Urinary excretion of sex steroid hormone metabolites after consumption of cow milk: a randomized crossover intervention trial

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ABSTRACT

Background: Current cow milk production practices introduce considerable levels of pregnancy hormones into the milk. Humans are exposed to these hormones when cow milk is consumed, and this may explain the observed association between cow milk consumption and several hormone-sensitive cancers.

Objectives: The aim of the study was to evaluate whether cow milk consumption is associated with an increase in urinary excretion of sex steroid hormones and their metabolites in humans.

Methods: We conducted a randomized crossover intervention feeding experiment. A total of 109 postmenopausal women consumed 1 L of semiskimmed milk (1.5% fat) per day for 4 d and 1 L of whole milk (3.5% fat) per day for 4 d, intersected by 4-d wash-out periods. Sex steroid hormone levels were measured in 24-h urine samples collected at the end of each intervention and wash-out period.

Results: Estrogens, androgens, and progesterone were detected in the examined milk samples used for our intervention. Although a very high proportion of the estrogens were conjugated, only small proportions of the androgens and progesterone were conjugated. Milk consumption resulted in a significant increase in urinary estrone (E1) excretion, whereas estradiol (E2), estriol (E3), and 16ketoE2 excretion only increased after semiskimmed milk consumption. Urinary pregnanediol glucuronide excretion was not significantly affected.

Conclusion: Cow milk consumption increases urinary excretion of E1 in humans. Ingestion of semiskimmed milk appears also to raise E2, E3, and 16ketoE2 excretion, but future studies need to confirm these associations. This trial was registered at https://www.drks.de as DRKS00003377. *Am J Clin Nutr* 2019;108:1–9.

Keywords: milk, estrogen, estradiol, estrone, progesterone, breast cancer, urine

Introduction

The ever-increasing efficiency of cow milk production has introduced substantial changes in farming practices. The normal cycle of reproduction that intends pregnancy to be followed by a phase of lactation that suppresses ovulation and thus prevents conception is highly inefficient with respect to commercial milk productivity. Therefore, cows are artificially inseminated and kept pregnant most of the time with only short intervals between pregnancies. This practice allows harvesting of milk from cows on an almost continuous basis. Although this increases commercial output for farmers, it changes the composition of cow milk provided to consumers. Because cows are now mostly pregnant while they lactate, sex-steroid-related pregnancy hormones, particularly estrogens, androgens, and progesterone, accumulate in the milk and expose humans to these hormones once cow milk is consumed (1).

Uptake of bioavailable sex steroid hormones is of immense importance for human health and may affect the incidence of hormone-sensitive cancers. Regular consumption of cow milk has been associated with some of the most common hormone-sensitive cancers among both women and men. The epidemiologic evidence relating regular milk consumption to prostate cancer is supported by meta-analyses of case-control studies (2) and cohort studies (3, 4). For women who frequently consume cow milk, increased incidences of breast and endometrial cancer have been reported (5, 6). A link between milk consumption and breast cancer is also suggested by time trends observed in Japan (7, 8).

Assessment of sex steroid levels in cow milk is challenging because most steroids are bound to proteins including those of fat

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Abbreviations used: AED, androstenedione; An, androsterone; Crea, creatinine; EI, electroionization; E1, estrone; E2, estradiol; E3, estriol; Et, etiocholanolone; FFQ, food frequency questionnaire; 16ketoE2, 16-ketoestradiol; 2MeOE1, 2-methoxyestrone; 17α-MeT, 17α-methyltestosterone; 2OHE1, 2-hydroxyestrone; 4OHE1, 4-hydroxyestrone; 16aOHE1, 16a-hydroxyestrone; 2OHE2, 2-hydroxyestradiol; P, progesterone; PdG, pregnanediol glucuronide; HRP, horseradish peroxidase; T, testosterone.

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globules and have to be extracted from those to be measurable. In addition, steroid hormones are predominantly conjugated to glucuronic or sulfuric acid. Conjugated steroid hormones have to be enzymatically deconjugated for easy quantification. Few analytic methods with sufficient sensitivity are available to quantify the various steroid hormones and their metabolites in milk.

