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SYMPOSIUM REPORT

Workshop on normal reference ranges for estradiol in postmenopausal women, September 2019, Chicago, Illinois

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Abstract

The North American Menopause Society (NAMS) organized the Workshop on Normal Ranges for Estradiol in Postmenopausal Women from September 23 to 24, 2019, in Chicago, Illinois. The aim of the workshop was to review existing analytical methodologies for measuring estradiol in postmenopausal women and to assess existing data and study cohorts of postmenopausal women for their suitability to establish normal postmenopausal ranges. The anticipated outcome of the workshop was to develop recommendations for establishing normal ranges generated with a standardized and certified assay that could be adopted by clinical and research communities. The attendees determined that the term *reference range* was a better descriptor than *normal range* for estradiol measurements in postmenopausal women. Twenty-eight speakers presented during the workshop.

Key Words: Estradiol – Estradiol assays – Estradiol workshop – Estrogen – Menopause.

Drs. Matsumoto, Pinkerton, Liu, and Santen opened the workshop by discussing the rationale and goals for the workshop. They noted that this workshop resulted because estradiol levels reported for postmenopausal women were highly variable, with concentrations depending on the assay used. Accordingly, no universally accepted reference range for estradiol was available for clinicians and researchers. A major reason for this variability is that immunoassays for estradiol without one or more preceding purification steps (ie, those on automated platforms) lack sensitivity, specificity, accuracy, and standardization. In addition, differences exist in the characteristics of patient populations used to determine reference ranges.[1,2] Current state-of-the-art assays involve tandem mass spectrometry in combination with separation either by gas chromatography (GC) or liquid chromatography (LC). Contemporary approaches to developing reference ranges involve harmonization of various assays between laboratories using well-characterized and validated

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reference standards, similar to that used to develop a harmonized reference range for serum total testosterone.[3] Before the workshop, several factors in developing reference ranges were identified for discussion, including the type of population to be used; identification of sensitive, accurate, and standardized estradiol assays; and use of direct versus indirect methods for reference range development.[4]

The North American Menopause Society (NAMS) has spent considerable effort identifying the possible risks of low-dose vaginal estrogen administration. NAMS' guidelines for the treatment of menopause recommend the use of vaginally delivered estrogens to treat the genitourinary syndrome of menopause (GSM) for certain patients. Possible risks listed in boxed warning for low-dose vaginal estradiol include breast cancer, coronary artery disease, stroke, deep venous thrombosis (DVT), pulmonary embolism, and probably dementia. However, it is reasonable to conclude that if estrogen levels from low-dose vaginal estrogen do not exceed the reference range established for postmenopausal women, such risks would be rare, unlikely, and difficult to identify.

During extensive literature review, it was recognized that no reference ranges for postmenopause estradiol levels have been established and universally agreed on. Determination of accurate systemic levels of estradiol and estrone allows determination and standardization of the references range of postmenopausal estrogen levels to further research into the effects of exogenous and endogenous estrogens on important health concerns for postmenopausal women.

WORKSHOP GOALS

1. Develop a roadmap for the methodology necessary to develop accurate and precise standardized reference range for plasma estradiol in postmenopausal women.
2. Compile existing data regarding postmenopause estradiol levels using highly sensitive, specific, and precise mass spectrometry-based assays.
3. Address important questions related to the clinical use of serum and urinary estradiol assays.

APPROACH TO PREPARING FOR THE WORKSHOP

Before the workshop, each speaker was asked to 1) report the sensitivity, specificity, and precision of their assays; 2) report the characteristics of their populations, and 3) perform subgroup analysis on factors such as chronologic age, body mass index (BMI), age of menopause, duration of menopause, ethnicity, and use of alcohol or smoking behavior. Several speakers were asked to address whether measurement of plasma estradiol levels in postmenopausal women might help in assessing the risk of breast cancer, endometrial cancer, fracture susceptibility, dementia, and heart disease. Because published data suggested that plasma estrone might be biologically relevant, the question was posed about whether current data support the measurement of estrone as well as estradiol as a marker of estrogenic effects. Finally, reviews on current thinking regarding the establishment of reference ranges in select populations (such as menopause) were requested. Supporting companies did not participate in the writing, review, revisions, conclusions, or approval of this report.

ASSAY MEASUREMENT ISSUES

A thorough data review provided compelling evidence that mass spectrometry-based assays, after full validation and standardization, should be used in determining reference ranges for postmenopausal women and patients with clinical conditions characterized by low estradiol levels. The group agreed that certain highly characterized radioimmunoassays using extraction and column chromatography might be used but only after Centers for Disease Control and Prevention (CDC) certification for accuracy and standardization. A detailed review of existing data indicated that immunoassays detect higher levels of estradiol than do mass spectrometry assays, but the precise reasons for the overestimation are presently unknown.[5] The data presented suggested that it might be useful to measure estradiol and estrone levels in the same samples in situations in which prior data demonstrated that each estrogen provided independent clinical information. At the present time, cost limits the routine measurement of both estradiol and estrone by mass spectrometry; but assay capability is not at issue. Age, BMI, and body weight all influence plasma estradiol and estrone levels, as demonstrated in several studies, but alcohol, tobacco use, and age of menopause exert lesser or nonsignificant effects.[6]

DEVELOPING REFERENCE RANGES

A key goal of the workshop was to provide a roadmap for developing reference ranges for estradiol in postmenopausal women. Jones discussed two methods, one called *direct*, which uses well-characterized controls, and *indirect*, which employs large data sets and mathematically excludes outliers.[6] The speakers concluded that the direct method was both preferable and feasible. Miller presented a step-by-step approach to establish reference ranges (Table 1) using the direct method. Consensus was reached that this approach be used in determining normal postmenopausal estrogen ranges.

