Extreme Concentrations of Endogenous Sex Hormones, Ischemic Heart Disease, and Death in Women

Marianne Benn, Sidsel Skou Voss, Haya N. Holmegard, Gorm B. Jensen, Anne Tybjærg-Hansen, Børge G. Nordestgaard

Objective—Sex hormones may be critical determinants of ischemic heart disease and death in women, but results from previous studies are conflicting. To clarify this, we tested the hypothesis that extreme plasma concentrations of endogenous estradiol and testosterone are associated with risk of ischemic heart disease and death in women.

Approach and Results—In a nested prospective cohort study, we measured plasma estradiol in 4600 and total testosterone in 4716 women not receiving oral contraceptives or hormonal replacement therapy from the 1981 to 1983 examination of the Copenhagen City Heart Study. During ≤30 years of follow-up, 1013 women developed ischemic heart disease and 2716 died. In women with a plasma estradiol below the fifth percentile compared with between the 10th and 89th percentiles, multifactorially adjusted risk of ischemic heart disease was 44% (95% confidence interval, 14%–81%) higher; however, plasma estradiol concentrations did not associate with death. Also, in women with a plasma testosterone concentration at or above the 95th percentile compared with between the 10th and 89th percentiles, multifactorially adjusted risk was 68% (34%–210%) higher for ischemic heart disease, 36% (18%–58%) higher for any death, and 38% (15%–65%) higher for death from other causes than cardiovascular disease and cancer. These results were similar for postmenopausal women alone.

Conclusions—In women, extreme low concentrations of endogenous estradiol were associated with high risk of ischemic heart disease, and extreme high concentrations of endogenous testosterone were associated with high risk of ischemic heart disease and death. (Arterioscler Thromb Vasc Biol. 2015;35:471-477. DOI: 10.1161/ATVBAHA.114.304821.)

Key Words: death ■ epidemiology ■ estrogens ■ general social development and population ■ gonadal steroid hormones ■ myocardial infarction ■ myocardial ischemia ■ testosterone

The incidence of ischemic heart disease is lower in premenopausal than in postmenopausal women. Also, ischemic heart disease occurs in women at a later age than in men. Together this suggests that a low plasma concentration of estradiol and a low plasma estradiol:testosterone ratio may be an explanation for the increased risk of ischemic heart disease in postmenopausal women, as both these measurements decrease around menopause. Although evidence from animal studies have suggested that both estradiol and testosterone have atheroprotective effects, randomized clinical intervention trials and a national study have in contrast reported an increased risk of ischemic heart disease in women using oral contraceptives or hormonal replacement therapy. Also, previous studies investigating the associations between endogenous sex hormones and ischemic heart disease are few and results are conflicting with reports of high risk in women with both low and high concentrations of endogenous estradiol and testosterone.2–5

The objectives of this study were as follow: first, to examine the associations between concentrations of endogenous plasma estradiol and testosterone and factors that may contribute to risk of ischemic heart disease and death, that is, age, total cholesterol, high-density lipoprotein cholesterol, body mass index, glucose, and systolic and diastolic blood pressure; second, to examine the associations between extreme plasma concentrations of estradiol and testosterone and risk of ischemic heart disease and death. For these purposes, we examined women from the 1981 to 1983 examination of the Copenhagen City Heart Study not receiving oral contraceptives or hormonal replacement therapy, and followed them prospectively for ≤30 years. To reduce bias introduced by physiologically low plasma estradiol concentrations in older
compared with younger women, percentiles of sex hormones were generated in 10-year age groups.

**Materials and Methods**

Materials and methods are available in the online-only Data Supplement.

**Results**

In this nested cohort study, we included 4716 women from the 1981 to 1983 examination of the Copenhagen City Heart Study, which in total examined 12,698 individuals (7018 women and 5680 men) and is a prospective general population study, initiated in 1976 to 1978 with follow-up examinations in 1981 to 1983, 1991 to 1994, and 2001 to 2003. Among the 7018 women, plasma was available on 6023; however, as 247 women receiving oral contraceptives and 1060 receiving hormonal replacement therapy at baseline were excluded, plasma estradiol and testosterone measurements were available in 4600 and 4716 women for the present study. Baseline characteristics of these women are shown in the Table.

