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

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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.

The occurrence of CHD in middle-aged women is not preceded by a low premenopausal endogenous estrogen production or high androgen production. Anovulatory cycles appear more frequently in women who develop CHD many years later. J CLIN EPIDEMIOL 50;3:275–281, 1997. © 1997 Elsevier Science Inc.

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

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studies [5]. 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 in the cycle 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 with no specific notice of the cycle day. All urine samples were stored at -20°C in 250-ml plastic containers.

Case-Finding

Medical registries of all 10 hospitals within the recruitment area of the screening were searched for hospital admissions, with diagnosis codes 410–414 (ICD) [12], of women who had originally participated in the screening. Follow-up period was until July 1, 1991, one hospital until January 1, 1990 and two hospitals until January 1, 1991. Medical records were reviewed to verify the diagnosis. CHD cases were defined as subjects suffering from either acute myocardial infarction ($n = 29$), according to WHO criteria [13], or angiographically proven coronary artery disease causing precordial pain (angina pectoris). A stenosis of $>50\%$ in at least one of the main coronary arteries had to be present ($n = 16$). Inclusion was decided by a medical doctor and

confirmed by two independent colleagues, a specialist in internal medicine and a cardiovascular epidemiologist. Only subjects, who had their first hospital admittance on basis of CHD after the moment of screening, were eligible.

Sampling

Referent subjects were randomly sampled out of the cohort (nested case-referent design [14]) and individually matched for age (± 6 months) and date of urine storage (± 6 months) to cases in a 3:1 ratio. Referent subjects of cases born in 1942–1945 were additionally matched for the cycle day of urine collection.

Data Collection

Data pertinent to the study aim were selected from the baseline questionnaire, including prevalence of diabetes and hypertension and smoking habit. Subjects were considered to have irregular cycles if the average cycle length of three cycles exceeded 35 days or was less than 21 days or if the length of separate cycles differed more than 7 days. Eighty-three percent of the subjects (only women born in 1932–1941) were addressed by means of a yearly questionnaire to establish the age at menopause. Natural menopause was defined as the absence of menstrual bleeding, not surgically induced, for at least 12 months.

The urine samples of cases and referents were thawed overnight at room temperature, homogenized by gentle manual shaking and poured out in polystyrene tubes that were refrozen at -20°C . Creatinine concentrations were measured by an automated Jaffé reaction using Boehringer (Mannheim, Germany) reagents, preceded by centrifugation (3,000 rpm, 10 min).

Assessment of Steroid Glucuronides

Concentrations of pregnanediol glucuronide and estrone glucuronide were assessed by direct specific radioimmunoassays of urine samples diluted 1:1,000. 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 $\mu\text{mol/l}$, respectively ($n = 20$). The inter-assay CV was 40% and 10.4%, respectively, at 0.46 and 22.4 $\mu\text{mol/l}$. As more than 98% of the results obtained was between 3 and 22.7 $\mu\text{mol/l}$, the high CV at the very low level of 0.5 $\mu\text{mol/l}$ 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 $\mu\text{mol/l}$, respectively ($n = 20$). The inter-assay CV was 13.8% and 6.6%, respectively, at 0.040 and 0.938 $\mu\text{mol/l}$.

The concentration of testosterone glucuronide was assessed as 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 μ l urine for 20 hrs at 37°C. This enzyme preparation is sulphatase free. After hydrolysis, the solution was neutralized with 50 μ l 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 μ mol/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 Logrank test. SPSS [16], EGRET [17], and NCSS [18] statistical packages were used. Urine samples were considered luteal when pregnanediol glucuronide excretion exceeded 0.5 μ mol/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-

val, or cycle regularity and cycle length at the time of baseline, were different between the two groups.

For both cases and referents, characteristics of the distribution of the subjects' mean estrone-glucuronide/creatinine ratio (EG/C), pregnanediol-glucuronide/creatinine ratio (PG/C), and testosterone-glucuronide/creatinine ratio (TG/C) are shown (Table 1). Figures 1, 2, and 3 show the individual values of cases and referents of EG/C, PG/C, and TG/C, respectively. No statistical differences at group level were observed (EG/C: $p = 0.83$; PG/C: $p = 0.09$; TG/C: $p = 0.92$; Wilcoxon test).