STEP-BY-STEP APPROACH TO ESTABLISHING REFERENCE RANGES FOR ESTRADIOL

Step 1. Describe characteristics of a reference individual

A key step in developing reference ranges is to define the characteristics of the population to be used to determine inclusion or exclusion of patients, including key patient characteristics of obesity, age, ethnicity, and other factors. Estrogen production through aromatase is increased as a function of BMI. The main source of estradiol in postmenopausal women is the aromatization of testosterone to estradiol and androstenedione to estrone with conversion to estradiol via 17β-hydroxylation of estrone. An important consideration is how well estradiol correlates with BMI and whether separate reference ranges should be developed for lean versus obese individuals.

TABLE 1. *Steps to develop reference interval for estradiol*

1. Describe characteristics of a reference individual
a. Consider comorbidities for exclusion, including diabetes, chronic kidney disease, cardiovascular disease, cancer, medications, etc
b. Is more than one partition needed?
2. Qualify measurement procedures with suitable performance for a reliable estradiol measurement to include selectivity, precision, sensitivity, proportionality
3. Remeasure frozen samples to develop a correction for bias to the CDC reference assay
4. Identify cohorts with the clinical characteristics and suitable measurement procedures
5. Aggregate estradiol results from cohorts and apply bias corrections if needed
6. Calculate reference interval and statistical uncertainty
7. Examine the reference interval for its relationship to clinical outcomes
8. Rationalize the reference interval for its intended clinical use
9. Determine applicability to results by measurement procedures that conform to the CDC standardization program

CDC, Centers for Disease Control.
This methodology was presented at the workshop by Dr. Greg Miller and has not been published elsewhere.

Correlation between body mass index and estradiol

Several detailed studies, as reviewed by Lee, demonstrated a positive correlation between BMI and plasma estradiol levels in postmenopausal women (Figure 1).[5] The best correlations were observed with GC and LC mass spectrometry assays and radioimmunoassays, which included extraction and column chromatography before radioimmunoassay. Additional data presented by Richardson reported a correlation coefficient between BMI and estradiol of 0.45 in more than 3,500 women. The median levels of estradiol within the BMI parameters assessed ranged from 3.5 pg/mL, with a BMI of 25 kg/m^2 or less ($n = 1,154$), to 8.0 pg/mL, with BMIs of 30 kg/m^2 or more ($n = 1,506$; $P < 0.01$). Ingle and Constantine presented similar results in women with breast cancer undergoing adjuvant hormone therapy (HT). Both Ingle and Constantine presented data demonstrating that estradiol levels increase as a function of BMI. Specifically, Ingle reported that baseline median estradiol levels were 3.0 pg/mL in women with a BMI less than 25 kg/m^2 and increased to 8.1 pg/mL for women with a BMI greater than 30 kg/m^2. Similarly, Constantine reported estradiol levels of 4.8 pg/mL for women with a BMI less than 25 and increasing to 7.7 pg/mL in women with a BMI greater than 30 kg/m^2.

Richardson examined subgroups of women aged younger than 50, 50 to 59, 60 to 74, and older than 74 years. Estradiol gradually decreased as a function of age with levels of 7.3, 5.7, 5.2, and 5.6 pg/mL respectively ($P = 0.01$). Constantine found similar results with levels of 8.3 pg/mL in women aged younger than 50 years and 4.2 pg/mL in women aged older than 60 years.

Richardson also examined the effects of duration of menopause, ethnicity, alcohol intake, and smoking on estrogen levels in a large group of participants using the high sensitivity Mayo Clinic tandem mass spectrometry assay. The baseline estradiol level in 4,066 women was 5.4 pg/mL, with a median of 4.0 pg/mL. The results were truncated for values below the 2.5th percentile and higher than the 97.5th percentile. One percent of the samples (39 participants) had a level that was undetectable (< 0.3 pg/mL); 10.6% of samples were greater than 10 pg/mL. Because of the number of participants, several of these analyses resulted in statistically significant but probably not clinically significant differences. For example, when comparing white (mean estradiol, 5.3 pg/mL) with African American (mean estradiol, 6.8 pg/mL), and Asian (mean estradiol, 4.3 pg/mL) women, the magnitude of differences among these groups was a maximum of 2.5 pg/mL. These differences were statistically significant ($P < 0.01$). Constantine found similar differences with mean estradiol levels of 5.8, 7.0, and 4.2 pg/mL, respectively, for these three groups. Small but likely not clinically significant differences were also observed for duration of menopause and effect of smoking, with differences ranging from 0.5 pg/mL to 1.2 pg/mL.