**Plasma Estradiol and Testosterone**

For plasma estradiol and testosterone concentrations, the median value was 445 pmol/L (interquartile range, 280–760; 95% confidence interval, 160–2170) in premenopausal and 150 pmol/L (120–180; 60–240) in postmenopausal women (Figure 1). For plasma testosterone concentrations, the corresponding values were 1.9 nmol/L (1.6–2.3; 1.1–3.1) in premenopausal and 1.9 nmol/L (1.5–2.3; 1.1–3.1) in postmenopausal women. Plasma estradiol and testosterone concentrations were relatively high in both pre- and postmenopausal women and we therefore tested the reproducibility of the measurements using another immunoassay on a different autoanalyzer. $R^2$ for comparison between the 2 assays were $R^2=0.87$ for estradiol (bias: 33.3 pmol/L) and $R^2=0.92$ for testosterone (bias: −0.76 nmol/L; Figure I in the online-only Data Supplement). A 10% lower plasma estradiol concentration was associated with slightly higher plasma total cholesterol (<0.001 mmol/L), plasma glucose (<0.001 mmol/L), and systolic and diastolic blood pressure (<0.001 mm Hg; Figure 2). Also, a 10% higher plasma testosterone concentration was associated with a 0.05-year higher median age at menopause, a 0.02-mmol/L higher plasma total cholesterol, a 0.03-kg/m² higher body mass index, a 0.01-mmol/L higher plasma glucose, a 0.38-mm Hg higher systolic blood pressure, and a 0.19-mm Hg higher diastolic blood pressure.

**Risk of Ischemic Heart Disease**

For plasma estradiol concentrations, the multifactorially adjusted risk of ischemic heart disease was increased by 44% (95% confidence interval, 14%–81%) in women below the fifth percentile compared with women in the 10th to 89th percentiles; for estradiol:testosterone ratios, the corresponding risk was 61% (24%–208%; Figure 3). These values translate into a population attributable fraction for risk of ischemic heart disease of 12%.

<table>
<thead>
<tr>
<th>Table. Characteristics From Baseline at the 1981 to 1983 Examination of the Copenhagen City Heart Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Women</td>
</tr>
<tr>
<td>No. of participants</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
</tr>
<tr>
<td>Menopause</td>
</tr>
<tr>
<td>Age at menopause, y</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
</tr>
<tr>
<td>Current smokers</td>
</tr>
<tr>
<td>Alcohol consumption, units/wk</td>
</tr>
</tbody>
</table>

Women using hormonal replacement therapy or oral contraceptives were excluded. Continuous data are reported as median and interquartile range and categorical variables in percent. One unit alcohol corresponds to 12 g alcohol. HDL indicates high-density lipoprotein.
heart disease of 1.6% (0.6%–2.2%) for plasma estradiol and of 1.9% (1.0%–2.6%) for the estradiol:testosterone ratio. Results were similar for postmenopausal women alone.

For plasma testosterone concentrations, the multifactorially adjusted risk of ischemic heart disease was increased by 68% (34%–210%) in women at or above the 95th percentile compared with women in the 10th to 89th percentiles (Figure 3). This value translates into a population attributable fraction for risk of ischemic heart disease of 2.0% (1.3%–2.6%) for plasma testosterone. Results were similar for postmenopausal women alone.

Risk of ischemic heart disease was not higher in women with extreme high plasma concentrations of endogenous estradiol, extreme low plasma concentrations of testosterone, or in women with extreme high estradiol:testosterone ratios (Figure 3).

**Risk of Death**

Plasma estradiol concentration did not associate with risk of death (Figure 4). In contrast, for plasma testosterone concentrations the multifactorially adjusted risk of death was 36% (18%–58%) higher in women at or above the 95th percentile compared with women with a concentration in the 10th to 89th percentiles. This result translates into a population attributable fraction for risk of death of 1.3% (0.8%–1.8%) for plasma testosterone. Results were similar for postmenopausal women alone.

Plasma estradiol concentrations did not associate with risk of death from cardiovascular disease, cancer, breast cancer, or other causes (Figure II in the Data Supplement). In contrast, for plasma testosterone concentrations, the multifactorially adjusted risk of death from other causes was 38% (15%–65%) higher in women at or above the 95th percentile when compared with women in the 10th to 89th percentiles. Also, for estradiol:testosterone ratios, the multifactorially adjusted risk of death from other causes was 23% (0%–52%) higher in women below the fifth percentile compared with women in the 10th to 89th percentiles.

**Discussion**

The main findings of this study of women are that extreme low plasma concentrations of endogenous estradiol were associated with high risk of ischemic heart disease, and that extreme high plasma concentrations of endogenous testosterone were associated with high risk of ischemic heart disease and death.

Major hormonal changes occur during menopause, including a profound fall in circulating estradiol concentrations of ovarian origin. In cross-sectional studies, concentrations of total testosterone have been reported to be lower in postmenopausal compared with premenopausal women, whereas in longitudinal studies, either no change or a 15% to 30% fall have been reported. In the present study of women from the general population, we observed the expected fall in estradiol coinciding with menopause, whereas testosterone concentrations were largely unchanged from the age of 20 to >80 years.

Median plasma estradiol and testosterone concentrations were in the present study relatively high in both pre- and postmenopausal women. The reason for this is unknown; however,
all plasma samples were analyzed between 2009 and 2011 using the same immunoassays with a high accuracy; samples were only thawed once (freezing may reduce estradiol concentrations); a subset of samples reanalyzed in a different laboratory using other immunoassays on a different autoanalyzer showed a good reproducibility; and the physiological changes in estradiol levels during life were preserved in the samples. Importantly, as we have used percentiles generated within 10-year age groups, the relatively high median concentrations do not invalidate the results and conclusions of the present article, although they might limit the generalizability of our results to other populations.

