Urinary Sex Hormone Excretions in Premenopausal Women and Coronary Heart Disease Risk: A Nested Case-Referent Study in the DOM-Cohort

W. J. M. J. Gorgels,1 Y. v. d. Graaf,2 M. A. Blankenstein,3 H. J. A. Collette,1 D. W. Erkelens,1 and J. D. Banga1,*

1Department of Internal Medicine, Academic Hospital Utrecht, 2Department of Epidemiology and Health Care, University of Utrecht, and 3Department of Endocrinology, Academic Hospital Utrecht, 3508 GA Utrecht, The Netherlands

ABSTRACT. The low incidence of coronary heart disease (CHD) in premenopausal women is partly ascribed to protection by endogenous estrogen production. As a consequence, we hypothesized that premenopausal women with low endogenous estrogen production or high androgen production might be at increased risk for CHD.

We studied the relationship between urinary sex hormone excretions and CHD risk by means of a nested case-referent study within a cohort of premenopausal (ages 40—49 yrs) women (n = 11,284). This cohort was formed at a breast cancer screening project in 1982–1986 (The Diagnostisch Onderzoek Mammacarcinoom [DOM] Project). Baseline data included self-administered questionnaires and anthropometric measurements. At the time of screening the women were instructed to collect an overnight urine sample on day 22 of three separate cycles. These urine samples were stored at —20°C. Up to June 1991, 45 subjects were admitted to local hospitals on diagnosis of CHD (29 with myocardial infarction, and 16 with angiographically confirmed coronary disease). Referents were sampled from the cohort, matched for age and year of screening in a 1:3 ratio. In a follow-up study, menopausal state of the subjects was assessed yearly by mailed questionnaires.

Urinary excretions of estrone-glucuronide, pregnanediol-glucuronide, and testosterone-glucuronide adjusted by creatinine were similar for cases and referents. Cases had no earlier menopause than referents, although cases had more anovulatory cycles.


KEY WORDS. Sex hormones, women, coronary disease, urine, risk factor

INTRODUCTION

Endogenous estrogens may protect women from coronary heart disease (CHD). Age-standardized CHD mortality appears to be twice as high in men as in women, and this same ratio is observed in countries with substantial differences in CHD mortality rates [1]. Furthermore, the male/female ratio in CHD mortality declines from about 5 at age thirty to less than 2 at age 75 [1,2]. Ovariectomized women have a higher CHD risk, unless they receive estrogen replacement therapy [3]. Natural menopause changes CHD risk factors such as blood pressure and plasma lipoproteins in an unfavorable direction, which can be attenuated by exogenous estrogens [1,4]. Finally, post-menopausal estrogen replacement therapy is associated with a 50% lower risk for cardiovascular events [5].

The relationship between endogenous estrogens and CHD risk has been intensively investigated in men [1]. In cross-sectional and case-control studies increased or normal plasma estrogen levels were reported in men with CHD [1]. In two prospective studies no relationship was observed between CHD and plasma estrogens [6,7]. In women, endogenous sex hormones in relation to CHD risk have been investigated mainly indirectly by comparison of reproductive histories, including age at menarche, number of pregnancies, age at first delivery, cycle regularity, and age at menopause. High parity and first child birth at an early age has been reported to increase the risk [5,8–10]. Interrelationships between reproductive factors and confounding by social status and pregnancy loss may bias the results of these
If estrogens protect from CHD, women with low endogenous estrogen or high testosterone levels would be at higher risk for this disease. This hypothesis was tested in a nested case-referent study of urinary sex hormone excretions and CHD risk within a cohort of premenopausal women from a breast cancer screening project (The Diagnostisch Onderzoek Mammacarcinoom [DOM] Project [11]).

**METHOD**

**Study Population and Baseline Data Collection**

All women from the city of Utrecht and vicinity, aged 40–49, were invited to participate in a research screening program for early detection of breast cancer in 1982–1986 (The Diagnostisch Onderzoek Mammacarcinoom [DOM] Project [11]). The response rate was 44% (n = 15,483). Exclusion of subjects reporting a history of myocardial infarction (49), angina pectoris and taking medication for this (80), current use of steroid hormones (572), and menopausal status at entry (4,199) reduced the number to 10,583, constituting the cohort of this study. Participants filled out questionnaires covering medical history, use of medication, smoking history, menarche, menstrual cycle, fertility, and pregnancies. Length and weight were measured. Permission was obtained to use the data for future research purposes.