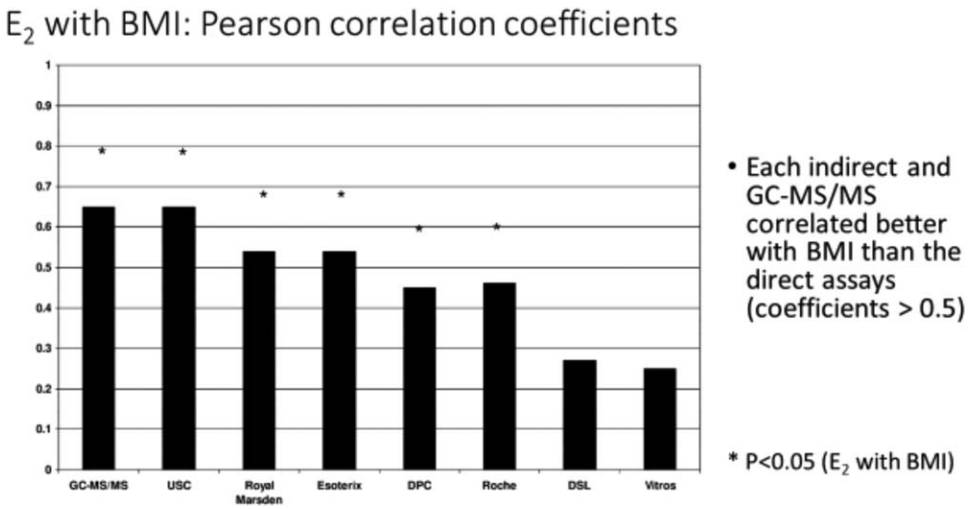

FIG. 1. The relationship of estradiol with body mass index expressed as the Pearson correlation coefficient as reported with several estradiol assays. Figure reproduced from Lee JS et al[5] with permission from Oxford University Press © 2006. All rights reserved.

The group discussed exclusion criteria from groups constituting reference populations. No data were reported, but the general consensus was to exclude individuals with chronic illnesses such as diabetes, chronic kidney disease, cardiovascular disease (CVD), cancer, and those taking medication that could affect estradiol levels.

A great deal of discussion addressed whether separate reference ranges should be established for various subgroups of women and particularly subgroups based on BMI and age. The data clearly indicated a substantial effect of age and BMI on plasma estradiol levels and a minimal effect of ethnicity, smoking, and duration of menopause. Whereas reference ranges for subgroups of women based on age and BMI may be useful in some situations, the consensus was that there should be a single harmonized estradiol reference range for postmenopausal women. The rationale for this decision was one of simplicity and the comment that clinicians could judge the effect of BMI and age in individual patients and that older or obese women should not be excluded in the standardized reference range.

Step 2. Criteria for assays to be used to determine reference range

The roadmap for developing normal ranges suggested by Miller was to require assays to be selective for estradiol, have appropriate precision, have proportionality over the measuring interval, and be sufficiently sensitive to measure most patient samples. The liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) assay methods presented by Singh, Danilenko, Mesaros, and Straseski each met these criteria and measured estradiol with virtually no interfering peaks nor interfering compounds.[7,8] The lower limit of quantitation of the assays ranged from 0.3 pg/mL (Singh), 0.71 pg/mL (Danilenko), and 0.8 pg/mL (Mesaros) to 0.5 pg/mL (Straseski). In Richardson's cohort of 4,066 patients, only 1% ($n = 39$) had levels of estradiol below the limit of detection (< 0.3 pg/mL). At estradiol levels within the range of 0.3 pg/mL to 10 pg/mL, coefficients of variation for the various assays were uniformly below 13%, and most were below 5%. Based on the general discussion, the consensus was that either LC or GC/MS/MS assays were preferable for determining reference ranges.

Step 3. Remeasure a subset of frozen samples to develop a correction for bias relative to the Centers for Disease Control and Prevention reference program values

The group concurred that each GC or LC/MS/MS assay should be standardized using the CDC Hormone Standardization (HoSt) Program and cross-calibrated to CDC values in the United States. This will involve reassaying frozen samples and comparing measured values with the CDC results. This process will identify the degree of bias for each assay and also will identify outliers as a result of sample specific influences. The model for this type of assay harmonization was the published successful methodology for harmonizing a reference range for total testosterone in men.[2]

Step 4. Identify cohorts with suitable characteristics

Several databases were identified at the workshop that could potentially be used as cohorts for use in developing a harmonized estradiol reference range in postmenopausal women. These include the National Health and Nutrition Examination Survey (NHANES), Mammary Prevention-3 (MAP-3), Imvexxy study baseline data, Mayo Clinic data on patients with known clinical characteristics, data from Brigham and Women's Hospital from Bhasin, baseline data from dehydroepiandrosterone (DHEA) studies, the Study of Women's Health Across the Nation (SWAN) study database, Women's Health Initiative (WHI) study baseline data, data from University of Pennsylvania from Blair and Mesaros and data from older women from the Aspirin in Reducing Events in the Elderly Study.[9] Other databases may be identified as investigators search further literature.

Step 5: Aggregate results from cohorts

The need for biostatistics expertise was identified, but no details were considered. Vesper from the CDC reviewed how this process was approached when developing the CDC HoSt Program and harmonized reference range for total testosterone in men.