The third and fourth column in Table 1 present the same variables, after exclusion of all nonluteal urine samples (PG/C < 0.5 μ mol/mmol). In total, 123 of 457 samples (27%) were excluded (36% of cases and 24% of referents). For all subjects new mean excretions were counted using only the luteal samples. Subjects not having at least one urine sample with PG/C > 0.5 μ mol/mmol, were completely (for all variables) excluded. By this criterion, significantly more cases than referents were excluded because of anovulatory cycles (29% versus 14%; $p = 0.02$). Again no differences between cases and referents were observed in the hormonal parameters (EG/C: $p = 0.15$; PG/C: $p = 0.64$; TG/C: $p = 0.78$; Wilcoxon test). In conditional logistic regression analysis the three hormone/creatinine ratios were entered in tertiles, leaving the first (lowest) tertile as reference. The crude and smoking adjusted odds ratios for the tertiles are shown in Table 2. None of the odds ratios was significantly different from 1. Additional adjustment for Quetelet Index, hypertension, cycle regularity, parity, and number of days till next menstrual blood loss did not result in substantially different odds ratios.

Follow-up of Age at Menopause

Kaplan-Meier survival curves for both cases and referents, with menopause as defined endpoint, were equal ($p = 0.4$, logrank test) (Fig. 4). The median menopausal age was 52.3 (interquartile range 50.3–53.7) for referents and 53.3 (interquartile range 51.3–54.8) for cases. Earlier menopause was observed among smokers (yes/no, $p = 0.02$), subjects with irregular cycles ($p = 0.01$). (Subjects with nonovulatory cycles: $p = 0.07$).

DISCUSSION

Urinary excretions of sex hormones and subsequent risk of CHD were not related in this prospective study among premenopausal women. Consequently the hypothesis that low endogenous estrogen production, or high production of androgens, may render premenopausal women at increased risk for CHD could not be confirmed. The use of urinary excretions of steroid sex hormones is valid, as these are representative of plasma levels of these hormones [20].

The higher prevalence of anovulatory cycles in cases could suggest an association of CHD risk with early meno-

TABLE 1. Nested case-referent study of urinary sex hormone excretions in premenopausal women and coronary heart disease risk

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

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 $\geq 0.5 \mu\text{mol}/\text{mmol}$). Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986. Categorical data are expressed in percentages. Continuous variables are expressed in mean (SD).

^aDifference cases - referents $p = 0.02$.

^bDifference cases - referents $p < 0.05$.

^cOf parous women only.

^dOf women with regular cycle lengths only.

^eSE.

pause [19]. 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 pregnanediolglucuronide/creatinine ratio $\geq 0.5 \mu\text{mol}/\text{mmol}$, a criterion for recent ovulation [19]. Even in this selected group no relationship