Steroid hormones pass the blood–milk barrier, and progesterone assessments in milk have been used for detecting pregnancy in cattle (9). Few studies have examined the oral bioavailability of sex steroid hormones consumed with cow milk or other dairy products. Whether these hormones are indeed bioactive and have pharmacologic effects is unclear. Little is known on the impact of cow milk or dairy product consumption on estrogen- and/or progesterone levels in humans.

We therefore conducted an intervention study to evaluate the impact of cow milk ingestion on the estrogen and progesterone levels of human bodily fluids. We used a crossover design to differentiate the effect of semiskimmed (1.5% fat) and whole (3.5% fat) milk. Our study population consisted of postmenopausal women to avoid endogenous sex hormone fluctuation during the menstrual cycle.

Materials and Methods

Study design

We conducted a randomized crossover intervention study in Freiburg, Germany. Bovine growth hormone is not used in the cattle industry in Europe. The interventions randomized were semiskimmed (1.5% fat) and whole (3.5% fat) milk consumption. The study was composed of 5 study periods: wash-out period 1 (4 d), consumption of 1 L of milk per day (4 d), wash-out period 2 (4 d), consumption of 1 liter of milk per day (4 d), and wash-out period 3 (4 d) (**Figure 1**). Study participants were randomly assigned to consume semiskimmed milk during the first intervention period and whole milk during the second intervention period (group A) or vice versa (group B). On the last day of each of the 5 study units, participants collected all void urine over 24 h. Because sex steroid hormone levels were assessed at 5 time points during the 20-d study period, we decided on measurements in urine samples rather than blood samples to optimize participation and compliance.

Study participants

Our study population consisted of postmenopausal women 55–70 y of age who were generally healthy. Women were included if their last menstrual period was at least 12 mo in the past. In women with hysterectomy who were less than 60 y of age with no or single oophorectomy, we assessed their 17β-estradiol (17β-E2), 17-OH progesterone, and progesterone levels to ensure that they were postmenopausal. Women were excluded if they smoked, had used hormone replacement therapy within the past 6 mo, were lactose-intolerant, consumed phytoestrogens, or had cancer, type 2 diabetes, diseases of the digestive tract (such as Morbus Crohn or celiac disease), or liver or gall bladder disorders.

The number of individuals to be recruited was determined by a sample size calculation indicating that 55 individuals per group would be sufficient for detecting an effect with a Cohen's $d = 0.28$ with a power of 80% at a significance level of 5%, assuming 10% drop-out. Participants were recruited via an advertisement in the local newspaper. Participants received €150 if they completed all aspects of the study.

Participants completed a questionnaire inquiring about their age, number of children, history of breastfeeding, medical history, medication, habitual diet, food allergies or intolerances, and level of physical activity (self-assessed low, moderate, or high). Participants' weight, height, waist, and hip measurements were assessed during their visit to the study center. The characteristics of the study participants are provided in **Table 1**.

For logistical reasons, the study was conducted in 2 phases: the first phase included 48 women, of whom 25 were randomly assigned to group A and 23 to group B; the second phase included 62 women, with 31 randomly assigned to group A and 31 to group B. One study participant in the second phase randomly assigned to group A missed one urine sample in one of the five 24-h urine collections and had to be excluded from the study; all remaining 109 study participants completed all five 24-h urine collections and constitute our study population (**Figure 2**).

Intervention

We selected one brand of milk widely consumed in this area of Germany (Schwarzwaldmilch = "black forest milk"). This brand and most other brands in Germany are now only available as an extended shelf-life version, which prolongs the shelf-life (compared with that of pasteurized milk: 5–7 d) but is still considerably less than that of ultrahigh-temperature milk (3–6 mo). The extended shelf-life in Schwarzwaldmilch is accomplished by preceding a 15-s heating at 72°C by a microfiltration step that mechanically separates out a large proportion of microorganisms. The Schwarzwaldmilch we selected is available with 1.5% and 3.5% fat content (semiskimmed and whole milk, respectively). It is sold in Tetra Paks. Milk is purchased by the University Medical Center from the manufacturer on a daily basis. We obtained the relevant number of milk Tetra Paks for this study from the Medical Center central dining facilities on the first day of every intervention period. The manufacturer had no knowledge that the delivered milk charges were used for a scientific study.

