SUPPLEMENTAL DATA

Comparison of immunoassay and mass spectrometry for the measurement of serum estradiol levels in men

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Supplemental Text (METHODS)

The MrOS Sweden study was considered to be the primary cohort and was used as a screening cohort for standard population characteristics which were differentially associated with immunoassay and MS E2 values. Next, the associations between immunoassay and MS E2 levels and clinical skeletal (MrOS Sweden, EMAS) and cardiovascular (MrOS Sweden) phenotypes were evaluated, taking C-reactive protein (CRP) levels into account.

Study subjects

The MrOS study is a multicenter, prospective study including older men in Sweden, Hong Kong and the United States. The ***MrOS Sweden*** cohort (n = 3014) consists of three sub-cohorts from three different Swedish cities (n = 1005 in Malmö, n = 1010 in Gothenburg, and n = 999 in Uppsala) and the study subjects (men aged 69–81 years) were randomly identified using national population registers. A total of 45% of the subjects who were contacted participated in the study. To be eligible for the study, the subjects had to be able to walk without aids and not have bilateral hip prostheses. There were no other exclusion criteria (1). The study was approved by the ethics committees at the Universities of Gothenburg, Uppsala and Lund. Informed consent was obtained from all study participants. A total of 2599 subjects with both immunoassay- and MS-based E2 levels available were included in the analyses. These subjects were slightly older (75.5±3.2 versus 75.1±3.0 years of age, $P<0.05$), weighed less (80.5±12.1 versus 82.3±12.0 kg, $P<0.01$) and had lower body mass index (BMI) (26.4±3.6 versus 26.8±3.4 kg/cm2, $P<0.05$) compared to subjects who did not participate.

The ***MrOS US*** study enrolled 5994 participants from March 2000 through April 2002 as previously described (2, 3). Community-based recruitment occurred at six US academic medical centers in Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR; and San Diego, CA. Eligible participants were at least 65 years old, could walk without assistance, and had not had bilateral hip replacement surgery. The institutional review board at each center approved the study protocol, and written informed consent was obtained from all participants. A total of 688 randomly

selected subjects (77% Caucasian, 8% African American, 7% Asian, 6% Hispanic, and 2% other) with both immunoassay- and MS-based E2 levels available were included in the analyses.

The European Male Aging Study (***EMAS***) is a multicenter population-based study of the determinants of male aging. Detailed study design and methodologies of EMAS are described elsewhere (4). Briefly, a total of 3369 community-dwelling men aged 40–79 yr were recruited from population registers in eight European centers: Manchester (UK), Leuven (Belgium), Malmö (Sweden), Tartu (Estonia), Łódz´ (Poland), Szeged (Hungary), Florence (Italy), and Santiago de Compostela (Spain). The men were invited to attend by letter of invitation for an interviewer-assisted questionnaire, assessment of height and weight, several performance measures, and a fasting blood test. Ethical approval for the study was obtained in accordance with local institutional requirements in each center, and participants gave informed consent. A total of 2908 subjects with both immunoassay- and MS-based E2 levels available were included in the analyses.

Serum analyses

Immunoassay E2

MrOS Sweden: Total E2 was measured using a direct radioimmunoassay (RIA) (Orion Diagnostica, Espoo, Finland) with a limit of detection of 1.4 pg/ml. The intraassay coefficients of variation (CVs) are 2.8%, 3.5% and 5.0% at 23.7, 84.7 and 278.2 pg/ml, respectively; the interassay CVs are 5.8%, 8.1% and 9.7% at 25.6, 70.8 and 490.2 pg/ml, respectively (Supplemental Table 4). Only the serum samples from the Gothenburg subcohort were all fasting morning samples whereas the other two subcohorts also included non fasting samples.

EMAS: Fasting blood samples were assayed for E2 by an electrochemiluminescence immunoassay on the Modular E170 platform (Roche Diagnostics, Mannheim, Germany) with a limit of detection of 5.0 pg/ml. The intraassay CVs are 6.1%, 3.5% and 1.3% at 39.1, 79.6 and 878 pg/ml, respectively; the interassay CVs are 7.0%, 4.5% and 1.9% at 39.1, 79.6 and 878 pg/ml, respectively (Supplemental Table 4).