Several studies have reported effects of estrogen and testosterone on cardiovascular risk factors. Low plasma estradiol concentrations have been generally associated with high concentrations of total cholesterol and low concentrations of high-density lipoprotein cholesterol.1,12; however, a recent study of women with ovarian failure and low estradiol concentrations also reported high total cholesterol and low-density lipoprotein cholesterol concentrations.13 In the present study using percentiles of sex hormones generated in 10-year age groups and thus circumventing the age-dependent changes in sex hormones, we observed that low plasma concentrations of endogenous estradiol were associated with modest adverse effects on cardiovascular risk factors, and that high plasma concentrations of endogenous testosterone were associated with adverse effects on plasma total cholesterol, body mass index, plasma glucose, and blood pressure in women. This suggests that low estradiol is directly associated with cardiovascular risk, whereas the risk observed for high testosterone may be mediated through other cardiovascular risk factors.

Mechanistically our findings of risk of ischemic heart disease and death may be explained by multiple effects of sex hormones on the cardiovascular system.14–17 Thus, androgens including testosterone are precursors for estradiol biosynthesis14 and estradiol and testosterone exert important actions in all body cells through interaction with estradiol and androgen receptors: binding of estradiol and testosterone to their receptors may either mediate a direct action on target cells,18 may act as transcription factors,19 or have other nongenomic actions in the cardiovascular system.20 Indeed, both estradiol and testosterone have effects on endothelial cells, cardiovascular smooth muscle cells, myocardial fibers, macrophages, and platelets.14–17

Results from previous conventional epidemiological studies investigating the association between plasma concentrations of endogenous estradiol and testosterone and risk of ischemic heart disease are conflicting.2–4 In the present study, we observed that extreme low, but not high plasma concentrations of endogenous estradiol were associated with higher risk of ischemic heart disease; and, as opposed to a previous study,3 that extreme high, but not low plasma concentrations of endogenous testosterone were associated with high risk of ischemic heart disease. Strengthening the present findings, the risk was intermediate in women with a plasma estradiol in the fifth to ninth percentiles and a testosterone in the 90th to 94th percentiles, and even higher in women with a plasma estradiol...
below the fifth percentile and a plasma testosterone at or above the 95th percentile, suggesting a continuum of risks as function of low estradiol and high testosterone concentrations. To define thresholds at which the risk increases and to translate these thresholds into clinical relevant recommendations for risk prediction and potential treatment would require a large study or meta-analysis of large studies.

Population wise, the fraction of women at risk of ischemic heart disease and death because of extreme levels of endogenous sex hormones is small, and the proportion of risk potentially prevented by a normalization of plasma estradiol and testosterone concentrations is <2%. Also, the 44% higher risk of ischemic heart disease observed in women with a plasma estradiol below the fifth percentile compared with those in the 10th to 89th percentiles is relatively modest, taking into account that, for example, smoking is associated with a 65% higher risk of ischemic heart disease in the same population.

Early case-control studies and a meta-analysis of such studies suggested that hormone replacement with estrogen might reduce the risk of ischemic heart disease and death because of extreme levels of endogenous sex hormones is small, and the proportion of risk potentially prevented by a normalization of plasma estradiol and testosterone concentrations is <2%. Also, the 44% higher risk of ischemic heart disease observed in women with a plasma estradiol below the fifth percentile compared with those in the 10th to 89th percentiles is relatively modest, taking into account that, for example, smoking is associated with a 65% higher risk of ischemic heart disease in the same population.

Early case-control studies and a meta-analysis of such studies suggested that hormone replacement with estrogen might reduce the risk of ischemic heart disease and death because of extreme levels of endogenous sex hormones is small, and the proportion of risk potentially prevented by a normalization of plasma estradiol and testosterone concentrations is <2%. Also, the 44% higher risk of ischemic heart disease observed in women with a plasma estradiol below the fifth percentile compared with those in the 10th to 89th percentiles is relatively modest, taking into account that, for example, smoking is associated with a 65% higher risk of ischemic heart disease in the same population.

