrOS Sweden	−0.007 ± 0.002 (<0.01)	0.004 ± 0.002 (NS)	−0.017 ± 0.002 (<0.001)

Abbreviation: hsCRP, high-sensitivity C-reactive protein. Age, BMI, and study center-adjusted multiple linear regression analyses with lumbar spine BMD, total hip BMD, or ABI as the dependent variable and immunoassay E2, MS E2, or hsCRP (model 1), immunoassay E2 and hsCRP (model 2), or MS E2 and hsCRP (model 3) as independent variables were conducted. Immunoassay E2, MS E2, and hsCRP were included as quintiles in the models. SE and β-values are given, with P values in parentheses. The BMD analyses included 2560 subjects from MrOS Sweden and 750 from EMAS. The ABI analyses included 2471 subjects from MrOS Sweden.

ously (12). Evaluation in each cohort of the association between the respective immunoassay and the MS technique showed a poor to moderate correlation, confirming previous reports comparing immunoassay and MS measurements of low serum E2 levels (1, 3). Therefore, the MS technique is considered the method of choice for the measurement of E2 levels in men and postmenopausal women. We went on to evaluate whether certain population characteristics associated differentially with immunoassay and MS E2 values to clarify the moderate association found between the assay techniques. Serum CRP levels, a general marker of inflammation, associated strongly with immunoassay E2 levels but not with MS E2 values. Mean immunoassay E2 levels also increased with each quintile increment in serum CRP, whereas MS E2 levels were unaffected. This suggests interference with the immunoassay-based measurements of serum E2, possibly by CRP or a CRP-associated factor. Previous studies investigating the relation between serum CRP and E2 levels in men provided conflicting results, probably because of different assay methods used (14–17).

We then assessed the impact of this interference on the putative associations between serum E2 levels and clinical phenotypes. Peripheral arterial disease has been reported to be associated with endogenous sex hormone levels (8, 9). In the present study, immunoassay E2 but not MS E2 levels associated inversely with ABI. Importantly, this association was lost after adjustment for serum CRP, suggesting interference with the E2 immunoassay, possibly by CRP or a CRP-associated factor. This implies that the observed inverse relation between serum immunoassay E2 levels and ABI in the present study may be misleading. Also, previous findings of associations between high serum E2 levels and inflammation-related phenotypes such as atherosclerotic CVD (7, 8, 10) may have been the result

of interference with the immunoassay-based E2 levels. Indeed, similar to the lack of association between MS-based E2 levels and ABI in the present study, we found no association between MS-based E2 levels and cardiovascular events in the Swedish MrOS study (11). Therefore, it seems appropriate to suggest a reevaluation of previous association studies between immunoassay-based E2 levels and CVD phenotypes or other inflammation-related end points in studies with MS E2 levels available. In this respect, a recent study by Yeap et al (18) reported a lack of association of low E2 levels measured by liquid chromatography tandem MS and CVD, diabetes, and frailty.

As an alternative sex steroid-dependent phenotype, the relation between serum E2 and BMD was evaluated. We found similar associations between immunoassay E2 or MS E2 and lumbar spine and total hip BMD, regardless of CRP levels. Moreover, Khosla et al (19) confirmed that serum E2 levels analyzed by MS showed a similar correlation with BMD parameters, as previously found for levels measured by a validated high-sensitive RIA. This suggests that there is no detected interference in the relation between serum E2 levels measured by immunoassay and clinical phenotypes when the investigated phenotype is BMD.

We can only speculate on the nature of the interference in the E2 immunoassay. Conventional immunoassay methodology is well known for its limitations, which include the risk of interference from antireagent antibodies, antianalyte antibodies, cross-reactivity with structurally related compounds, matrix effects, and the high-dose hook effect (20). Many naturally occurring proteins in serum such as albumins, complement factors, and CRP can interfere with immunoassays by binding other proteins or substances. The observed interference can be, but does not need to be, related to inflammation. It is also

Table 1. Continued

Model 2		Model 3	
Immunoassay E2	hsCRP	MS E2	hsCRP
0.017 ± 0.003 (<0.001)	-0.004 ± 0.003 (NS)	0.019 ± 0.003 (<0.001)	0.001 ± 0.003 (NS)
0.014 ± 0.004 (0.001)	-0.007 ± 0.005 (NS)	0.012 ± 0.005 (0.01)	-0.006 ± 0.005 (NS)
0.012 ± 0.002 (<0.001)	-0.007 ± 0.002 (<0.001)	0.012 ± 0.002 (<0.001)	-0.004 ± 0.002 (NS)
0.009 ± 0.003 (<0.01)	-0.006 ± 0.004 (NS)	0.009 ± 0.004 (<0.05)	-0.006 ± 0.004 (NS)
0.003 ± 0.002 (NS)	-0.016 ± 0.002 (<0.001)	0.004 ± 0.002 (NS)	-0.017 ± 0.002 (<0.001)

possible that both the E2 and CRP immunoassays were affected by a separate or common interfering or cross-reacting substance or by matrix effects, unrelated to CRP or inflammation. We are, to the best of our knowledge, not aware of any previous reports of CRP-related or inflammation-related interference of immunoassays for other analytes.