Step 6: Calculate reference intervals and their statistical uncertainty

Bhasin and Vesper discussed the specifics of the process and statistical methodology used to establish a harmonized reference range for total testosterone in men using existing cohorts of community-dwelling men of various age and BMI. The group generally agreed that use of the specific approach used previously for establishing the harmonized reference range for total testosterone in men would be most appropriate to use for postmenopausal women.

Step 7: Examine the reference interval for its relationship to clinical outcomes

Consensus was reached that an important goal is to use a generally agreed on, rigorously established estradiol reference range to determine whether low-dose vaginal estrogen formulations are associated with sufficient systemic absorption to cause estradiol values to rise above the upper limit of a newly identified postmenopause reference range. Further clinical validation will be necessary to assess risk of breast and endometrial cancers, fracture rate, or risk of cardiovascular or cognitive effects using standardized estradiol assays.

Step 8: Rationalize the reference interval for its intended clinical use

One pertinent issue raised was to determine the level of sensitivity required for clinical assays to include the ability to detect the lowest possible range. Ingle provided evidence that an assay needs to measure estradiol down to at least 0.3 pg/mL to be used to determine degree of suppression of estradiol in women with breast cancer treated with aromatase inhibitors (AIs). Ingle's data indicate that women on AIs with estradiol

levels above 0.5 pg/mL have a 30% greater chance of breast cancer recurrence, which needs to be confirmed by additional studies. If confirmed, this clinical finding would suggest the need for a very sensitive estradiol assay to monitor women taking AIs. Because low estradiol levels have been suggested to predict fracture risk, it is possible that very sensitive assays would also be needed for fracture risk prediction. The role of estrone measurement in fracture risk prediction merits further study. For other health concerns, such as determining whether vaginal estradiol or DHEA causes estradiol levels to increase beyond the postmenopausal reference range, less sensitive methods would likely suffice.

Step 9: Determination of applicability to results by measurement procedures that conform to the CDC standardization program

After extensive discussion, it was agreed that assays for estradiol should be standardized in an accuracy-based quality control program, preferably to the CDC HoSt Program, or the equivalent for non-US countries. Jones emphasized that several international guidelines have been published such as the International Federation of Clinical Chemistry and Laboratory Medicine, the Clinical and Laboratory Standards Institute, and the Tietz Textbook guidelines that address reference range development. During the discussion, the term normal range was felt to be an inaccurate descriptor, because some normal persons would fall out of this range. By consensus, the term reference range was preferred.

CONTRIBUTION OF ESTRONE TO ESTROGEN BIOLOGIC EFFECTS

Data presented at the workshop provided strong support for the concept that estrone can exert clinical effects independent of estradiol. These data included in vitro studies of breast cancer, prediction of risk of breast and endometrial cancer, and recurrence rate of breast cancer in women treated with AIs. Based on potential clinical health risk data, it was considered reasonable to assay both estradiol and estrone in the same GC or LC/MS/MS assays in postmenopausal women. Assay data were presented for plasma estrone levels as a function of age. In contrast to estradiol levels, estrone increased with age from 23.5 pg/mL for ages 50 to 59 years, 24.5 pg/mL for ages 60 to 74 years, and 26.8 pg/mL for age older than 74 years, as presented by Richardson. Davis reported data for estrone levels in 5,326 community-dwelling postmenopausal women aged 70 to 94 years without cardiovascular disease or cognitive impairment measured by liquid chromatography/tandem mass spectrometry.[10] The median plasma estrone was 49 pg/mL (181 pmol/L; interdecile range 88.7-347.6 pmol/L or 24.0-94.0 pg/mL). In this older cohort estrone concentrations increased with age and BMI. In this cohort, 66% of the women had plasma estradiol concentrations below the limit of detection of 3 pg/mL, consistent with older postmenopausal women having lower estradiol concentrations than younger postmenopausal women.

DETERMINATION OF NORMAL REFERENCE POSTMENOPAUSE LEVELS

Data were presented by Constantine on estradiol levels from two randomized, blinded, phase 3 trials providing a large database of healthy postmenopausal women, which used gas chromatography/tandem mass spectrometry estradiol assay (lower limit of quantification of 2.0 pg/mL and sensitivities for estradiol of 4.83% for precision and -8.00% for accuracy). The overall mean estradiol level at screening was 5.6 pg/mL to 6.5 pg/mL in the vasomotor symptom population and 3.6 pg/mL to 4.9 pg/mL in the vulvovaginal atrophy (VVA) population. Mean levels of estradiol decreased with increasing age, ranging from 8.2 pg/mL in women 40 to 45 years to 3.5 pg/mL in women aged 70 years and older. Estradiol concentrations also increased with increasing BMI from 4.8 pg/mL for BMI less than 25 kg/m^2 to 7.8 pg/mL for BMI 30 kg/m^2 and higher. Black women had the highest estradiol levels, followed by white and Asian women. Smoking, alcohol use, and numbers of pregnancies or live births did not appear to influence screening estradiol levels.