A known limitation of prospective studies using a single baseline measurement for classification is misclassification because of regression dilution bias because extreme values at baseline tend to attenuate over time, regress toward the mean value of measurements, cause an underestimation of the association between sex hormone and risk, and thus give more conservative risk estimates. For estradiol this may introduce a bias in premenopausal women where estradiol concentrations fluctuate during the menstrual cycle, cause random variation, and thus wider confidence intervals on risk estimates. For testosterone, a single measurement has been suggested to be fairly representative for the long-term hormonal milieu in men; however, little is known about estradiol and testosterone concentrations over time in women. As mentioned above, the median plasma concentrations of

<table>
<thead>
<tr>
<th>Percentiles</th>
<th>No. of participants</th>
<th>No. of events</th>
<th>HR (95% CI)</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>243</td>
<td>170</td>
<td>1.03 (0.88-1.20)</td>
<td>0.46</td>
</tr>
<tr>
<td>5-9</td>
<td>276</td>
<td>267</td>
<td>1.09 (0.95-1.26)</td>
<td>0.46</td>
</tr>
<tr>
<td>10-89</td>
<td>3,591</td>
<td>2,506</td>
<td>1 (reference)</td>
<td>0.46</td>
</tr>
<tr>
<td>90-94</td>
<td>256</td>
<td>182</td>
<td>0.89 (0.85-1.15)</td>
<td>0.46</td>
</tr>
<tr>
<td>95-100</td>
<td>232</td>
<td>136</td>
<td>1.10 (0.92-1.31)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Testosterone</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>232</td>
<td>155</td>
<td>0.69 (0.79-1.10)</td>
<td>0.24</td>
</tr>
<tr>
<td>5-9</td>
<td>233</td>
<td>148</td>
<td>0.82 (0.78-0.99)</td>
<td>0.24</td>
</tr>
<tr>
<td>10-89</td>
<td>3,797</td>
<td>2,658</td>
<td>1 (reference)</td>
<td>0.24</td>
</tr>
<tr>
<td>90-94</td>
<td>199</td>
<td>143</td>
<td>1.25 (1.00-1.49)</td>
<td>0.24</td>
</tr>
<tr>
<td>95-100</td>
<td>255</td>
<td>196</td>
<td>1.38 (1.18-1.58)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Estradiol/testosterone ratio</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>100</td>
<td>141</td>
<td>1.17 (0.89-1.13)</td>
<td>0.04</td>
</tr>
<tr>
<td>5-9</td>
<td>232</td>
<td>175</td>
<td>1.10 (0.94-1.26)</td>
<td>0.04</td>
</tr>
<tr>
<td>10-89</td>
<td>3,077</td>
<td>2,506</td>
<td>1 (reference)</td>
<td>0.04</td>
</tr>
<tr>
<td>90-94</td>
<td>224</td>
<td>140</td>
<td>0.93 (0.70-0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>95-100</td>
<td>272</td>
<td>172</td>
<td>0.86 (0.64-1.15)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Figure 4. Risk of death as a function of percentiles of plasma estradiol, plasma total testosterone, and the estradiol:testosterone ratio in women from the Copenhagen City Heart Study. Total number of women in groups may vary slightly because of availability of sex hormone measurements and covariates. CI indicates confidence interval; and HR, hazard ratio.
both estradiol and testosterone measured in the present study were relatively high and there was an ≈20% bias on plasma testosterone values >25 nmol/L between the 2 methods used, potentially limiting the generalizability of our results to other study populations. Another potential limitation of the present study is that we measured total testosterone, which includes the biologically active-free testosterone and testosterone bound to sex hormone binding globulin. It is not known how well the concentration of total testosterone correlates with the concentration of free testosterone, and whether the association of testosterone concentrations with risk of ischemic heart disease and death is because of high total or free testosterone concentrations. Furthermore, because testosterone can be aromatized to estradiol in endothelial cells, plasma concentrations of estradiol and testosterone provide only a partial indication of the potential concentrations at the target tissue. This variation in bioavailability may influence the strength of the associations of testosterone with other cardiovascular risk factors. Finally, it is a strength, as well as a potential limitation that we studied white women only; however, we are not aware of data to suggest that our results might not apply to women of all races.

In conclusion, in women, extreme low plasma concentrations of endogenous estradiol were associated with high risk of ischemic heart disease and death is because of high total or free testosterone concentrations. Furthermore, because testosterone can be aromatized to estradiol in endothelial cells, plasma concentrations of estradiol and testosterone provide only a partial indication of the potential concentrations at the target tissue. This variation in bioavailability may influence the strength of the associations of testosterone with other cardiovascular risk factors.

Acknowledgments

We are indebted to the participants and the staff of the Copenhagen City Heart Study for their important contributions. Rikke Aamo is thanked for expert technical assistance.

Sources of Funding

We thank The Research Fund of the Capital Region of Denmark and Herlev Hospital, Copenhagen University Hospital for support to this project and The Danish Heart Foundation for support to the Copenhagen City Heart Study.

Disclosures

None.

References


Sex hormones may be critical determinants of ischemic heart disease and mortality risk in women, but results from previous studies are conflicting. The present study tested the hypothesis that extreme plasma concentrations of endogenous estradiol and testosterone are associated with increased risk of ischemic heart disease and death in women from the general population. The study showed that in women, extreme low plasma concentrations of endogenous estradiol were associated with high risk of ischemic heart disease, and extreme high plasma concentrations of endogenous testosterone were associated with high risk of ischemic heart disease and death. These results suggest that the safety of testosterone supplementation resulting in extreme high testosterone concentrations in women might be a concern.