Of note, whereas organic milk is obtained from cows exposed to considerably lower levels of pesticides in their feed, organic farmers use the same reproductive practices as those used by conventional farmers, using artificial insemination and maximizing milk production efficiency through continuous lactation. Thus, organic milk likely contains the same concentrations of pregnancy hormones as conventional milk (10; Schätzle, personal communication, Schwarzwaldmilch, 2016).

The entire study lasted 20 d and consisted of five 4-d intervals. Before the start of the study, participants were invited to the study center and provided with detailed information about the study protocol and information on dietary items to avoid during the study. Participants also signed an informed consent form and received their study ID. Participants had to abstain from all other dairy product consumption (including dairy from cow, sheep, and goat) during the entire 20-d study period. They were also advised to abstain from foods containing phytoestrogens

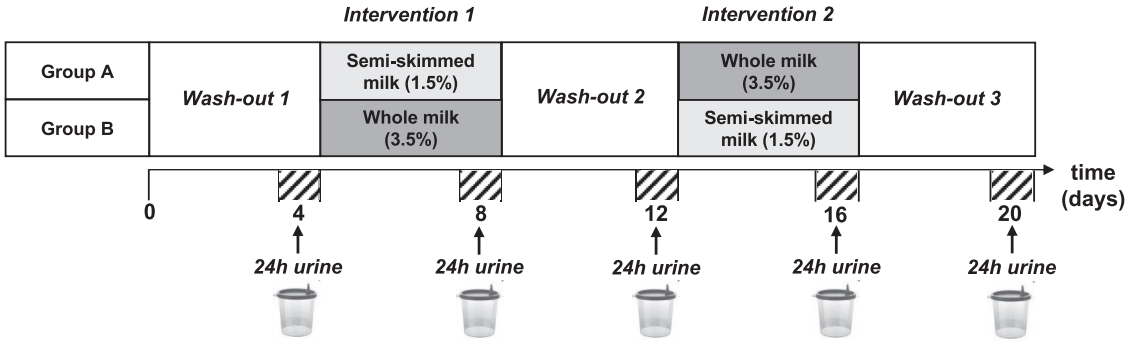

FIGURE 1 Study design of the randomized crossover intervention trial on the effects of milk consumption on steroid hormone excretion.

including legumes, asparagus, flax seeds, pumpkin seeds, and grain bran.

During the initial 4-d wash-out period, study participants began to abstain from the aforementioned food items. Following the wash-out period, study participants visited the study center and were given four 1-L milk packs in a cooler bag to take home. Depending on whether participants were randomly assigned to group A or B, they received semiskimmed or whole milk. One liter of milk was consumed during each of the 4 d of the intervention period following the initial wash-out period. Participants were asked not to heat the milk, but they were allowed to add it to foods or other cold beverages. The first intervention period was followed by a second 4-d wash-out period. For the second intervention period, participants again received four 1-L milk packs, and depending on whether they were randomly assigned to group A or B, they received whole or semiskimmed milk. The study finished with a final 4-d wash-out period.

Participants completed a food frequency questionnaire (FFQ) at baseline and again at the end of the 20-d study period. The FFQ captured the customary diet of the participants and was particularly detailed on meat consumption, asked about alcohol consumption, and allowed participants to note dairy consumption in addition to the intervention.