EFFECT OF VAGINAL ESTRADIOL ON SERUM ESTRADIOL LEVELS

Extensive review of data presented on vaginal estradiol led to several conclusions.[10-16] Absorption into the systemic circulation is higher when the vaginal mucosa is atrophic and much less so after chronic vaginal estrogen therapy, which thickens the mucosa. This occurs after 2 to 4 weeks generally but is slower in women on AIs.[17] Lower-dose vaginal estradiol formulations produce lower increments in plasma estradiol concentrations. The key findings were presented by several speakers. Lobo reviewed the data obtained during study of 10-μg and 25-μg vaginal estradiol tablets.[18] When first administered in women with VVA, average peak levels of estradiol at approximately 6 hours were 25 pg/mL after the 10-μg dose and 42 pg/mL after the 25-μg dose, with return to near baseline after 24 hours. By days 14 through 83, there was very little systemic absorption (an average change in concentration of 3.8 pg/mL for the 10-μg dose and 18.3 pg/mL for 25 μg). Simon reported on the systemic absorption of 4-μg and 10-μg vaginal estradiol inserts.[19] Acute absorption on the first dose caused a minimal average estradiol increment of 3 pg/mL with the 4-μg dose and 6 pg/mL on the 10-μg dose. During administration on the 4th and 18th day, no increments in estradiol occurred. Results were similar with the 10-μg vaginal estradiol suppository. With administration of intravaginal DHEA, plasma estradiol and estrone levels increased only minimally (1 pg/mL) and never exceeded a recently reported range of 0 to 10.7 pg/mL.[20-23] Similar low systemic levels have been seen with the vaginal estradiol ring releasing 7.5 μg and with vaginal conjugated estrogen and estradiol creams dosed at 0.5 mg or lower.[10,12] These data support the concept that vaginal estradiol can be dosed low enough to not increase systemic levels beyond putative

normal reference ranges in postmenopausal women. Observational data up to 3 years and endometrial safety studies up to 1 year reported no adverse effects with vaginal estradiol (Figure 2).[24,25] The preponderance of evidence on low-dose vaginal estrogen supports no increase in risk of endometrial cancer when estradiol is dosed within reference postmenopausal ranges, but further studies are needed to assess longer-term endometrial safety.

RELATIONSHIP OF PLASMA ESTRADIOL LEVELS TO COGNITIVE FUNCTION

Henderson reviewed multiple studies examining the correlations of plasma estradiol to various parameters of cognitive function.[26-37] Although selected studies demonstrated a few parameters correlating with plasma estradiol levels, the results were inconsistent. Henderson concluded that, at present, the results are inconclusive, and further studies are needed.