**Significance**

Sex hormones may be critical determinants of ischemic heart disease and mortality risk in women, but results from previous studies are conflicting. The present study tested the hypothesis that extreme plasma concentrations of endogenous estradiol and testosterone are associated with increased risk of ischemic heart disease and death in women from the general population. The study showed that in women, extreme low plasma concentrations of endogenous estradiol were associated with high risk of ischemic heart disease, and extreme high plasma concentrations of endogenous testosterone were associated with high risk of ischemic heart disease and death. These results suggest that the safety of testosterone supplementation resulting in extreme high testosterone concentrations in women might be a concern.
Extreme Concentrations of Endogenous Sex Hormones, Ischemic Heart Disease, and Death in Women
Marianne Benn, Sidsel Skou Voss, Haya N. Holmegard, Gorm B. Jensen, Anne Tybjærg-Hansen and Børge G. Nordestgaard

Arterioscler Thromb Vasc Biol. 2015;35:471-477; originally published online December 30, 2014;
doi: 10.1161/ATVBAHA.114.304821
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/2/471

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/12/30/ATVBAHA.114.304821.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Material and Methods:

**Extreme concentrations of endogenous sex hormones, ischemic heart disease and death in women**

Marianne Benn MD PhD DMSc\(^1,4,5\), Sidsel Skou Voss MD\(^2\), Haya N. Holmegard MSc PhD\(^1\), Gorm B. Jensen MD DMSc\(^4\), Anne Tybjærg-Hansen MD DMSc\(^3,4,5\), Børge G. Nordestgaard MD DMSc\(^2,4,5\).

\(^1\)Department of Clinical Biochemistry, Gentofte Hospital, Copenhagen University Hospital.
\(^2\)Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital.
\(^3\)Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital.
\(^4\)The Copenhagen City Heart Study, Frederiksberg Hospital, Copenhagen University Hospital.
\(^5\)Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.
Material and methods

Participants
Participants were from the 1981-1983 examination of the Copenhagen City Heart Study, a prospective general population study, initiated in 1976-1978 and with follow-up examinations in 1981-1983, 1991-1994 and 2001-2003. Individuals were selected from the national Danish Civil Registration System to reflect the adult Danish population aged 20-100 years and all were whites of Danish descent. Data were obtained from a self-completed questionnaire, a brief physical examination, and a non-fasting venous blood sample. Baseline plasma estradiol concentrations were measured in 4,600 women and testosterone concentration in 4,716 women; the estradiol to testosterone ratio was available in 4,593 women. None of the women was receiving oral contraceptives or hormonal replacement therapy. The study was approved by Herlev Hospital, Copenhagen University Hospital and by a Danish ethical committee (KF-100.2039/91), and was conducted according to the Declaration of Helsinki. Informed written consent was obtained from all participants at the time of examination and included permission to retrieve data from medical records and registries, and later use of stored blood samples.

Estradiol and testosterone
Plasma samples drawn at baseline in 1981-1983 were stored at −20 °C until analysis. Analyses were performed daily in batches from February 2009 through March 2011 using the “Estradiol-6 III” assay measuring total estradiol (reference interval for postmenopausal women: 27.2-118.2 pmol/L; analytical sensitivity of 27.2 pmol/L with intra-serial coefficient of variation of 5.6% and inter-serial coefficient of variation of 1.9% at estradiol concentrations of 244 pmol/L, and calibrator target values of respectively 118 pmol/L and 8322 pmol/L) and the “Testosterone” assay measuring total testosterone (reference interval in women: 0.5-2.6 nmol/L; analytical sensitivity of 0.35 nmol/L with intra-serial coefficient of variation of 6.2% and inter-serial coefficient of variation of 4.4% at testosterone concentrations of 3.31 nmol/L, and calibrator target values of respectively 2.78 nmol/L and 27.76 nmol/L). Both assays are competitive immunoassays and are routinely analyzed on an ADVIA Centaur XP using two calibrators traceable to isotope dilution-gas chromatography/mass spectroscopy for each assay (all Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Analyses were followed for precision using daily internal controls and 12 times a year for accuracy with a Scandinavian external control program (coefficients of variation were in 2009-2011 all ≤ 7.2%).

Because population median concentrations of estradiol and testosterone from 1981-1983 were relatively high in both pre- and postmenopausal women compared to previous reports, the accuracy of the estradiol and testosterone measurements were further validated in a subset of respectively, 159 and 191 samples, by analysis in another laboratory, using different immunoassays on a different autoanalyzer (Estradiol II and Testosterone II on a Cobas e411; Roche Diagnostics, IN, USA). R² for comparison between the two assays were R²=0.87 for estradiol (bias: 33.3 pmol/L) and R²=0.92 for testosterone (bias: -0.76 nmol/L)(Supplementary Figure 1).