Before examination the women were asked to deliver a urine sample, collected overnight on day 22 of three consecutive menstrual cycles (thus resulting in three luteal samples). Women with irregular cycles had to bring a urine sample at a random day (when the start of the actual cycle could not be defined). The women were asked to keep a menstrual calendar for at least three months, providing information about cycle length and regularity, the exact moment of urine collection, and days of menstrual blood loss. The women born in 1942–1945 (n = 2,528) were screened in 1985 and 1986 and collected one overnight urine sample on day 22 of three consecutive menstrual cycles. Length and weight were measured. Permission was obtained to use the data for future research purposes.

**Assessment of Steroid Glucuronides**

Concentrations of pregnanediol glucuronide and estrone glucuronide were assessed by direct specific radioimmunoassays of urine samples diluted 1:10,000. Reagents were obtained from Dr. P. Samarajeewa, Department of Biochemistry, University College of London, London, U.K.

The intra-assay coefficient of variation (CV) for pregnanediol glucuronide was 31%; 5.2% and 6.7% at concentrations of 0.51; 3.1 and 22.7 μmol/l, respectively (n = 20). The inter-assay CV was 40% and 10.4%, respectively, at 0.46 and 22.4 μmol/l. As more than 98% of the results obtained was between 3 and 22.7 μmol/l, the high CV at the very low level of 0.5 μmol/l was judged not to influence the final outcome. The intra-assay CV for oestrone glucuronide was 12.8%; 6.9% and 6.3% at concentrations of 0.032; 0.102 and 1.02 μmol/l, respectively (n = 20). The inter-assay CV was 13.8% and 6.6%, respectively, at 0.40 and 0.93 μmol/l.

The concentration of testosterone glucuronide was assessed by testosterone by radioimmunoassay after enzymatic
hydrolysis of the urine (diluted 1:20) with 0.32 Units of Escherichia coli β-glucuronidase (Boehringer Mannheim, Germany) /50 µl urine for 20 hrs at 37°C. This enzyme preparation is sulphatase free. After hydrolysis, the solution was neutralized with 50 µl sodium hydroxide and extracted with 5 volumes diethyl ether. The extracts were evaporated to dryness and the residue was dissolved in ethanol. Appropriate aliquots were used for the testosterone radioimmunoassay, which was carried out as described before [15]. The intra-assay CV was 3.5% and 8.8% at 4.6 and 23.8 µmol/l, respectively, whereas the inter-assay CV was found to be 11.7% and 12.6% at the same levels (n = 10).

Analysis
Statistical procedures for matched data were used. Crude analysis was performed with the Wilcoxon test for paired data. For this test the matched pairs contain the value of the case and the mean value of the corresponding referents of the matched set. Hormone excretion per sample was adjusted by division by creatinine. The hormone excretion level of a subject was computed as the mean of these creatinine adjusted values of all available samples per subject. Adjustment for potential confounders was performed by conditional logistic regression. For this analysis, hormone/creatinine ratios were divided in tertiles. Odds ratios were computed of second and third tertiles with the first tertile as reference. The multivariate models included terms in the following form: smoking (nonsmoker, 1–10 cigarettes a day, more than 10 cigarettes a day); hypertension (drug treatment yes/no), diabetes mellitus (diet- or drug treatment yes/no); Quetelet Index (continuous); number of days till next menstrual bleeding (continuous); cycle regularity (yes/no); menarcheal age (continuous); cycle length (continuous); and parity (number of live births).

The follow-up of menopausal age was analyzed with survival analysis. As failure event we defined surgical menopause or the date of last menstrual bleeding followed by a period of amenorrhea of at least 12 months. Subjects not fulfilling this criterion were censored at the date of the last known menstrual bleeding. Furthermore, non-responding subjects were censored at the date of screening. Differences in curves were tested with the non-parametric Log-rank test. SPSS [16], EGRET [17], and NCSS [18] statistical packages were used. Urine samples were considered luteal when pregnanediol glucuronide excretion exceeded 0.5 µmol/mmol creatinine [19]. For nine out of 468 urine samples hormone levels were not determined for reasons of nonretrieval of the sample or leaking containers.