TABLE 1
Baseline characteristics of the study population ($n = 109$) participating in a randomized crossover intervention trial on the effects of milk consumption on steroid hormone excretion

Characteristics	Mean (SD)
Age, y	61.17 (4.97)
Height, m	1.63 (0.06)
Weight, kg	70.40 (15.00)
BMI, kg/m^2	26.38 (5.53)
Waist–hip ratio	0.85 (0.08)
	n (%)
Menopause	
Natural	105 (96.3)
Surgical	4 (3.7)
Children	
No	23 (21.1)
1	22 (20.2)
2	42 (38.5)
3	22 (20.2)
Breastfeeding	
No	36 (33.0)
Yes	73 (67.0)
Thyroid disease	
No	63 (57.8)
Yes, thyroxin	33 (30.3)
Yes, no medication	13 (11.9)
Hypertension	
No	81 (74.3)
Yes, medication	26 (23.9)
Yes, no medication	2 (1.8)
Physical activity	
Low	40 (36.7)
Moderate	60 (55.0)
High	9 (8.3)
Diet	
Omnivore	95 (87.2)
Vegetarian	14 (12.8)

24-hour urine collection

24-h urines were collected during the last day of each of the 5 study units: the 2 intervention and 3 wash-out periods. Participants were supplied with five 24-h urine containers made from polyethylene (Sarstedt). Urine was collected over 24 h, stored at 4°C, and transported to the laboratory on ice packs within 5 h after the last urine collection. Urine samples were mixed on a vortex, divided into aliquots, and stored at −80°C.

Steroid hormone assessment in study milk

Levels of sex steroid hormones in the milk used in our intervention were assessed in the laboratory of Dr Frédérique Courant at Laboratoire d'Etude des Résidus et Contaminants dans les Aliments in Nantes, France. We sent 8 samples from the intervention milk (4 samples with semiskimmed milk and 4 samples with whole milk) to Dr Courant's laboratory. These milk samples were analyzed for quantification of free and total forms (free plus deconjugated forms) of 17β-E2, 17α-estradiol (17α-E2), estrone (E1), 17β-testosterone (17β-T), 17α-testosterone (17α-T), etiocholanolone (Et), androsterone (An), and progesterone (P). For androstenedione (AED), only the free form was measured, because this metabolite cannot be conjugated. Two aliquots (3 mL) of each sample were analyzed in order to determine separately free and total steroids. Each