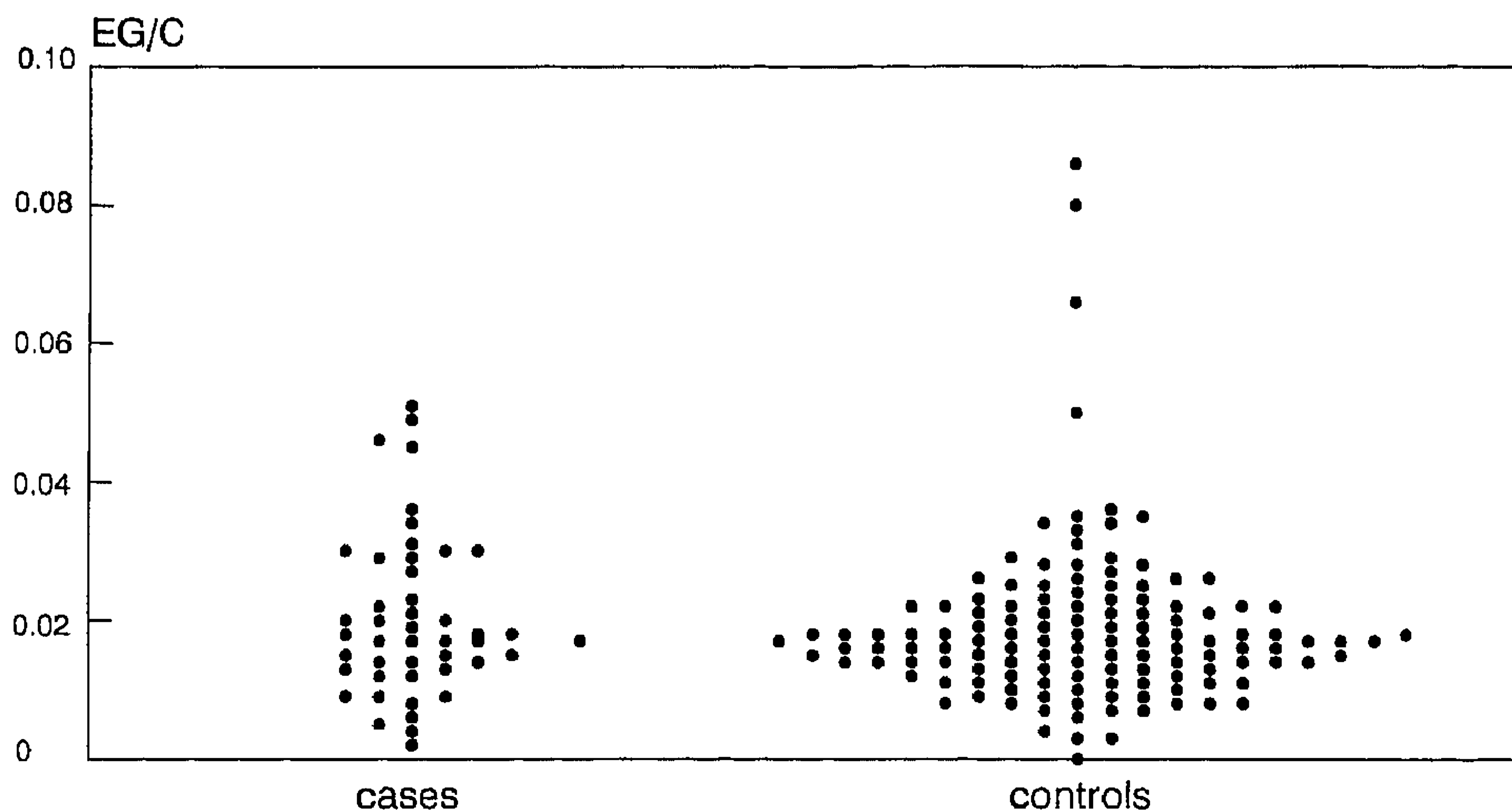


FIGURE 1. Estrone-glucuronide/creatinine ratio (individual values for cases and controls). Nested case-referent study of urinary sex hormone excretions in premenopausal women and CHD risk. Scatterplot of estrone-glucuronide/creatinine ratio distribution. Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986. Abbreviation: EG/C-estrone-glucuronide/creatinine ($\mu\text{mol}/\text{mmol}$).

FIGURE 2. Pregnanediol-glucuronide/creatinine ratio (individual values for cases and controls). Nested case-referent study of urinary sex hormone excretions in premenopausal women and CHD risk. Scatterplot of pregnanediol-glucuronide/creatinine ratio distribution. Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986. Abbreviation: PG/C-pregnanediol-glucuronide/creatinine ($\mu\text{mol}/\text{mmol}$).