MrOS US: Baseline fasting morning blood was collected. Serum total E2 was measured with a direct RIA [Beckman Coulter (formerly Diagnostic Systems Laboratories), Brea, CA] with a detection limit

of 2.5 pg/ml. The intraassay CVs are 6.5%, 7.6% and 6.9% at 24.9, 40.4 and 92.6 pg/ml, respectively; the interassay CVs are 9.7%, 8.0% and 12.2% at 28.0, 42.3 and 108.7 pg/ml, respectively (Supplemental Table 4).

MS E2

In *MrOS Sweden and EMAS*, the validated GC-MS system at the Laboratory of Molecular Endocrinology and Oncology, Laval University, Québec, Canada (5-8) was used for the analysis of E2 with a limit of detection of 2 pg/ml. The intraassay CVs are 3.5%, 1.2% and 1.4% at 19.9, 199.9 and 299.9 pg/ml, respectively; the interassay CVs are 3.7%, 2.7% and 3.2% at 19.9, 199.9 and 299.9 pg/ml, respectively (Supplemental Table 5). Analytes and internal standard were detected using a HP5973 quadrupole mass spectrometer equipped with a chemical ionization source. Serum samples for sex steroid levels in the MrOS Sweden cohort were available for 99% of the subjects in the Gothenburg cohort and 96% of the subjects in the Malmö cohort, while the one ml required for the GC-MS analyses was only available for 68% of the subjects in the Uppsala cohort. These subjects who had sex steroids analyzed by MS were older (75.7±3.2 versus 74.8±3.0 years of age, $P<0.001$) and weighed less (80.0±11.7 versus 82.2±12.3 kg, $P<0.01$) compared to subjects who did not have sex steroids analyzed by MS.

MrOS US: Serum E2 levels were analyzed using a combined gas chromatographic-negative ionization tandem MS and liquid chromatographic electrospray tandem MS bioanalytical method (Taylor Technology, Princeton, NJ) with a limit of detection of 0.625 pg/ml, as previously described (7, 9). The intraassay CVs are 7.2%, 3.0% and 2.8% at 1.9, 25 and 61 pg/ml, respectively; the interassay CVs are 7.9%, 3.7% and 2.7% at 1.9, 25 and 61 pg/ml, respectively (Supplemental Table 5).

C-reactive protein (CRP)

Serum levels of high-sensitivity CRP (hsCRP) in the *MrOS Sweden* cohort were measured by an ultrasensitive immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland) on a Konelab 20 autoanalyzer (Thermo Fisher Scientific) with an interassay CV below 5%.

In *EMAS*, hsCRP levels were determined using a solid-phase, chemiluminescent immunometric assay [Immulite 2000 hsCRP assay; Siemens Healthcare Diagnostics (formerly Diagnostics Products Corporation), Surrey, UK] with an interassay CV below 3%.

Assessment of bone mineral density (DXA)

MrOS Sweden: Areal bone mineral density (BMD, g/cm^2) of the lumbar spine (L1 to L4) and the proximal femur (total region) was assessed using the Lunar Prodigy DXA (n=2004 from the Uppsala and Malmö cohorts; GE Lunar Corp., Madison, WI, USA) or Hologic QDR 4500/A-Delphi (n=1010 from the Göteborg cohort; Hologic, Waltham, MA, USA). The CVs for the aBMD measurements ranged from 0.5% to 3%. To be able to use DXA measurements performed with equipment from two different manufacturers, a standardized BMD was calculated, as previously described (1). The analyses were also adjusted for study center.

EMAS: aBMD at the lumbar spine (L1 to L4) and the proximal femur (total region) was measured using DXA (QDR 4500 Discovery, Hologic, Inc., Bedford, MA, USA) in two centers (Manchester and Leuven, n=750). The precision errors of these measurements in Leuven were 0.57% and 0.56% at the lumbar spine and total femur region respectively. In Manchester, these precision errors were 0.97% and 0.97% respectively. Both devices were cross-calibrated with the European Spine Phantom.