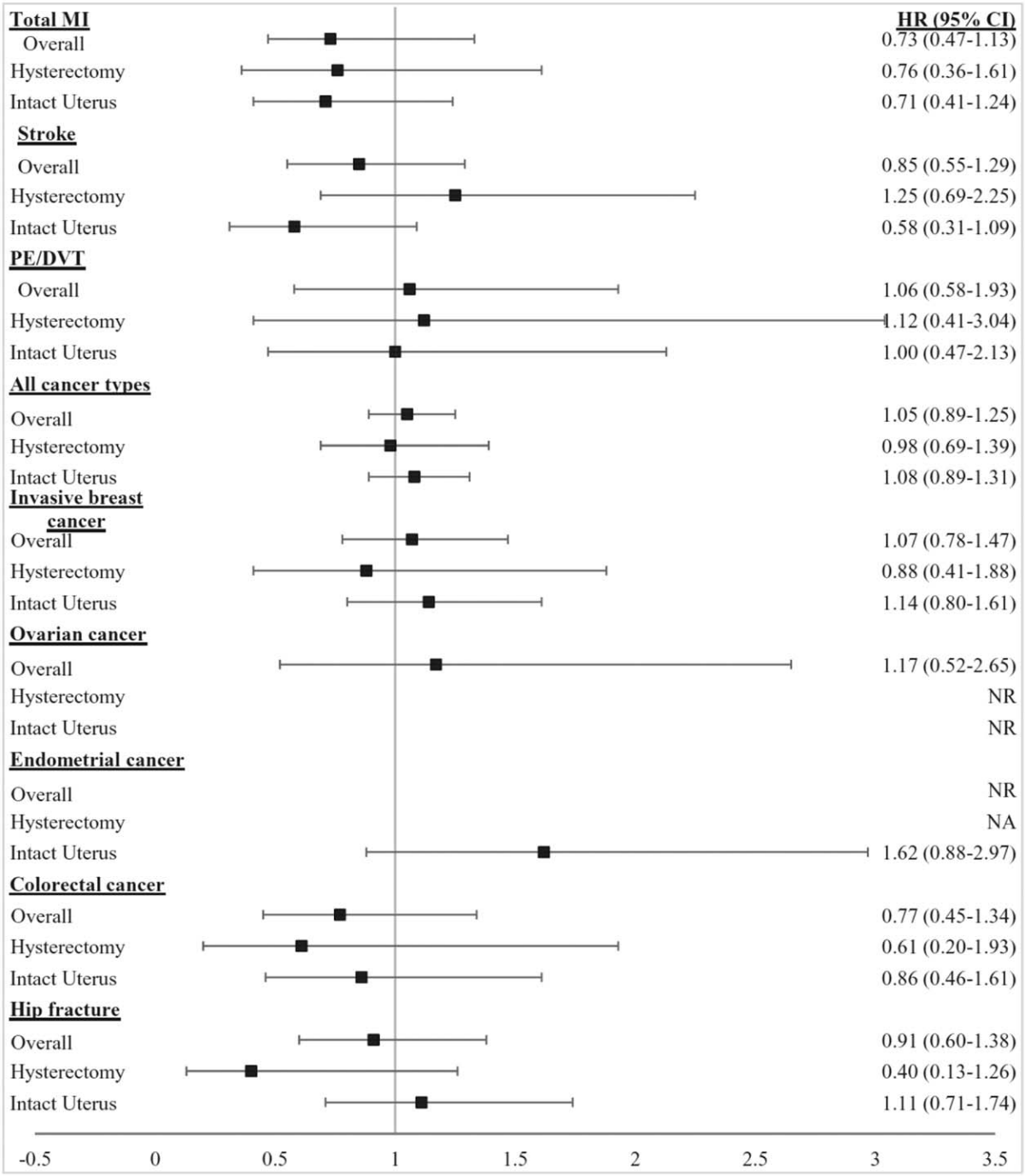

FIG. 2. Hazard ratios for several clinical parameters during administration of vaginal estradiol. Data reproduced from Bhupathiraju SN et al.[24] Permission from The North American Menopause Society ©2019. All rights reserved.

USE OF PLASMA ESTRADIOL MEASUREMENTS TO PREDICT RISK OF ENDOMETRIAL NEOPLASIA, BREAST CANCER, AND FRACTURE

In the workshop discussion, the group generally agreed that additional studies need to be performed with state-of-the-art estradiol assays with sufficient sensitivity to uncover possible groups in which systemic levels of estradiol and estrone could predict a high risk of endometrial cancer and breast cancer or a high risk of fracture. The existing data provided substantial evidence to support using estradiol to predict risk.

ENDOMETRIAL CANCER

Brinton reviewed the literature and her own data indicating that increases in endogenous levels of plasma estradiol predict the risk of developing endometrial cancer.[38-48] For unconjugated estradiol (estradiol cleaved from its sulfated and glucuronidated form), the hazard ratio (HR) was 6.19 and for estrone corrected for estradiol, 2.13 ($P < .001$). This correlation existed for Type I (estrogen responsive) but not Type II endometrial cancer and was independent of the effects of BMI.

BREAST CANCER

Manson reviewed the recently published observational study[49] that found an increased risk of breast cancer with oral estrogen replacement treatment (HR, 1.33) and compared estradiol (HR, 1.32; 95% confidence interval [CI], 1.25-1.39) with that of conjugated equine estrogen (HR, 1.38; 95% CI, 1.30-1.46). For estrogen plus a progestogen, the HR for breast cancer was 2.08 (95% CI 2.02-2.15). There was no increase in breast cancer risk with vaginally administered estradiol.

Hankinson examined the effect of adding endogenous plasma estrone, estradiol, or estrone sulfate to established risk models for breast cancer. For both the Gail and Rosner-Colditz models, adding estrone, estradiol, or estrone sulfate increased the area under the risk prediction curve (AUC). When adding all of these measurements together, the AUC increased from 50.4 to 60.1.[50,51]

FRACTURE

Cauley reviewed the literature on the relationship between plasma estradiol and fracture risk.[52] Low estradiol concentration (<5 pg/mL) was associated with a relative risk for fracture of 2.51 (95% CI, 1.4-4.6) in one study, and several others supported the concept that low estradiol levels might be used as one component in the assessment of risk of fracture. The mechanisms for this effect appear to be both direct (effect of estradiol on bone) and indirect related to weight loss, lean mass loss, falls, and frailty, which in turn can be influenced by estradiol or estrone levels.

DATABASES CONTAINING NORMAL POSTMENOPAUSAL WOMEN

Vesper described the NHANES database with approximately 7,000 well-characterized women. His laboratory at the CDC is currently measuring estradiol in these patients with their routine standardized assay, not the reference assay as described by Danilenko. The cohort described by Richardson appears ideal for constructing reference ranges. Additional databases include the Imvexxy placebo group, Mayo Clinic, Brigham and Women's Hospital, intravaginal DHEA data, and SWAN studies, the WHI database, and the results from the University of Pennsylvania. Additional databases could be identified with additional searching, but these were felt to be sufficiently robust to determine a normal postmenopause reference range.

FIRST PASS FROM VAGINA TO UTERUS

Liu reviewed data on the first-pass effect of vaginal estradiol into the uterus via local circulatory pathways. He also presented data suggesting that estradiol, present in the upper one-third of the vagina, may pass directly via venous and lymphatic channels to the uterus.[53] This concept has practical implications regarding the safety of vaginal estradiol administration. In the discussion, the need for further studies was identified, and the experiments to examine messenger RNA signatures characteristic of an estrogen effect in the human uterus after vaginal estradiol exposure.