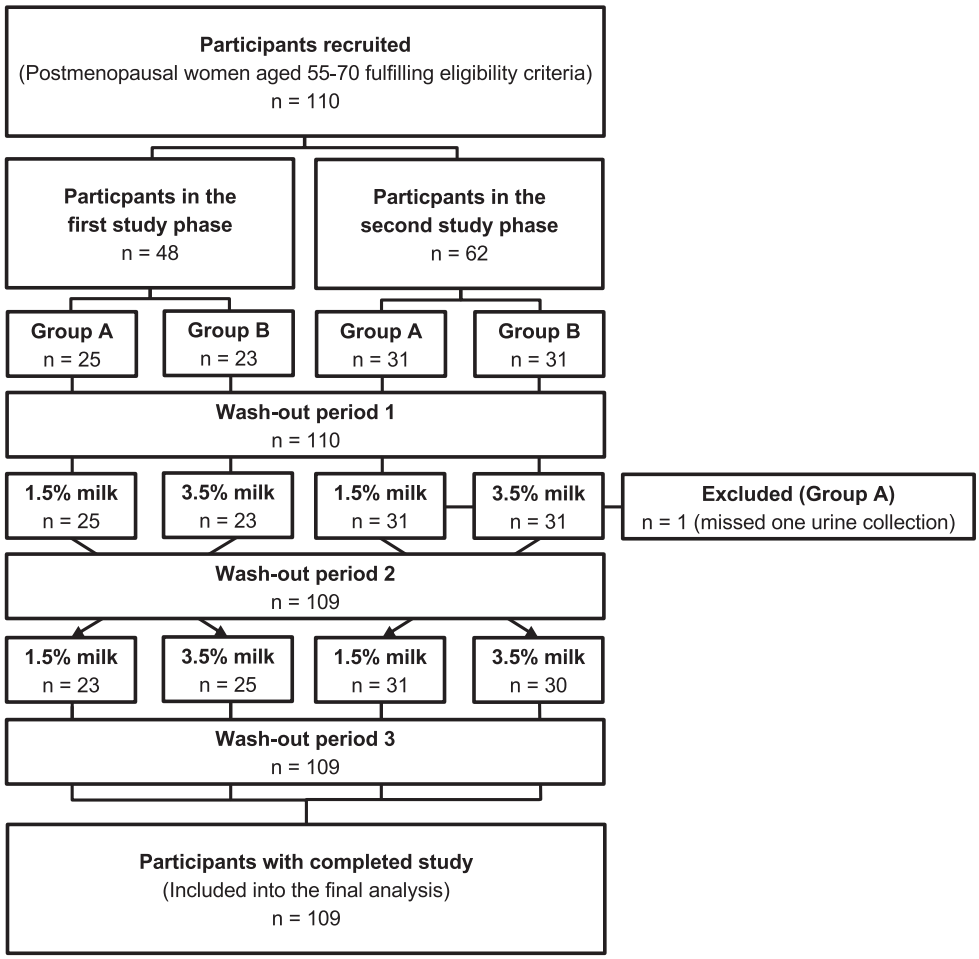

FIGURE 2 Flow chart illustrating inclusion and exclusion of study participants and definition of the study cohort. For logistical reasons, the study was conducted in 2 phases, with the second phase starting 1 month after the beginning of the first phase. Wash-out and intervention periods comprised 4 d each.

aliquot was spiked with 150 pg of 17β-E2-d3, Et-d5, 17α-T-d3, and 17α-methyltestosterone-d3 (17α-MeT-d3). Briefly, an enzymatic hydrolysis was performed (aliquots analyzed for determination of total steroids). Then, for all aliquots, a liquid–liquid extraction with diethylether was performed before a first purification step using a Chrom P SPE cartridge. This was followed by a liquid/liquid partitioning with *n*-pentane in order to separate estrogens from androgens and progesterone. Both fractions were then purified on a silica SPE cartridge before a final fractionation using semipreparative HPLC. After MSTFA/TMIS/DTT derivatization for androgen and progesterone, and PFBBr/BSTFA derivatization for estrogens, measurements were carried out by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) in electroionization (EI) mode and negative chemical ionization mode, respectively.

Quantification was performed by isotopic dilution. Standard extracted calibration curves were established by adding a fixed amount (150 pg) of 17β-E2-d3, Et-d5, 17α-T-d3, and 17α-MeT-d3 to 3 mL water and increasing quantities of the target analytes (17β-E2, 17α-E2, E1, 17β-T, 17α-T, Et, An, AED, and P) from 30 pg to 1.5 ng. The response linearity (R^2 values) was satisfying for all analytes. The accuracy of the quantification method was estimated to $\pm$15%. It was possible to quantify each analyte in all 8 analyzed milk samples.

Assessment of estrogen levels in urine samples

We aimed to assess a large range of estrogens and estrogen metabolites in the 24-h urine samples. In particular, we were interested in assessing catechol estrogens due to their carcinogenic profile. We therefore selected a liquid chromatography–mass spectrometry (LC-MS) method that quantifies up to 16 steroidal estrogens including oxidized metabolites in urine (11). Analyses were conducted at the University of Hawai'i Cancer Center, Honolulu, Hawaii, USA, in the laboratory of Adrian A. Franke. Urine samples (1 mL per sample) were shipped to Hawaii frozen via World Courier Service. For cost reasons, urine samples of the last wash-out period (wash-out 3) were assessed in only 50 participants. Of the total 486 samples, 49 randomly assigned samples were run in duplicate. To control for potential batch effect, corresponding samples were analyzed on the same day. All urine samples were corrected for creatinine (crea) levels to adjust for variations in the volume and concentration of the collected urine samples. Estrogen values were expressed as mass/mg crea.