Assessment of ankle-brachial index

Ankle-brachial index in *MrOS Sweden* was measured as described previously (10). Briefly, with a standard mercury sphygmomanometer and a Doppler probe, we conducted duplicate measures of supine blood pressure in the right arm and both ankles after subjects rested in a quiet room for at least 10 min. The study coordinator placed appropriately sized cuffs over the right upper arm and around each ankle, proximal to the malleolus, rapidly inflated them to 30 mm Hg above the audible systolic pressure, and then slowly deflated them over each artery. A hand-held Doppler (Huntleigh Mini Dopplex Model D900, Huntleigh Healthcare AB, Limhamn, Sweden) recorded the pressure in each artery as the first audible systolic pressure. After performing 2 measurements at each site, we used the mean value of systolic pressure taken first in the right brachial artery and then in the right and left

posterior tibial artery. We calculated ABI for each leg by dividing the posterior tibial systolic pressure by the upper extremity pressure and used the lowest ABI. Because ABI >1.40 might represent falsely high values due to incompressible arteries (11), we excluded subjects with ABI>1.40 from analyses including ABI.

Assessment of covariates

In *MrOS Sweden*, height was measured using a Harpenden stadiometer and weight was measured by an electric scale. In the *EMAS* study, height and weight were measured using standard, calibrated instruments. In *MrOS US*, height was measured using a Harpenden stadiometer and weight was measured by a balance beam or digital scale. BMI was calculated in all three cohorts according to the formula BMI (kg/m^2) = weight (kg)/height $(m)^2$.

In the initial screening in the MrOS Sweden cohort, we also used the following variables:

Fasting serum insulin was measured with an immunometric method based on chemiluminescence technology on a ADVIA Centaur (Bayer AB, Solna, Sweden; interassay CV <10%). Fasting plasma glucose was quantitated by an enzymatic method on a Modular (Roche, Gothenburg, Sweden; interassay CV <4%). Serum lipid analysis was performed on a Konelab 20 autoanalyzer (Thermo Electron Corp., Vantaa, Finland). Total cholesterol and triglyceride levels were determined in human fasting serum by fully enzymatic techniques. High density lipoprotein (HDL) was determined after precipitation of apolipoprotein (Apo) B-containing lipoproteins with magnesium sulfate and dextran sulfate. Low-density lipoprotein (LDL) was calculated using Friedewald's formula. ApoB and ApoA1 were determined by immunoprecipitation enhanced by polyethylene glycol at 340 nm. Interassay CVs were less than 5% for all Konelab analyses. We used a standardized questionnaire to gather information about amount of physical activity (12) and smoking (13). Physical activity was the subject's average total daily walking distance, including both walking as a means of exercise and leisure and as a means of outdoor transportation in activities of daily life.

Supplemental Text (RESULTS)

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

Serum E2 concentrations were measured in the same individual by both immunoassay and MS for a large number of subjects in all cohorts. The two 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<0.001; mean E2 Québec laboratory 19.8 ± 6.6 pg/ml versus mean E2 Taylor laboratory 21.8 ± 7.7 pg/ml) (Supplemental Fig. 1A and (7)). Comparing the Québec E2 MS with the Taylor E2 MS, Bland-Altman analysis revealed a mean bias of 1.9 pg/ml for the Taylor E2 MS (Supplemental Fig. 1B), which proved significantly different (P value for difference from paired sample T-test <0.001).

SUPPLEMENTAL TABLE 1. Basic study subject characteristics in the three cohorts.

	MrOS Sweden (n = 2599)	**MrOS US (n = 688)**	**EMAS (n = 2908)**
Age (years)	75.5 ± 3.2	72.9 ± 5.7	60.0 ± 11.0
Height (cm)	174.7 ± 6.5	174.2 ± 6.8	173.1 ± 7.3
Weight (kg)	80.5 ± 12.1	83.3 ± 13.4	83.3 ± 14.1
BMI (kg/m^2)	26.4 ± 3.6	27.4 ± 3.8	27.8 ± 4.1
Serum immunoassay E2 (pg/ml)	26.8 ± 11.2	17.9 ± 6.0	25.2 ± 7.8
	(25.2, 19.5-32.6, 1.4-112.6)#	(17.3, 14.3-20.5, 3.1-74.7)	(24.4, 19.9-29.3, 5.1-79.1)
Serum MS E2 (pg/ml)	21.0 ± 8.0	23.1 ± 8.9	20.1 ± 6.9
	(20.3, 15.9-25.3, 2.0-55.3)*	(21.8, 17.4-27.4, 1.3-90.6)	(19.1, 15.4-23.7, 2.7-59.6)

Values are given as mean ± SD; for E2 (estradiol), median, interquartile range, and observed range are also given between brackets. MS = mass spectrometry.
Two subjects had serum E2 levels below the limit of detection.
* Twenty-two subjects had serum E2 levels below the limit of detection.