Endpoints
Individuals were followed from baseline in 1981-1983 until the occurrence of ischemic heart disease, death, or end of follow-up May 2011, whichever came first. Follow-up for death was not censored by non-fatal ischemic heart disease. Follow-up was 100% complete, that is, we
did not lose track of any individuals. Median follow-up time was 19 (range: 0-30) years. Information on a diagnosis of ischemic heart disease (WHO; International Classification of Diseases (ICD), ICD-8: 410-414, and ICD-10: I20-I25) was retrieved from the national Danish Patient Registry and causes of death entered in the national Danish Causes of Death Registry, and diagnoses were verified by reviewing hospital admissions and medical records from hospitals and general practitioners\textsuperscript{2}. Ischemic heart disease was fatal and non-fatal myocardial infarction or characteristic symptoms of angina pectoris based on location, character and duration of pain, and relation of pain to exercise; myocardial infarction required the presence of characteristic chest pain, electrocardiographic changes, and/or elevated cardiac enzymes following changing criteria over time\textsuperscript{3}. Primary causes of death were categorized as cardiovascular death (ICD-8: 410-414, 430-439; and ICD-10: I20-I25, I60-I69), cancer death (ICD-8: 140-209; and ICD-10: C0-C97), and other causes of death (all other codes not included in the two previous categories).

**Other covariates**

Body mass index was measured weight (kg) divided by measured height squared (m\textsuperscript{2}). Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured using colorimetric assays (Boehringer Mannheim, Manheim, Germany). Information on menopausal status, age at menopause, use of oral contraceptives and hormone replacement therapy (the latter two were exclusion criteria) was self-reported. Hypertension was systolic blood pressure $\geq 140$ mmHg ($\geq 135$ mmHg for diabetics), diastolic blood pressure $\geq 90$ mmHg ($\geq 85$ mmHg for diabetics)$^4$, and/or use of antihypertensive medication prescribed specifically for hypertension. Diabetes mellitus was defined according to the current WHO criteria as a non-fasting blood glucose concentration $>11$ mmol/L$^5$, use of anti-diabetic medication and/or a diagnosis of diabetes mellitus type 1 or 2 (ICD-8: 249-250; and ICD-10: E10, E11, E13, E14). Smokers were current smokers. Weekly alcohol intake was self-reported in units, 1 unit $\sim 12$ gram of alcohol. None received lipid-lowering therapy at baseline.

**Statistical analyses**

Data were analyzed using STATA/S.E.12.1. To test for systematic and stepwise changes in risk for increasingly extreme values of sex hormones, trend across ordered groups was tested using the nonparametric Cuzick’s extension of a Wilcoxon rank-sum test. Continuous variables were summarized by median and interquartile range. Categorical variables were expressed as number and percentage.

Correlation of plasma sex hormones with known risk factors for ischemic heart disease was examined using linear regression, and reported as the change in the risk factor for a 10% lower plasma estradiol, or a 10% higher plasma testosterone.

Finally, the associations of plasma concentrations of estradiol and plasma concentrations of testosterone, and estradiol to testosterone ratio with risk of ischemic heart disease and death were explored by strata of median age, body mass index (normal weight versus overweight/obese), hypertension (no/yes), median total cholesterol, menopausal status (no/yes), smoking (no/yes), and median alcohol intake using Cox regression analyses. Multifactorial adjustment was as described below, with the parameter stratified on excluded from the adjustment.

To test whether extreme plasma concentrations of estradiol, extreme plasma concentrations of testosterone, and extreme estradiol to testosterone ratio were associated with increased
risk of ischemic heart disease and death, we defined ordered categories of sex hormones \textit{a priori} on the basis of extreme percentiles of the distribution, as previously done for other biomarkers\textsuperscript{6,7}. To reduce bias due to age dependent variation in sex hormone concentrations, percentiles were generated in 10 year age groups. The percentile groups were 10\textsuperscript{th}-89\textsuperscript{th} (reference) versus 5\textsuperscript{th}-10\textsuperscript{th} and 0\textsuperscript{th}-4\textsuperscript{th}, or 90\textsuperscript{th}-94\textsuperscript{th}, and 95≥\textsuperscript{th}percentile (categories coded as 1, 2, and 3).

Cox regression models with age as time scale and left truncation (delayed entry at the 1981-1983 examination) were used to estimate hazard ratios for endpoints. Risk of endpoints was estimated as a function of plasma estradiol, plasma total testosterone, and estradiol to testosterone ratio in groups of percentiles. Individuals diagnosed with an endpoint before study entry were excluded from Cox regression analyses, and those dying during follow-up were censured at their death date. Statistical analyses were adjusted for 1) age or 2) multifactorially for age, body mass index, total cholesterol, HDL cholesterol, menopausal status, age at menopause, hypertension, diabetes mellitus, current smoking, and alcohol intake. To test for trend in risk across increasingly extreme categories of sex hormones, trend was tested for by including each sex hormone ordered category as a continous variable into the Cox regression model.