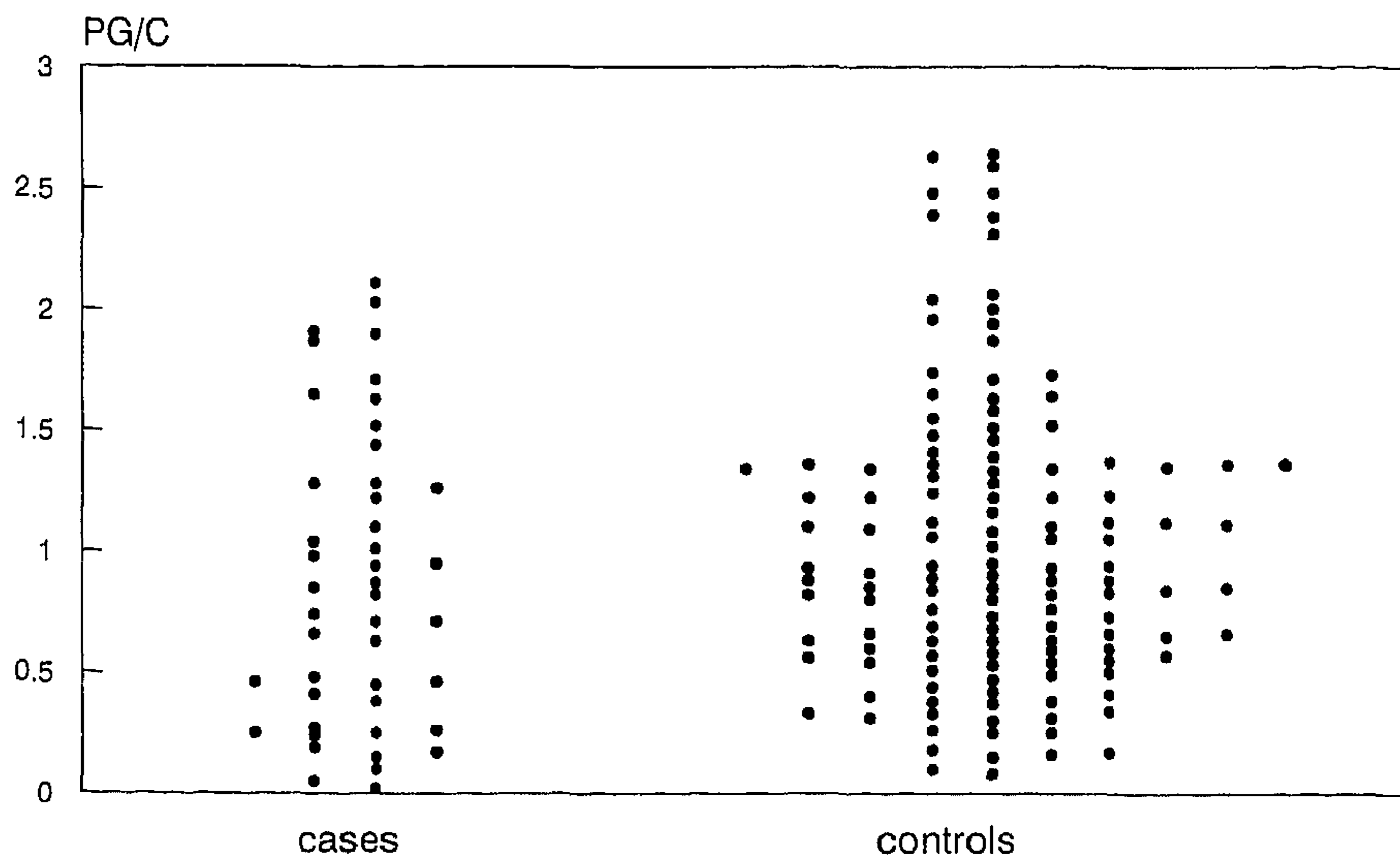


FIGURE 3. Testosterone-glucuronide/creatinine ratio (individual values for cases and controls). Nested case-referent study of urinary sex hormone excretions in premenopausal women and CHD risk. Scatterplot of testosterone-glucuronide/creatinine ratio distribution. Urine samples from DOM Cohort, Utrecht, Netherlands, collected in 1982–1986. Abbreviation: TG/C-testosterone-glucuronide/creatinine ($\mu\text{mol}/\text{mmol}$).