SUPPLEMENTAL TABLE 2. Detailed study subject characteristics in MrOS Sweden.

	MrOS Sweden (n = 2599)
n = 2599	
Smoking (%)	8.6
Physical activity (km/day)	3.9 ± 3.1
Grip strength (kg)	39.9 ± 7.6
Serum hsCRP (mg/l)	4.7 ± 9.9 (2.1, 1.6-3.2)
Lumbar spine BMD (g/cm^2)	1.14 ± 0.20
Total hip BMD (g/cm^2)	0.93 ± 0.14
ABI	1.1 ± 0.2
n = 968	
Serum insulin (mU/ml)	10.6 ± 11.0
Serum glucose (mmol/l)	5.8 ± 1.4
Serum cholesterol (mmol/l)	5.44 ± 1.04
Serum triglycerides (mmol/l)	1.46 ± 0.79
Serum HDL (mmol/l)	1.27 ± 0.36
Serum LDL(mmol/l)	3.51 ± 0.96
Serum ApoA1 (g/l)	1.62 ± 0.32
Serum ApoB (g/l)	1.07 ± 0.24
Serum ApoB/ApoA1	0.68 ± 0.18

Values are given as mean ± SD unless indicated otherwise; for hsCRP (high-sensitivity C-reactive protein), median and interquartile range are also given between brackets. BMD = bone mineral density, ABI = ankle-brachial index.

SUPPLEMENTAL TABLE 3. Detailed study subject characteristics in EMAS.

	EMAS
Serum hsCRP (mg/l) (n = 2908)	4.5 ± 8.4 (2.3, 1.2-4.8)
Lumbar spine BMD (g/cm^2) (n = 750)	1.05 ± 0.18
Total hip BMD (g/cm2) (n = 750)	1.01 ± 0.15
ABI	NA

Values are given as mean ± SD; for hsCRP (high-sensitivity C-reactive protein), median and interquartile range are also given between brackets. BMD = bone mineral density, ABI = ankle-brachial index, NA = not available.

SUPPLEMENTAL TABLE 4. Methodology and characteristics of the E2 immunoassays.

	MrOS Sweden	*MrOS US*	*EMAS*
E2 Immunoassay			
Type of immunoassay	ultrasensitive RIA (competition immunoassay)	ultrasensitive RIA (competition immunoassay)	ECLIA (sandwich immunoassay)
Manufacturer	Orion Diagnostica	Beckman Coulter (formerly Diagnostics Systems Laboratories)	Roche Diagnostics
Sample volume	200 µl	200 µl	100 µl
Measurement range	2.7-545 pg/ml	2.5-750 pg/ml	5.0-4298 pg/ml
Limit of detection	1.4 pg/ml	2.5 pg/ml	5.0 pg/ml
Intra-assay CV			
low concentration, number of samples, CV	23.7 pg/ml, 10 replicates, 2.8%	24.9 pg/ml, 12 replicates, 6.5%	39.1 pg/ml, 20 replicates, 6.1%
medium concentration, number of samples, CV	84.7 pg/ml, 10 replicates, 3.5%	40.4 pg/ml, 12 replicates, 7.6%	79.6 pg/ml, 20 replicates, 3.5%
high concentration, number of samples, CV	278.2 pg/ml, 10 replicates, 5.0%	92.6 pg/ml, 12 replicates, 6.9%	878 pg/ml, 20 replicates, 1.3%
Inter-assay CV			
low concentration, number of runs, CV	25.6 pg/ml, duplicates in 10 runs, 5.8%	28.0 pg/ml, duplicates in 8 runs, 9.7%	39.1 pg/ml, 20 samples, 7.0%
medium concentration, number of runs, CV	70.8 pg/ml, duplicates in 10 runs, 8.1%	42.3 pg/ml, duplicates in 8 runs, 8.0%	79.6 pg/ml, 20 samples, 4.5%
high concentration, number of runs, CV	490.2 pg/ml, duplicates in 10 runs, 9.7%	108.7 pg/ml, duplicates in 8 runs, 12.2%	878 pg/ml, 20 samples, 1.9%
Sample preparation	none	none	none
Linearity with and without dilution	Serum samples with high E2 concentrations may be diluted up to 1:100.	Serum samples with high E2 concentrations may be diluted up to 1:64.	Serum samples with high E2 concentrations may be diluted up to 1:100
Recovery	Known amounts (2.5 - 480 pg/ml) of estradiol were added to serum samples which contained 22 - 120 pg/ml estradiol. Recoveries ranged from 88% to 117%, with a mean value of 102%.	Three samples containing 9.5 - 121 pg/ml estradiol were spiked with known quantities of estradiol (5.0 - 125 pg/ml). The recovery percentages ranged from 86% to 126%.	NA
Interferences	Serum bilirubin concentrations ≤ 170 µmol/l do not interfere, serum haemoglobin concentrations up to 10 g/l do not interfere, highly lipemic samples are not recommended.	Haemolyzed and lipemic specimens should not be used.	Serum bilirubin concentrations ≤ 1129 µmol/l do not interfere, serum haemoglobin concentrations up to 1 g/dl do not interfere, intralipid <1000mg/dl do not interfere.
Cross-reactivity	Estrone, 0.97%; estriol, 0.44%, other endogenous steroids tested were undetectable	Estrone, 2.4%; estriol, 0.64%; other endogenous steroids tested were undetectable (Endocrinol 2011 + guide)	Estrone, 0.81%; estriol, 0.22%, other endogenous steroids tested were undetectable.