RESULTS
The two groups differed significantly in smoking behavior, and prevalence of hypertension and diabetes (Table 1). None of the reproductive factors like menarcheal age, parity, age at first delivery, menarche till first-childbirth inter-
### TABLE 1. Nested case-referent study of urinary sex hormone excretions in premenopausal women and coronary heart disease risk

<table>
<thead>
<tr>
<th></th>
<th>Total Cases</th>
<th>Total Referents</th>
<th>Non-luteal samples excluded Cases</th>
<th>Non-luteal samples excluded Referents</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>135</td>
<td>32 (71%)*</td>
<td>116 (86%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.6 (3.2)</td>
<td>45.7 (3.2)</td>
<td>45.4 (3)</td>
<td>45.8 (3.1)</td>
</tr>
<tr>
<td>Year of investigation</td>
<td>1984.2 (1.1)</td>
<td>1984.2 (1.1)</td>
<td>1984 (1.0)</td>
<td>1984 (1.0)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.2 (3.3)</td>
<td>24.3 (3.7)</td>
<td>25.4 (3.6)</td>
<td>24.3 (3.9)</td>
</tr>
<tr>
<td>Smoking (% yes)*</td>
<td>60</td>
<td>38</td>
<td>59</td>
<td>38</td>
</tr>
<tr>
<td>Hypertension (% yes)*</td>
<td>24</td>
<td>11</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Diabetes (% yes)*</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Menarcheal age (years)</td>
<td>13.4 (1.6)</td>
<td>13.5 (1.5)</td>
<td>13.2 (1.2)</td>
<td>13.5 (1.4)</td>
</tr>
<tr>
<td>Childless (% yes)</td>
<td>4</td>
<td>15</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Parity</td>
<td>2.4 (1.2)</td>
<td>2.5 (1.7)</td>
<td>2.3 (1.1)</td>
<td>2.5 (1.4)</td>
</tr>
<tr>
<td>Age at first delivery³</td>
<td>25.0 (3.7)</td>
<td>25.1 (3.3)</td>
<td>25.4 (3.0)</td>
<td>25.2 (3.2)</td>
</tr>
<tr>
<td>Time from menarche to first delivery³</td>
<td>11.7 (3.4)</td>
<td>11.6 (3.3)</td>
<td>12.2 (3.3)</td>
<td>11.7 (3.3)</td>
</tr>
<tr>
<td>Subjects with regular cycles (%)</td>
<td>65</td>
<td>75</td>
<td>71</td>
<td>76</td>
</tr>
<tr>
<td>Average cycle length (days)⁴</td>
<td>26.0 (2.3)</td>
<td>26.8 (2.3)</td>
<td>26.4 (1.9)</td>
<td>26.6 (2.3)</td>
</tr>
<tr>
<td>Estrone-gluc./creatinine</td>
<td>0.021 (0.002)*</td>
<td>0.019 (0.001)*</td>
<td>0.024 (0.002)*</td>
<td>0.019 (0.001)*</td>
</tr>
<tr>
<td>50th percentile</td>
<td>0.018</td>
<td>0.017</td>
<td>0.019</td>
<td>0.017</td>
</tr>
<tr>
<td>Pregnanediol-gluc./creatinine</td>
<td>0.877 (0.090)*</td>
<td>1.018 (0.052)*</td>
<td>1.270 (0.084)*</td>
<td>1.238 (0.050)*</td>
</tr>
<tr>
<td>50th percentile</td>
<td>0.83</td>
<td>0.89</td>
<td>1.24</td>
<td>1.12</td>
</tr>
<tr>
<td>Testosterone-gluc./creatinine</td>
<td>3.247 (0.331)*</td>
<td>3.014 (0.141)*</td>
<td>3.436 (0.460)*</td>
<td>3.004 (0.155)*</td>
</tr>
<tr>
<td>50th percentile</td>
<td>2.52</td>
<td>2.62</td>
<td>2.46</td>
<td>2.59</td>
</tr>
</tbody>
</table>

Baseline characteristics from cases and referents. The first two columns present data from all subjects (total group), the third and fourth columns present data from subjects with at least one luteal sample (pregnanediol-glucuronide/creatinine ratio ≥ 0.5 µmol/mmol). Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986. Categorical data are expressed in percentages. Continuous variables are expressed in mean (SD).

* Difference cases - referents p = 0.02.
* Difference cases - referents p < 0.05.
³ Of parous women only.
⁴ Of women with regular cycle lengths only.
*SE.