To estimate the public health impact of having extreme plasma estradiol and testosterone concentrations, we calculated the category attributable fraction in women in the category at risk\textsuperscript{8,9}. This measure is calculated as the proportion of the risk category of the total population of women, multiplied by \(((\text{risk in the category}-1)/\text{risk in the category})\) and the estimate thus depends on both the multifactorially adjusted risk estimate in the risk category and the prevalence of the risk factor.
References for Materials and methods


2. Kamstrup PR, Tybjaerg-Hansen A, Steffensen R, Nordestgaard BG. Genetically elevated lipoprotein(a) and increased risk of myocardial infarction. JAMA. 2009; 301: 2331-2339.


Supplementary Material:

**Extreme concentrations of endogenous sex hormones, ischemic heart disease and death in women**

Marianne Benn MD PhD DMSc\(^1\), Sidsel Skou Voss MD\(^2\), Haya N. Holmegard MSc PhD\(^1\), Gorm B. Jensen MD DMSc\(^4\), Anne Tybjærg-Hansen MD DMSc\(^3\,\,\,^4\,\,\,^5\), Børge G. Nordestgaard MD DMSc\(^2\,\,\,^4\,\,\,^5\).

\(^1\)Department of Clinical Biochemistry, Gentofte Hospital, Copenhagen University Hospital.
\(^2\)Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital.
\(^3\)Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital.
\(^4\)The Copenhagen City Heart Study, Frederiksberg Hospital, Copenhagen University Hospital.
\(^5\)Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.
Supplementary Figure IA.

Estradiol

$Y = -20.4 + 0.94X$

$R^2 = 0.87$

$n=159$

Bias: 33.3 pmol/L (95% CI: 18.1 to 48.5 pmol/L)

Limits of agreement: -160.7 to 227.3
Supplementary Figure IB.

Testosterone

Y = 0.70 + 0.83X
R² = 0.92
n=191

Bias: -0.76 nmol/L (95% CI: -1.15 to -0.37 nmol/L)
Limits of agreement: -6.1 to 4.9
Association between sex hormone concentrations measured using two different immunoassays on two different autoanalyzers. Upper parts of the two panels show the association between, plasma estradiol (Panel A) and plasma total testosterone (Panel B) concentrations measured using Estradiol II and Testosterone II on a Cobas e411 (Roche Diagnostics, Indianapolis, IN, USA) autoanalyzer and Estradiol-6 III and Testosterone on an ADVIA Centaur XP (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Lower parts of each panel show the difference between paired results from the two methods plotted as a function of the average of the two methods. Broken black line denotes the reference line, solid black line the bias, and solid blue lines the upper and lower limits of agreement for the methods. Samples were a subset from the 1981-1983 and 1991-1994 examinations of the Copenhagen City Heart Study and from participants enrolled in the Copenhagen General Population Study from 2003-2011.


### Supplementary Figure II.

Risk of cardiovascular death, cancer death, death of breast cancer, or death of other causes as a function of percentiles of plasma estradiol, total testosterone, and the estradiol to testosterone ratio in women from the Copenhagen City Heart Study. HR=hazard ratio; CI=confidence interval.