RIA = radioimmunoassay, ECLIA = electrochemiluminescence immunoassay, CV = coefficient of variation, NA = not available.

SUPPLEMENTAL TABLE 5. Methodology and characteristics of the E2 MS assays.

	MrOS Sweden and EMAS	***MrOS US***
E2 MS		
Type of MS	GC-MS	GC-MS
Laboratory	Québec (CHUL)	Taylor Technology
Sample volume	0.75 ml	1 ml
Measurement range	2-400 pg/ml	0.625-80 pg/ml
Limit of detection	2 pg/ml	0.625 pg/ml
Intra-assay CV		
low concentration, number of samples, CV	19.9 pg/ml, 6 replicates, 3.5%	1.9 pg/ml, 6 replicates, 7.2%
medium concentration, number of samples, CV	199.9 pg/ml, 6 replicates, 1.2%	25 pg/ml, 6 replicates, 3.0%
high concentration, number of samples, CV	299.9 pg/ml, 6 replicates 1.4%	61 pg/ml, 6 replicates, 2.8%
Inter-assay CV		
low concentration, number of runs, CV	19.9 pg/ml, duplicates in 18 runs, 3.7%	1.9 pg/ml, duplicates in 17 runs, 7.9%
medium concentration, number of runs, CV	199.9 pg/ml, duplicates in 18 runs, 2.7%	25 pg/ml, duplicates in 18 runs, 3.7%
high concentration, number of runs, CV	299.9 pg/ml, duplicates in 18 runs, 3.2%	61 pg/ml, duplicates in 16 runs, 2.7%

GC-MS = gas chromatography – mass spectrometry, CV = coefficient of variation.

SUPPLEMENTAL TABLE 6. Association analysis between serum E2 levels measured by immunoassay or MS and standard population characteristics in MrOS Sweden.

	MrOS Sweden	
	Immunoassay E2	**MS E2**
n = 2599		
Age	-0.04 (NS)	-0.05 (<0.01)
Height	0.01 (NS)	0.02 (NS)
Weight	0.08 (<0.001)	0.04 (NS)
BMI	0.08 (<0.001)	0.04 (NS)
Serum hsCRP	0.29 (<0.001)	-0.01 (NS)
Smoking	0.04 (NS)	-0.02 (NS)
Physical activity	-0.03 (NS)	0.02 (NS)
n = 968		
Serum insulin	-0.02 (NS)	-0.06 (NS)
Plasma glucose	-0.02 (NS)	-0.03 (NS)
Serum cholesterol	0.01 (NS)	0.00 (NS)
Serum triglycerides	-0.01 (NS)	-0.03 (NS)
Serum HDL	-0.07 (NS)	0.01 (NS)
Serum LDL	0.03 (NS)	0.01 (NS)
Serum ApoA1	0.01 (NS)	0.04 (NS)
Serum ApoB	0.01 (NS)	-0.01 (NS)
Serum ApoB/ApoA1	0.01 (NS)	-0.03 (NS)