This study has several limitations. The results are based on a single measurement of serum E2; therefore, we were unable to test the reproducibility of these measurement techniques. Furthermore, we were not able to compare the more specific indirect, extraction-based immunoassay methods with the MS method. Still we evaluated the direct immunoassays which are most often used clinically and have been the measurement technique of choice in the vast majority of clinical epidemiological studies.

In conclusion, our findings suggest interference in the standard immunoassay-based E2 analyses, possibly by CRP or a CRP-associated factor. Although this interference does not seem to affect association studies between immunoassay E2 levels and skeletal parameters, we propose a reevaluation of previous association studies between immunoassay-based E2 levels and inflammation-related outcomes. In addition, MS-based assays are to be preferred for the quantification of E2 levels in men.

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References

1. **Lee JS, Ettinger B, Stanczyk FZ, et al.** Comparison of methods to measure low serum estradiol levels in postmenopausal women. *J Clin Endocrinol Metab.* 2006;91:3791–3797.

2. **Hsing AW, Stanczyk FZ, Belanger A, et al.** Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. *Cancer Epidemiol Biomarkers Prev.* 2007;16:1004–1008.

3. **Huhtaniemi IT, Tajar A, Lee DM, et al and the EMAS Group** Comparison of serum testosterone and estradiol measurements in 3174 European men using platform immunoassay and mass spectrometry: relevance for the diagnostics in aging men. *Eur J Endocrinol.* 2012; 166:983–991.

4. **Demers LM.** Testosterone and estradiol assays: current and future trends. *Steroids.* 2008;73:1333–1338.

5. **Vandenput L, Ohlsson C.** Estrogens as regulators of bone health in men. *Nat Rev Endocrinol.* 2009;5:437–443.

6. **Arnlov J, Pencina MJ, Amin S, et al.** Endogenous sex hormones and cardiovascular disease incidence in men. *Ann Intern Med.* 2006; 145:176–184.

7. **Tivesten A, Hulthe J, Wallenfeldt K, Wikstrand J, Ohlsson C, Fagerberg B.** Circulating estradiol is an independent predictor of progression of carotid artery intima-media thickness in middle-aged men. *J Clin Endocrinol Metab.* 2006;91:4433–4437.

8. **Tivesten A, Mellstrom D, Jutberger H, et al.** Low serum testosterone and high serum estradiol associate with lower extremity peripheral arterial disease in elderly men. The MrOS Study in Sweden. *J Am Coll Cardiol.* 2007;50:1070–1076.

9. **Haring R, Travison TG, Bhasin S, et al.** Relation between sex hormone concentrations, peripheral arterial disease, and change in ankle-brachial index: findings from the Framingham Heart Study. *J Clin Endocrinol Metab.* 2011;96:3724–3732.

10. **Corona G, Rastrelli G, Monami M, et al.** Hypogonadism as a risk factor for cardiovascular mortality in men: a meta-analytic study. *Eur J Endocrinol.* 2011;165:687–701.

11. **Ohlsson C, Barrett-Connor E, Bhasin S, et al.** High serum testosterone is associated with reduced risk of cardiovascular events in elderly men The MrOS (Osteoporotic Fractures in Men) Study in Sweden. *J Am Coll Cardiol.* 2011;58:1674–1681.

12. **Eriksson AL, Lorentzon M, Vandenput L, et al.** Genetic variations in sex steroid-related genes as predictors of serum estrogen levels in men. *J Clin Endocrinol Metab.* 2009;94:1033–1041.

13. **Pasternak RC, Criqui MH, Benjamin EJ, et al.** Atherosclerotic Vascular Disease Conference: Writing Group I: epidemiology. *Circulation.* 2004;109:2605–2612.

14. **Van Pottelbergh I, Braeckman L, De Bacquer D, De Backer G, Kaufman JM.** Differential contribution of testosterone and estradiol in the determination of cholesterol and lipoprotein profile in healthy middle-aged men. *Atherosclerosis.* 2003;166:95–102.

15. **Nakhai Pour HR, Grobbee DE, Muller M, van der Schouw YT.** Association of endogenous sex hormone with C-reactive protein levels in middle-aged and elderly men. *Clin Endocrinol (Oxf).* 2007; 66:394–398.

16. **Maggio M, Ceda GP, Lauretani F, et al.** Estradiol and inflammatory markers in older men. *J Clin Endocrinol Metab.* 2009;94:518–522.

17. **Kupelian V, Chiu GR, Araujo AB, Williams RE, Clark RV, McKinlay JB.** Association of sex hormones and C-reactive protein levels in men. *Clin Endocrinol (Oxf).* 2010;72:527–533.

18. **Yeap BB, Alfonso H, Chubb SA, et al.** Reference ranges and determinants of testosterone, dihydrotestosterone, and estradiol levels measured using liquid chromatography-tandem mass spectrometry in a population-based cohort of older men. *J Clin Endocrinol Metab.* 2012;97:4030–4039.

19. **Khosla S, Amin S, Singh RJ, Atkinson EJ, Melton LJ 3rd, Riggs BL.** Comparison of sex steroid measurements in men by immunoassay versus mass spectroscopy and relationships with cortical and trabecular volumetric bone mineral density. *Osteoporos Int.* 2008;19: 1465–1471.

20. **Sturgeon CM, Viljoen A.** Analytical error and interference in immunoassay: minimizing risk. *Ann Clin Biochem.* 2011;48:418–432.