However, with follow-up of menopausal age, no difference in time interval between baseline and menopause was observed between cases and referents, despite this higher prevalence of anovulatory cycles and smoking among cases. As smoking and prevalence of anovulatory cycles in this study were not related the occurrence and frequency of anovulatory cycles may independently indicate CHD risk rather than levels of hormone excretions measured in the luteal phase of ovulatory cycles. This was supported by separate analysis of the samples with a pregnanediol-glucuronide/creatinine ratio ≥ 0.5 µmol/mmol, a criterion for recent ovulation [19]. Even in this selected group no relationship

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of hormone excretions and CHD was present. Whether these findings suggest that the hormonal interactions associated with ovulation protect against coronary heart disease is of interest.

The findings from the present study are in accordance with results of hormone studies in men, indicating no relation of endogenous estrogen plasma levels and CHD risk in men. In prospective studies in men plasma estradiol levels in CHD cases were similar to controls [6,7]. Estrogen levels tend to increase after a myocardial infarction [1].

It is unlikely that inaccurate urine sampling and storage times would have obscured potential differences. The women had been instructed to collect the urine samples on day 22 of a cycle. Differences in cycle length resulted in variation of the urine collection time to the ovulation. Adjustment for differences in cycle length by “time until next

TABLE 2. Nested case-referent study of urinary sex hormone excretions in premenopausal women and coronary heart disease risk

<table>
<thead>
<tr>
<th></th>
<th>Second tertile</th>
<th>Third tertile</th>
<th>Second tertile</th>
<th>Third tertile</th>
<th>Second tertile</th>
<th>Third tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>0.8 (0.2-2.5)</td>
<td>1.6 (0.6-4.2)</td>
<td>1.0 (0.4-2.8)</td>
<td>1.3 (0.5-3.4)</td>
<td>0.6 (0.2-1.7)</td>
<td>0.9 (0.3-2.4)</td>
</tr>
<tr>
<td>Adjusted</td>
<td>0.7 (0.2-2.4)</td>
<td>1.8 (0.7-4.7)</td>
<td>0.9 (0.3-2.6)</td>
<td>1.3 (0.4-3.7)</td>
<td>0.6 (0.2-1.7)</td>
<td>0.6 (0.2-1.7)</td>
</tr>
</tbody>
</table>

Odds ratios (and 95% confidence intervals) of coronary disease of second and third tertiles of classification for hormone/creatinine ratio, with the first (lowest) tertile as reference. All non-luteal samples were excluded. Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986.
Cholesterol values traced from the hospital records in 87% of cases indicated that 75% had a plasma cholesterol level above normal. This information was congruent with findings in men, where gradual differences in endogenous estrogen levels do not predict coronary heart disease risk. The relatively high frequency of anovulatory cycles in women who will develop CHD is of interest as it suggests a relationship of CHD with ovulation.

**CONCLUSION**

Premenopausal women at higher risk of coronary heart disease could not be identified by means of measurement of urinary sex hormone excretions. The results of this study are congruent with findings in men, where gradual differences in endogenous estrogen levels do not predict coronary heart disease risk. The relatively high frequency of anovulatory cycles in women who will develop CHD is of interest as it suggests a relationship of CHD with ovulation.

We are indebted to the participating cardiologists and hospital staff who made it possible for us to carry out this study: Dr. C.A.P.L. Ascoop (Hospital St. Anthonius, Nieuwegein), Dr. R. Bergsma (Hospital Overvecht, Utrecht), Dr. B.K. Bootsmma (Central Military Hospital, Utrecht), Dr. T.A.R. van der B. J. van Zoelen (Diakonessenhuis, Utrecht), Dr. Roebles de Medina (Academic Hospital, Utrecht), Dr. R. Schotman, Dr. F. A. van Erven, (Medical Centre Molendael, Baarn-Soest), Dr. J. Wisse-Smit (Eemland Hospital, Amersfoort), Dr. P. M. Kuizer (Hospital Berg en Bosch, Utrecht), Dr. E. P. Brinkman (Hofsuur Hospital, Woerden), and Dr. J. Visser (Lorentz Hospital, Zeist). Special thanks to Jacques Fracheboud, Bernhard Slotboom, and Leon Fontijn for assistance in data collection, Alie Algra for classification of cases and methodological advices, Eln van der Put for critical reading of the manuscript, Joop Faber for advices concerning non-parametric tests, Wouter Kortland for determination of creatinine, Gerard Graat and Winnie Veenman for determination of the hormone concentrations, and Isolde den Tonkelaar for the follow-up data of menopausal status.

**References**