Spearman Rank correlation coefficients (r_S) are shown, with p values between brackets, for serum estradiol (E2) levels measured by either immunoassay or MS, versus study subject characteristics. Because of multiple testing, the significance threshold was set at p=0.01. hsCRP = high-sensitivity C-reactive protein, HDL = high-density lipoprotein, LDL = low-density lipoprotein, ApoA1 = apolipoprotein A1, ApoB = apolipoprotein B. NS = non significant.

Supplemental references

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Supplemental Figure Legends

SUPPLEMENTAL FIGURE 1. Cross-calibration of the MS E2 measurements.
(A) Scatter plot of the correlation between MS E2 measurements at the Taylor and Québec laboratories (n = 50). The Spearman Rank correlation coefficient (r_S) is indicated, as well as the linear regression line (solid line) and the line of full agreement (dashed line). (B) Bland-Altman plot of MS estradiol measured at two sites. The difference between MS E2 at the Taylor and Québec laboratories is plotted against the mean of the two measurements. The limits of agreement, specified as the mean difference ± 1.96 SD (95% limits of agreement), are indicated by the light grey lines.

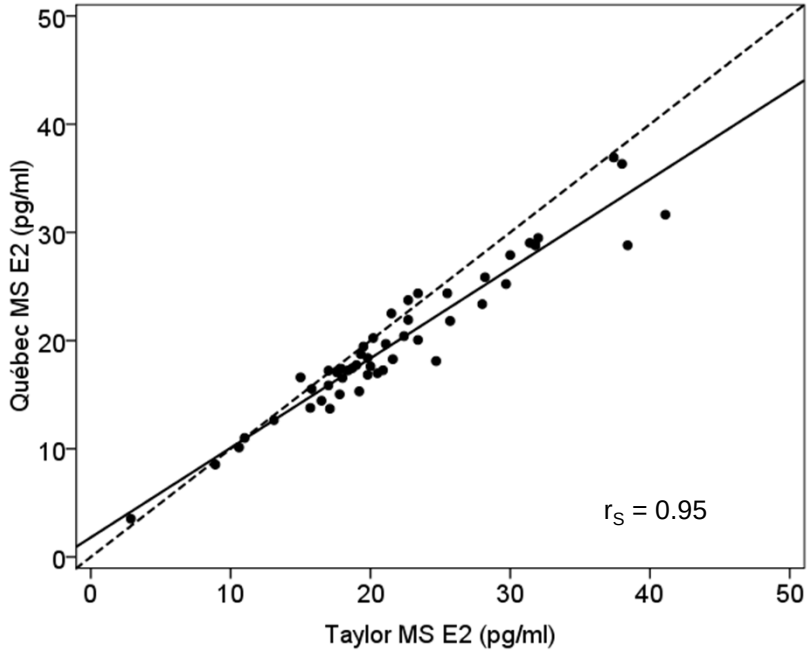
SUPPLEMENTAL FIGURE 2. Correlation between the E2 immunoassay and MS assay in MrOS US.
Scatter plot of the correlation between E2 measured by immunoassay and MS in MrOS US (n = 688). The Spearman Rank correlation coefficient (r_S) is indicated, as well as the linear regression line (solid line) and the line of full agreement (dashed line).

SUPPLEMENTAL FIGURE 3. Kernel density plots of immunoassay and MS E2.
Density plots of E2 measured by immunoassay (solid line) and MS (dashed line) in (A) MrOS Sweden, n = 2599 and (B) MrOS US, n = 688.

SUPPLEMENTAL FIGURE 4. Bland-Altman plots of immunoassay and MS E2.
Bland-Altman plots of the difference between immunoassay and MS E2 plotted against the mean in (A) MrOS Sweden, n = 2599 and (B) MrOS US, n = 688. The limits of agreement, specified as the mean difference ± 1.96 SD (95% limits of agreement), are indicated by the light grey box.