GOALS IDENTIFIED THROUGH THE WORKSHOP

Short-term goals

The group reached a consensus that harmonized reference ranges for both estradiol and estrone should be developed using standardized GC or LC/MS/MS assays with a sensitivity to at least 2 pg/mL, which would allow detection of estradiol levels in 98% of women. The assays to be used should be certified by the CDC HoSt Program. Construction of reference ranges would use the direct method, which involves identifying community-dwelling healthy postmenopausal women with full characterization of clinical features. Sample sizes should significantly exceed 120, the usually recommended but relatively arbitrary number for the population used to establish reference ranges. Normal women receiving systemic HT should stop this medication for at least 3 months if oral estrogen or 4 weeks if vaginal estrogen is being used before blood is drawn to establish a reference range.

Intermediate-term goals

1. Compile definitive evidence to examine whether vaginal estrogen causes short-term or long-term increases in plasma estradiol outside of the reference range for postmenopausal women and effects on the uterus using cDNA methodology to assess estrogen receptor responsive genes
2. Examine biologic relevance of reference ranges for estradiol and estrone concentrations for postmenopausal women of various BMIs and ages.
3. Demonstrate validity of adapting doses of estradiol therapy based on plasma levels in patients with selected clinical conditions.

Long-term goals

1. Conduct clinical studies to determine the relationship between estradiol and estrone levels on the risk of

breast cancer, endometrial cancer, and fractures, using standardized LC or GC tandem mass spectrometry assays. Cardiovascular risk and cognitive dysfunction were felt to be more complicated.
2. Measure estradiol and estrone levels in women with breast cancer receiving AI therapy to determine the relationship between adequately suppressed levels and recurrence rate in these women, to confirm the studies presented by Ingle.

HYPOTHESIS GENERATED DURING THE WORKSHOP

The effects of estrone sulfate and role of other estrogen metabolites on breast cancer risk independent of estradiol was postulated and supported by preliminary studies but need to be confirmed. Bioassays for measurement of estrogen activity in postmenopausal women may provide better evidence of biologic effects of estrogen as opposed to specific measurements of estradiol, estrone, or estrone-sulfate, a concept requiring further testing. Measurement of total body fat by dual-energy x-ray absorptiometry (DEXA) and its relationship with estradiol concentration may be more valid than using BMI as a surrogate marker for total body fat. Studies comparing estradiol levels with total body fat measured by DEXA are warranted in the future. It was suggested that low levels of estradiol in postmenopausal women reduced quality of life and parameters of sexuality. These hypotheses need to be confirmed in future studies.

CONCLUSIONS

1. Standardized LC- or GC-tandem mass spectrometry assays are needed to accurately measure the low levels of circulating estradiol and estrone in postmenopausal women.
2. Serum estradiol and estrone concentrations have been measured using various LC or GC tandem mass spectrometry assays in numerous cohorts of well-characterized, community-dwelling, postmenopausal women, but differences in assays preclude consensus of results.
3. A harmonized reference range for serum estradiol and estrone concentrations in postmenopausal women is needed to unequivocally interpret results of clinical studies and for translation of research findings into clinical practice.
4. A harmonized reference range for serum estradiol and estrone concentrations in postmenopausal women should be established by measurements in a subset of samples from existing cohorts in which levels were measured by tandem mass spectrometry that are cross-calibrated to measurements by the CDC hormone standardization program.
5. Standardized estradiol and estrone assays and a harmonized reference range in postmenopausal women should better inform changes in the low concentrations that occur with vaginal estrogen and AI treatment and health risks.
6. For postmenopause estradiol reference ranges using a large database of healthy volunteers, prestudy estradiol levels vary by enrollment for vasomotor symptoms (5.6-6.5 pg/mL) or for VVA (3.6-4.9 pg/mL), by age decreasing from 8.2 pg/mL in women aged 40 to 45 years and 3.5 pg/mL in women aged 70 and older; by BMI with increasing estradiol levels as BMI increases; and variable estradiol ranges depending on ethnicity.
7. The normal postmenopausal estradiol range is likely to be less than 10 pg/mL, such that the postmenopause reference range is likely to include levels below the limit of detection up to 10 pg/mL.
8. Vaginal estrogen dosed within normal reference postmenopausal range is unlikely to cause increased risk of coronary heart disease (CHD), DVT, stroke, pulmonary embolism, breast cancer, or probable dementia. More studies are needed on longer-term endometrial safety.
9. Further studies are needed to assess the effects of endogenous estradiol and estrone on major health concerns of postmenopausal women and the use of systemic serum levels for risk calculations for breast and endometrial cancer and osteoporotic fracture.

EXECUTIVE SUMMARY

Assay measurement

- Gas chromatography and liquid chromatography tandem mass spectrometry assays for estradiol and estrone can provide greater accuracy and sensitivity than immunoassays when evaluating postmenopausal women as well as persons with clinical conditions associated with low estrogen levels such as amenorrhea, puberty, hypopituitarism, and ovarian failure.
- The CDC HoSt Program provides a mechanism for standardization of estradiol assays.
- Not all GC or LC tandem mass spectrometry assays are CDC certified, and estradiol measurements may vary from assay to assay.
- Plasma or serum estradiol levels measured by most immunoassays are higher than those measured by GC or LC tandem mass spectrometry assays, but the specific cross-reacting substances or other factors contributing to the differences are not well established.
- Estrone and estradiol can be measured together by LC or GC tandem mass spectrometry on a single sample.
- Age and BMI or body weight are positively correlated with plasma estradiol and estrone levels, which may substantially influence important biologic actions.
- Existing studies demonstrate no biologically meaningful effects of alcohol, tobacco, ethnicity, or age of onset of menopause on reported postmenopause estradiol levels.
- Workshop speakers identified several available cohorts and databases of postmenopausal women that could be used to establish reference ranges for serum estrone and estradiol concentrations.