TABLE 2
Mean levels (±SD) of sex steroid hormones in 4 semiskimmed and 4 whole milk samples used in this intervention study

		Estrogens, pg/mL			Androgens, pg/mL					Progestogens, ng/mL
		17β-E2	17α-E2	E1	17β-T	17α-T	Et	An	AED	P
Semiskimmed milk	Mean total	8.9 (±1.4)	32.8 (±4.2)	116.6 (±35.1)	5.4 (±1.7)	79.2 (±9.5)	35.9 (±5.6)	66.9 (±10.5)		4.56 (±0.48)
	Mean free	1.3 (±0.2)	1.2 (±0.3)	9.3 (±2.5)	2.1 (±0.7)	64.1 (±8.5)	32.0 (±5.1)	67.1 (±12.8)	370.3 (±23.0)	4.03 (±0.36)
	Conjugates, %	85	96	92	62	19	11	0		12
Whole milk	Mean total	10.0 (±1.1)	35.1 (±4.4)	148.9 (±36.7)	6.8 (±0.8)	120.4 (±12.5)	61.1 (±6.1)	147.8 (±20.0)		9.65 (±0.89)
	Mean free	1.6 (±0.4)	1.9 (±0.6)	13.6 (±4.0)	3.9 (±0.7)	107.3 (±20.7)	53.3 (±6.8)	125.4 (±12.6)	512.7 (±130.8)	7.75 (±0.23)
	Conjugates, %	84	95	91	41	11	13	15		20

Results for estrogens and androgens are expressed in pg/mL, those for progestogens in ng/mL. For AED, androstenedione, only the free form was determined because this metabolite cannot be conjugated. 17β-E2, 17β-estradiol; 17α-E2, 17α-estradiol; 17β-T, 17β-testosterone; 17α-T, 17α-testosterone; An, androsterone; E1, estrone (E1); Et, etiocholanolone; P, progesterone.

Assessment of progesterone levels in urine samples

We analyzed urine samples for immunoreactive pregnanediol glucuronide (PdG; major urinary metabolite of progesterone) excretion in our laboratory using a microtiter plate competitive enzyme immunoassay according to Munro et al. (12). The method is widely used (13–15), and its validity to capture the 24-h excretion of PdG has been reported previously (12, 16). The PdG (R13904) assay utilizes a polyclonal capture antibody (supplied by Coralie Munro at the University of California, Davis). The competitor for the assay is PdG conjugated to horseradish peroxidase (PdG-HRP) (also supplied by Coralie Munro). An endpoint substrate color reaction was developed with azino-bis-ethylbenzthiazoline sulfonic acid and peroxidase. PdG standard (Sigma) was used for the standard curve, and high- and low-value quality controls from in-house samples were utilized. All standards, blanks, and controls were run in duplicate on every plate, with samples in triplicate. Samples were added to the assay prediluted (1:50). Intra- and interassay coefficients of variations, calculated from high- and low-value quality controls, were between 4.4% and 13.5%, and 4.6% and 16.8%, respectively. All urine samples were corrected for crea levels to adjust for variations in the volume and concentration of the collected urine samples. PdG values were expressed as mass/mg crea.

Statistical analysis

Triplicate measurements of PdG were performed for all participants, and the mean of the 3 values was calculated for further analyses. Duplicate measurements of estrogen metabolites were performed for a random sample of individuals. We had sufficient statistical power to detect meaningful differences. To analyze the effects of milk consumption (semiskimmed or whole milk, before and after consumption) a linear mixed effects model was used, where repeated measurement, corresponding to the crossover design, was taken into account by a random intercept term. This also allowed duplicate values to be incorporated, to include measurements from individuals where some values were missing, and provided estimates of the mean difference due to consumption, for both semiskimmed and whole milk. A mixed effects model without the baseline ("before") measurements was used for directly comparing semiskimmed with whole milk. To decrease variability due to fluctuations in baseline measurements, we only considered data from individuals where the difference between the 2 available baseline values was smaller than one standard deviation of the first baseline value. Carryover effects and deviations from normality were checked by visual inspection, and no strong effects/deviations were found. All statistical analyses were performed in the R statistical environment (version 3.1.2).