Supplemental Figure 1

A



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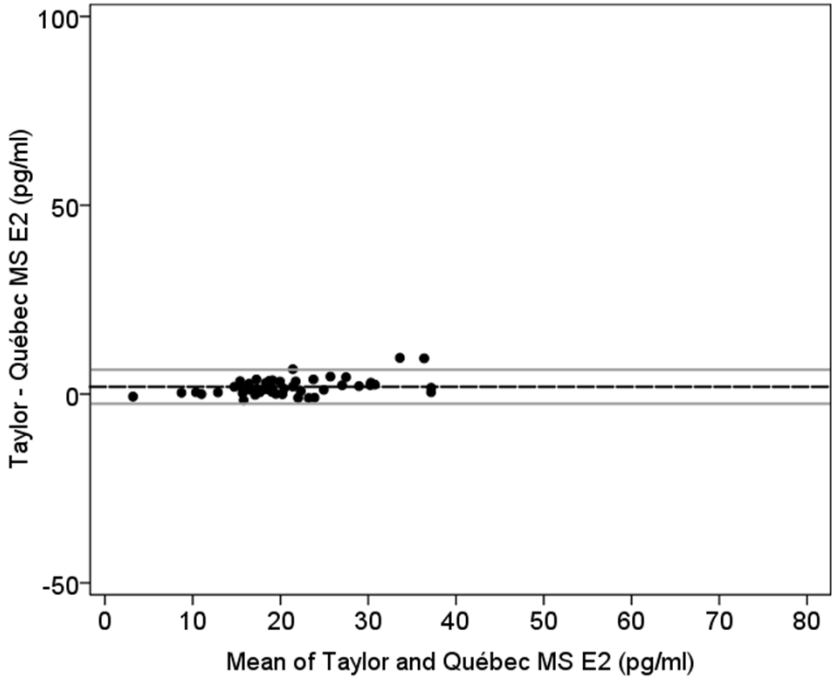
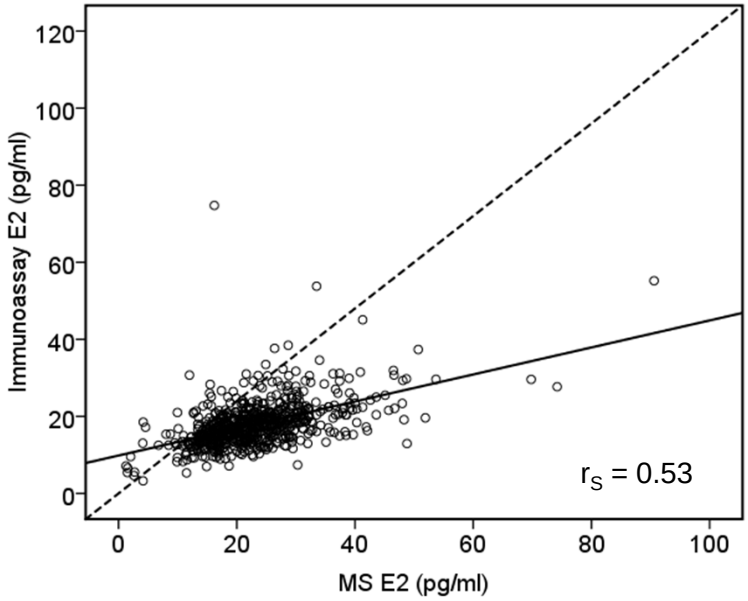
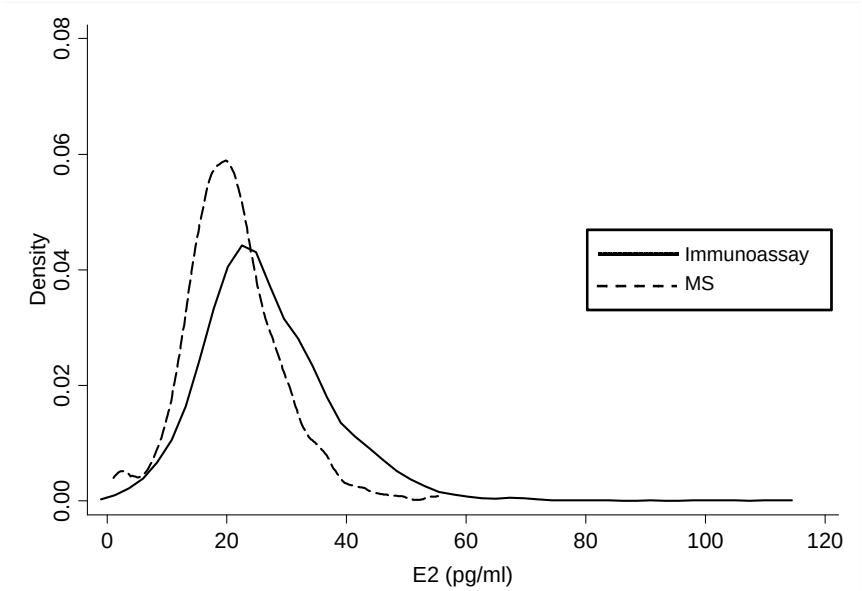