<table>
<thead>
<tr>
<th>Percentiles</th>
<th>No. of participants</th>
<th>No. of events</th>
<th>HR (95%CI)</th>
<th>P-trend</th>
<th>No. of events</th>
<th>HR (95%CI)</th>
<th>P-trend</th>
<th>No. of events</th>
<th>HR (95%CI)</th>
<th>P-trend</th>
<th>No. of events</th>
<th>HR (95%CI)</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estradiol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>243</td>
<td>34</td>
<td>1.03 (0.72-1.47)</td>
<td>0.54</td>
<td>21</td>
<td>0.72 (0.46-1.11)</td>
<td>0.12</td>
<td>6</td>
<td>0.82 (0.36-1.88)</td>
<td>0.27</td>
<td>115</td>
<td>1.12 (0.92-1.35)</td>
<td>0.16</td>
</tr>
<tr>
<td>5-9</td>
<td>276</td>
<td>44</td>
<td>1.17 (0.86-1.60)</td>
<td>0.27</td>
<td>31</td>
<td>0.94 (0.65-1.36)</td>
<td>0.16</td>
<td>4</td>
<td>0.48 (0.18-1.30)</td>
<td>0.21</td>
<td>132</td>
<td>1.11 (0.93-1.32)</td>
<td>0.29</td>
</tr>
<tr>
<td>10-89</td>
<td>3,591</td>
<td>507</td>
<td>1 (reference)</td>
<td>0.12</td>
<td>414</td>
<td>1 (reference)</td>
<td>0.05</td>
<td>113</td>
<td>1 (reference)</td>
<td>0.60</td>
<td>1,585</td>
<td>1 (reference)</td>
<td>0.29</td>
</tr>
<tr>
<td>90-94</td>
<td>258</td>
<td>34</td>
<td>0.91 (0.64-1.29)</td>
<td>0.60</td>
<td>25</td>
<td>0.84 (0.56-1.26)</td>
<td>0.27</td>
<td>2</td>
<td>0.24 (0.06-0.97)</td>
<td>0.95</td>
<td>123</td>
<td>1.06 (0.88-1.27)</td>
<td>0.95</td>
</tr>
<tr>
<td>95-100</td>
<td>232</td>
<td>25</td>
<td>1.13 (0.75-1.70)</td>
<td>0.95</td>
<td>27</td>
<td>1.00 (0.66-1.50)</td>
<td>0.60</td>
<td>9</td>
<td>1.13 (0.55-2.34)</td>
<td>0.29</td>
<td>84</td>
<td>1.12 (0.90-1.40)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Testosterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>232</td>
<td>35</td>
<td>1.04 (0.73-1.47)</td>
<td>0.72</td>
<td>21</td>
<td>0.84 (0.54-1.31)</td>
<td>0.38</td>
<td>3</td>
<td>0.43 (0.14-1.36)</td>
<td>0.15</td>
<td>99</td>
<td>0.92 (0.75-1.12)</td>
<td>0.21</td>
</tr>
<tr>
<td>5-9</td>
<td>233</td>
<td>34</td>
<td>1.08 (0.76-1.53)</td>
<td>0.15</td>
<td>24</td>
<td>0.92 (0.61-1.39)</td>
<td>0.15</td>
<td>6</td>
<td>0.87 (0.38-1.99)</td>
<td>0.15</td>
<td>90</td>
<td>0.88 (0.71-1.09)</td>
<td>0.21</td>
</tr>
<tr>
<td>10-89</td>
<td>3,797</td>
<td>531</td>
<td>1 (reference)</td>
<td>1.12</td>
<td>426</td>
<td>1 (reference)</td>
<td>1.12</td>
<td>112</td>
<td>1 (reference)</td>
<td>1.70</td>
<td>1,701</td>
<td>1 (reference)</td>
<td>0.21</td>
</tr>
<tr>
<td>90-94</td>
<td>199</td>
<td>22</td>
<td>1.06 (0.69-1.63)</td>
<td>0.81</td>
<td>29</td>
<td>1.35 (0.92-1.99)</td>
<td>0.81</td>
<td>9</td>
<td>1.77 (0.89-3.50)</td>
<td>0.45</td>
<td>92</td>
<td>1.27 (1.03-1.57)</td>
<td>0.05</td>
</tr>
<tr>
<td>95-100</td>
<td>255</td>
<td>37</td>
<td>1.36 (0.97-1.90)</td>
<td>0.11</td>
<td>31</td>
<td>1.26 (0.87-1.83)</td>
<td>0.07</td>
<td>10</td>
<td>1.55 (0.81-2.97)</td>
<td>0.06</td>
<td>128</td>
<td>1.38 (1.15-1.65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Estradiol/testosterone ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>188</td>
<td>28</td>
<td>1.18 (0.80-1.73)</td>
<td>0.57</td>
<td>18</td>
<td>0.91 (0.57-1.47)</td>
<td>0.95</td>
<td>5</td>
<td>1.02 (0.41-2.50)</td>
<td>0.81</td>
<td>95</td>
<td>1.23 (1.00-1.52)</td>
<td>0.03</td>
</tr>
<tr>
<td>5-9</td>
<td>232</td>
<td>29</td>
<td>0.94 (0.64-1.36)</td>
<td>0.57</td>
<td>33</td>
<td>1.20 (0.83-1.73)</td>
<td>0.95</td>
<td>8</td>
<td>1.21 (0.59-2.48)</td>
<td>0.81</td>
<td>113</td>
<td>1.12 (0.92-1.36)</td>
<td>0.03</td>
</tr>
<tr>
<td>10-89</td>
<td>3,677</td>
<td>519</td>
<td>1 (reference)</td>
<td>0.95</td>
<td>414</td>
<td>1 (reference)</td>
<td>0.95</td>
<td>111</td>
<td>1 (reference)</td>
<td>1.63</td>
<td>1,635</td>
<td>1 (reference)</td>
<td>0.03</td>
</tr>
<tr>
<td>90-94</td>
<td>224</td>
<td>37</td>
<td>1.06 (0.76-1.48)</td>
<td>0.78</td>
<td>22</td>
<td>0.81 (0.52-1.24)</td>
<td>0.78</td>
<td>5</td>
<td>0.67 (0.27-1.64)</td>
<td>0.78</td>
<td>81</td>
<td>0.76 (0.61-0.95)</td>
<td>0.16</td>
</tr>
<tr>
<td>95-100</td>
<td>272</td>
<td>30</td>
<td>0.89 (0.61-1.29)</td>
<td>0.78</td>
<td>31</td>
<td>1.04 (0.71-1.51)</td>
<td>0.78</td>
<td>5</td>
<td>0.59 (0.24-1.46)</td>
<td>0.15</td>
<td>111</td>
<td>1.00 (0.82-1.21)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

**Hazard ratio (95% confidence interval)**