Results

The characteristics of our study participants are provided in Table 1. Overall, 109/110 participants (>99%) completed all aspects of the study. The mean age of the study population was 61.2 y with a mean BMI of 26.4 kg/m^2. Of the study participants, 96.3% had experienced natural menopause.

The mean levels of estrogens, androgens, and progesterone measured in semiskimmed (1.5%) and whole milk (3.5%) are provided in **Table 2**. Estrogens were found to be mostly conjugated in milk, whereas androgens and progesterone were found mainly as free form (except 17β-T). Concentration levels of androgens and progesterone were substantially higher in whole milk than in semiskimmed milk samples. Absolute levels of these hormones were in the same range as previously reported by Courant et al. (17).

Besides the determination of PdG in participants' urine samples in our laboratory, 9 urinary estrogens were assessed in Dr Adrian A. Franke's laboratory in Hawaii: E1, E2, estriol (E3), 16a-hydroxyestrone (16aOHE1), 16-ketoestradiol (16ketoE2), 2-hydroxyestrone (2OHE1), 2-hydroxyestradiol (2OHE2), 2-methoxyestrone (2MeOE1), and 4-hydroxyestrone (4OHE1). A graphical display of the impact of cow milk consumption on the urinary level of sex steroid levels is provided in **Figure 3**. The shift resulting from the intervention appears to be moderate for E1, E2, E3, and 16ketoE2. There is no obvious shift for 2OHE1, 2OHE2, 2OMeE1, 4OHE1, and 16aOHE1. Results from the corresponding mixed effects model are presented in **Table 3**. The beta values indicate the mean difference in the urinary baseline level of the analyte compared with its value after the intervention, respectively. For example, the mean value of E1 increased by 0.18 ng/mg crea (95% CI: 0.07, 0.28) after 4 d of daily consumption of 1 L of semiskimmed cow milk. Urinary levels of E1 rose