Supplemental
Figure 2

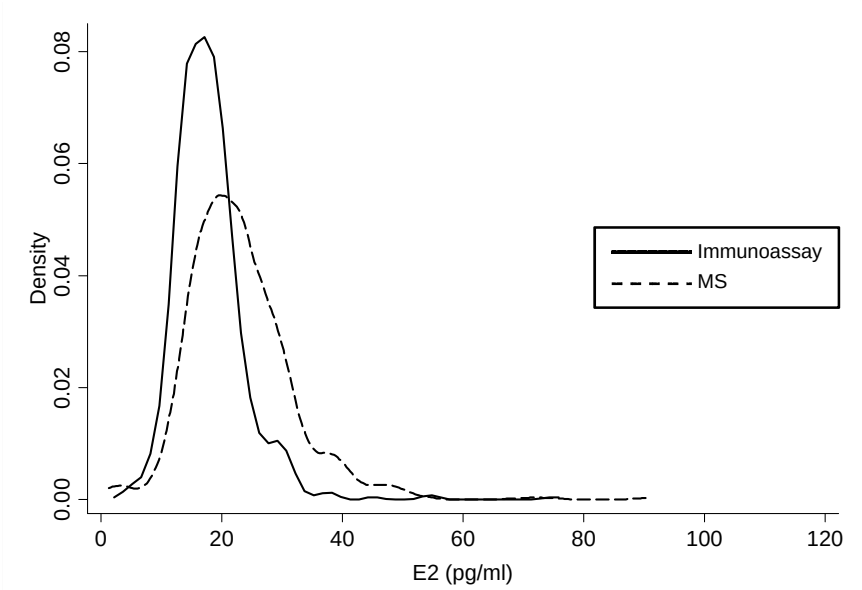


Supplemental Figure 3

A

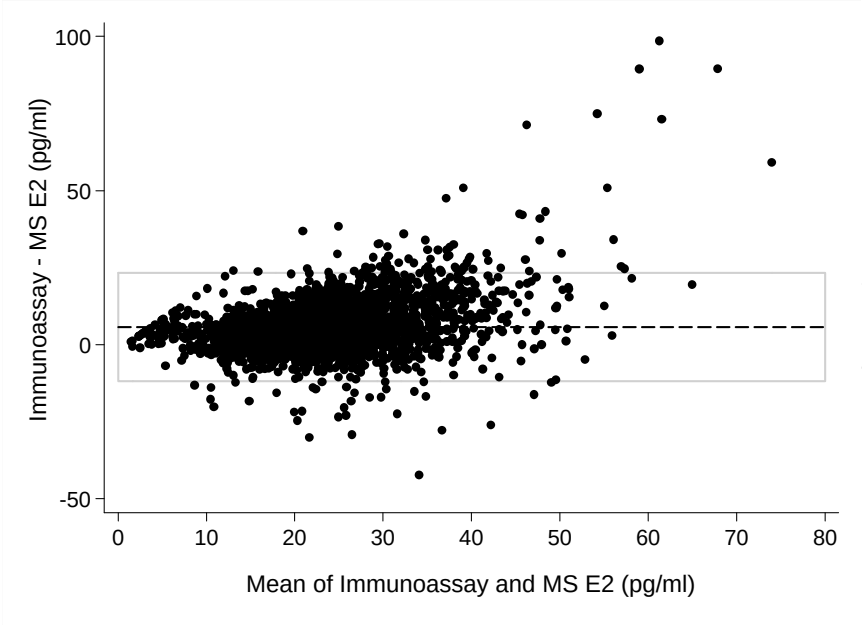


B



Supplemental Figure 4

A



B