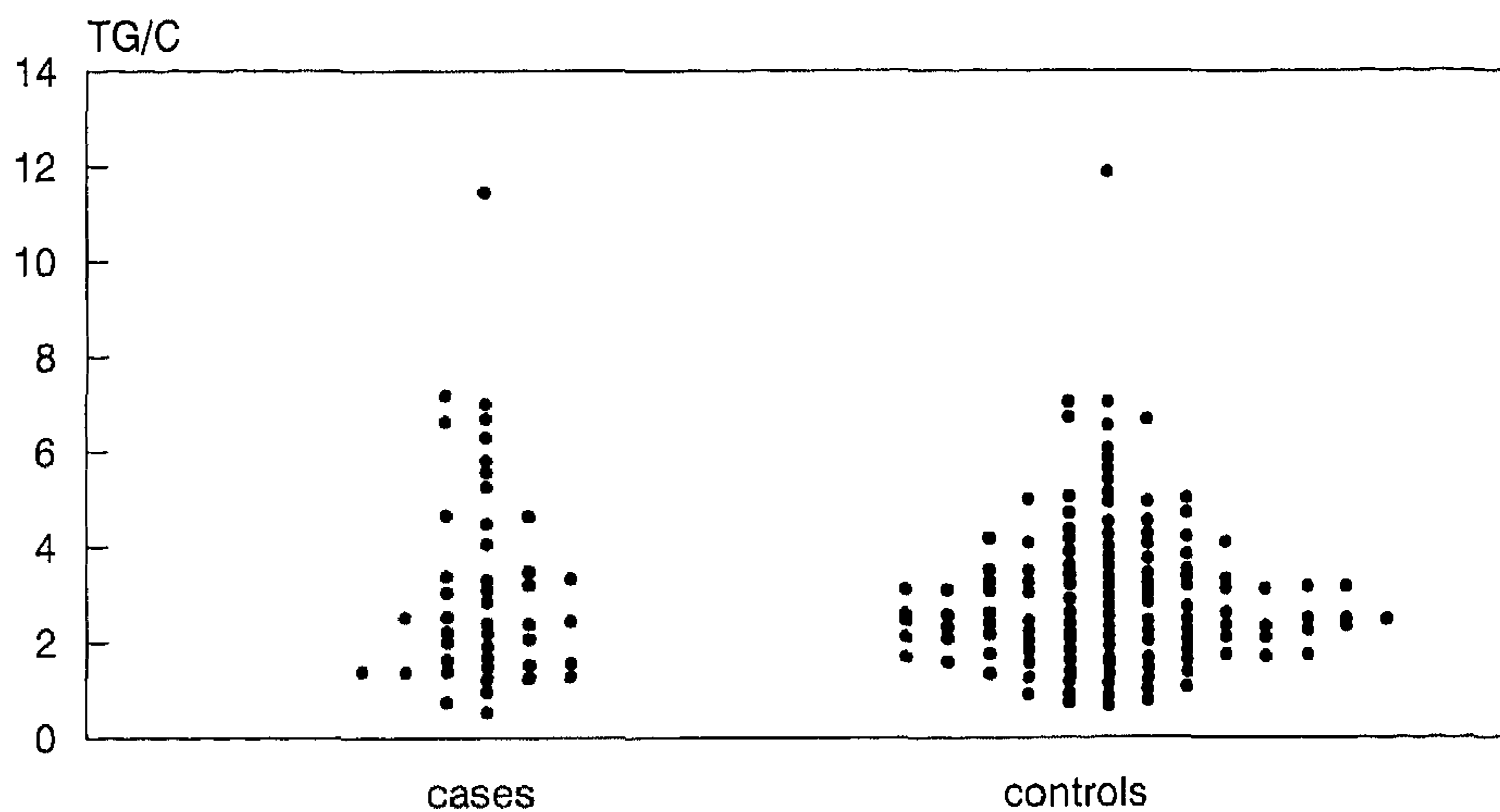


TABLE 2. Nested case-referent study of urinary sex hormone excretions in premenopausal women and coronary heart disease risk

	EG/C		PG/C		TG/C	
	Second tertile	Third tertile	Second tertile	Third tertile	Second tertile	Third tertile
Crude	0.8 (0.2–2.5)	1.6 (0.6–4.2)	1.0 (0.4–2.8)	1.3 (0.5–3.4)	0.6 (0.2–1.7)	0.9 (0.3–2.4)
Adjusted for smoking	0.7 (0.2–2.4)	1.8 (0.7–4.7)	0.9 (0.3–2.6)	1.3 (0.4–3.7)	0.6 (0.2–1.7)	0.6 (0.2–1.7)

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.