Establishment of reference ranges

- In order to translate research results to clinical care, establishment of a single harmonized reference range for estradiol concentrations in postmenopausal women should be developed.
- For practical reasons, data from subsets of postmenopausal women of varying age and BMI should be included in that range rather than having multiple ranges.

- Assays used for establishing reference ranges should be certified for accuracy by the CDC HoSt Program within the United States, and values obtained should be harmonized against the CDC ''gold standard'' reference assay. Ideally, international assay harmonization also should be achieved.
- Direct methods for determining reference ranges involving well-characterized groups of postmenopausal women are preferable to using the indirect method involving mathematical methods to identify outliers in less well-characterized groups of women.
- A step-by-step approach should be used for determining reference ranges for estradiol and estrone, with a logical scheme based on assay and patient characteristics.

Physiologic issues

- Multiple studies suggest that accurate measurement of estradiol could inform both clinical care and research into estrogen-mediated health concerns.
- Preliminary findings suggest that estrone has biologic activity independent of estradiol, but definitive, confirmatory studies are needed to determine whether clinical recommendations should include measurement of both estradiol and estrone routinely in clinical practice or only in certain circumstances.
- Because of their high degree of enzymatic conversion, plasma estradiol and estrone are highly correlated with Pearson coefficients, which range from the Pearson correlation coefficient $R = 0.7$ to 0.8.

Clinical issues

- The normal postmenopausal estradiol range is likely to be less than 10 pg/mL, such that the postmenopause reference range is likely to include levels below the limit of detection up to 10 pg/mL.
- Data on effects of estradiol, with less data available for estrone, suggest that measurement of estrogen levels could be beneficial in understanding the role of endogenous estrogens and the effects of endogenous estrogens on estrogen-sensitive tissues and medical concerns.
- After menopause, estradiol levels decline with age, and estrone levels increase in postmenopausal women.
- Evaluation of estrogen levels and CV risk in postmenopausal women should be interpreted in relationship to obesity, other comorbidities, degree of inflammation, insulin levels, and resistance, as well as other factors.
- The measurement of estradiol, estrone, and estrone-sulfate, as well as other hormones, would improve existing breast cancer risk-prediction models, which are currently based on clinical and genetic factors.
- Low levels of plasma estradiol correlate with fracture risk; exogenous estrogen reduces risk of fracture; the protective association between endogenous estrone and fracture risk is less clear.
- Existing data for relationships between estradiol and cognitive dysfunction are conflicting and no conclusions are possible at this time; no data is available for estrone.
- Existing observational data indicate no long-term adverse effects of low and ultra-low doses of exogenous vaginal estrogen administration on the risk of CHD, stroke, venous thromboembolic events, cognitive dysfunction, or breast cancer.
- Low-dose exogenous vaginal estrogen for the purposes of safety is defined as doses that do not raise the plasma levels of vaginal estradiol above the postmenopause reference range after the transient increases seen with first use of vaginal estrogen in atrophic vaginal tissues.
- One-year randomized studies demonstrate the safety of low-dose vaginally administered estradiol on endometrial hyperplasia and cancer and observational studies suggest longer-term safety. Nonetheless, longer-term randomized, placebo-controlled trials are needed to demonstrate longer-term safety and whether placement in the upper or lower vagina alters endometrial absorption or risk to the uterus.
- Since vaginally administered DHEA is converted locally to estradiol and estrone, longer-term endometrial safety data are needed for this agent as well.

FUTURE GOALS

Short-term goal

Establish harmonized reference ranges for estradiol and estrone in postmenopausal women using appropriate assays in adequately powered studies of well-characterized populations.

Intermediate-term goals

- Conduct studies of the association between the clinical measurement of serum estradiol and estrone levels and prespecified clinical outcomes.
- Obtain definitive evidence to examine the relationship between low and ultra-low dose vaginal estrogen formulations that induce short-term or long-term increases in plasma/serum estradiol levels.

Long-term goals

- Conduct additional clinical studies to determine the association between estradiol and estrone levels measured by using standardized LC or GC tandem mass spectrometry assays and the risk of breast cancer, endometrial cancer, CVD, cognitive dysfunction, and fractures.
- Measure estradiol and estrone levels in women taking AIs for breast cancer to determine the association between suppressed levels and recurrence rate in these patients.
- Encourage longer-term safety studies of vaginal estrogen preparations and the site of placement in the vagina to minimize any risk of endometrial neoplasia.

SUMMARY OF SPEAKERS' PRESENTATIONS

Although space limitations do not permit publication of individual speaker summaries of the presentations, they are available at Supplementary Digital Content 1, http://links.lww.com/MENO/A586.

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