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

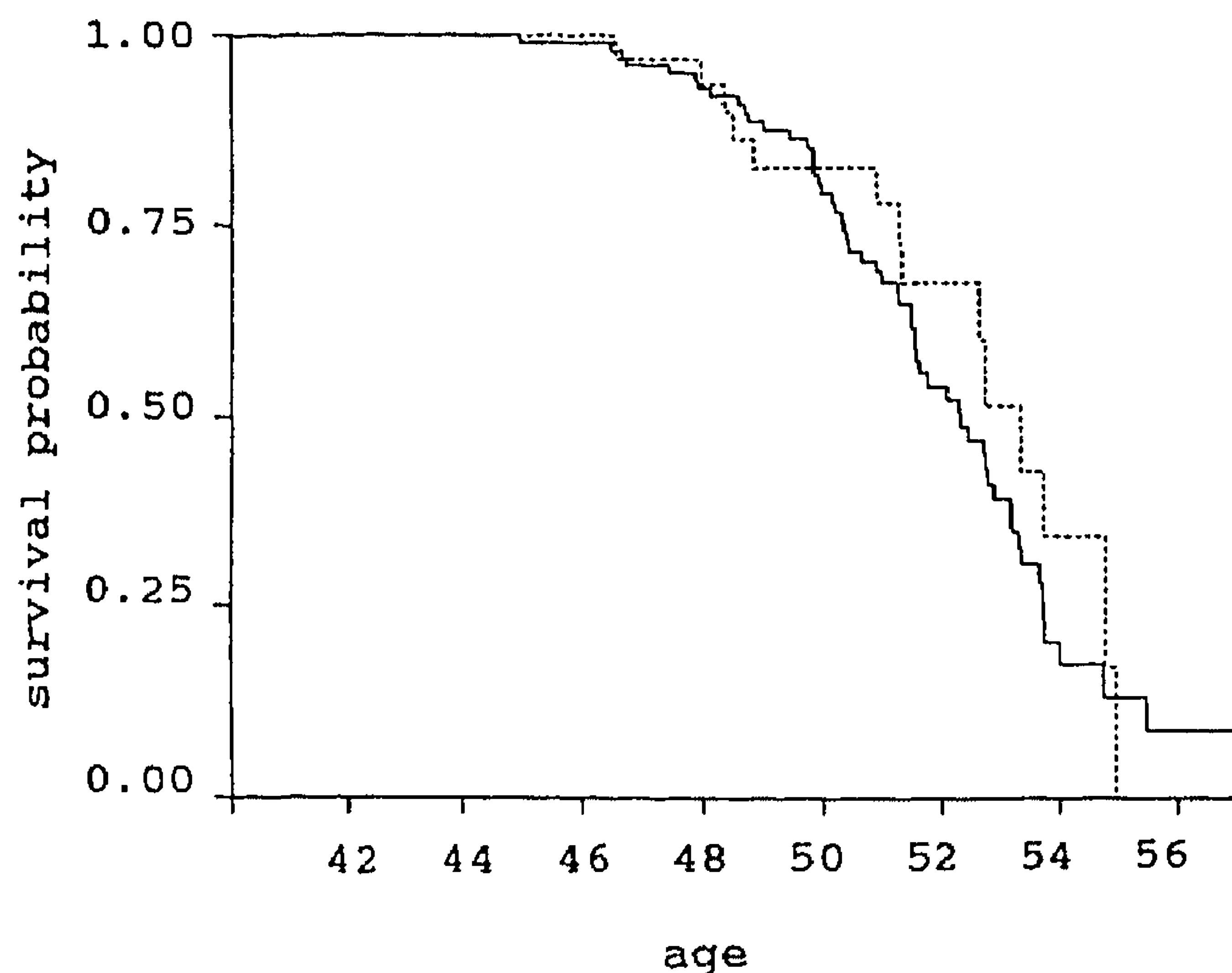


FIGURE 4. Nested case-referent study of urinary sex hormone excretions in premenopausal women and CHD risk. Age at menopause for cases (dotted line) and referents (solid line). Kaplan-Meier curves with menopause as endpoint. DOM Cohort, Utrecht, Netherlands. Difference between cases and referents: $p = 0.4$, Log-rank test.

menstrual bleeding," accounting for the more time constant luteal phase instead of the follicular phase, did not have a substantial effect on the odds ratios. The long duration of frozen storage is not a likely explanation for loss of differences, as steroid hormones are known to be stable under these conditions. Moreover, storage conditions were identical in cases and referents.

Of the reproductive factors, high parity, first childbirth at age <20 years have been related to coronary heart disease [5,8,9], but the reports are contradictory. In our study these factors did not predict coronary heart disease. Odds ratios for high parity and for young age at first childbirth were <1, but the 95% confidence intervals were wide as a result of the low power of this study to find associations for these factors. The median age for menopause was high for both cases and referents (52.3 and 53.3, respectively) [21], probably affected by the study design, excluding women being postmenopausal at entry. Therefore, the study does not allow conclusions on the relationship between age of menopause and subsequent cardiovascular disease risk.

Exogenous estrogen use as hormone replacement therapy (HRT) is associated with lower coronary disease incidence [5]. However, whether this association is causal is not yet clear. Furthermore, exogenous estrogens may influence coronary disease risk in a different way compared to endogenous estrogens.

Plasma samples were not prospectively collected in this cohort precluding case-control comparisons. Information on cholesterol values traced from the hospital records in 87% of cases indicated that 75% had a plasma cholesterol value >6.0 mmol/l, indicating a strong association of hyper-

cholesterolemia, similar to other "classical" risk factors as smoking, hypertension, and diabetes, with development of CHD in this cohort of women at premenopausal age.

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.

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