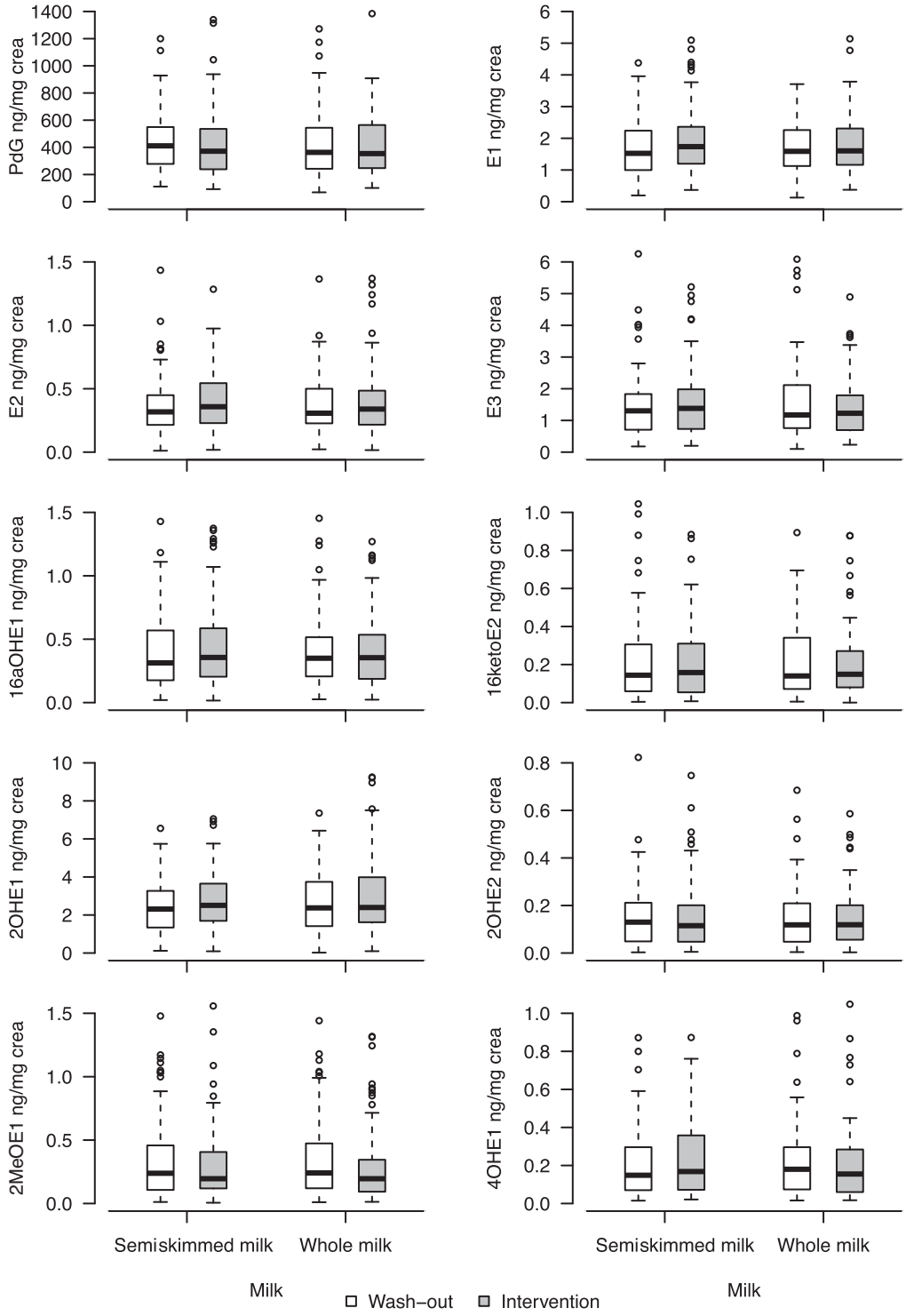

FIGURE 3 Impact of cow milk consumption on the urinary excretion of sex steroid levels among 109 women participating in a randomized crossover intervention trial. E1, estrone; E2, estradiol; E3, estriol, Intv, intervention; 16aOHE1, 16a-hydroxyestrone; 16ketoE2, 16-ketoestradiol; 2OHE1, 2-hydroxyestrone; 2OHE2, 2-hydroxyestradiol; 2MeOE1, 2-methoxyestrone; 4OHE1, 4-hydroxyestrone; PdG, pregnanediol glucuronide.

significantly after consumption of either semiskimmed or whole cow milk. The levels of E1 are somewhat higher after intake of semiskimmed milk compared with baseline (0.18 ng/mg crea; 95% CI: 0.07, 0.28) than after intake of whole milk compared with baseline (0.12 ng/mg crea; 95% CI: 0.01, 0.23). Urinary excretion of E2, E3, and 16ketoE2 increased significantly only after consumption of semiskimmed milk. Other estrogen metabolites and PdG levels were not significantly affected by cow milk consumption. Nevertheless, there was a trend to higher 2OHE1 excretion after intake of semiskimmed milk (0.26 ng/mg

TABLE 3
Estimated effects of milk consumption (semiskimmed or whole milk) on urinary sex steroid hormone excretion (post intervention versus baseline; ng/mg creatinine), and estimated effects of type of milk consumption (semiskimmed milk versus whole milk) on difference in hormone values (ng/mg creatinine) among 109 women participating in a randomized crossover intervention trial

	Semiskimmed milk (post intervention vs. baseline)				Whole milk (post intervention vs. baseline)				Type of milk (semiskimmed vs. whole)			
	N[1]	Baseline[2]	β (95% CI)[4]	P	N[1]	Baseline[2]	β (95% CI)[4]	P	N[1]	Baseline[3]	β (95% CI)[5]	P
Parent estrogens												
E1	116	1.78	0.18 (0.07, 0.28)	0.001	113	1.85	0.12 (0.01, 0.23)	0.03	102	0.12	0.09 (−0.1, 0.27)	0.36
E2	117	0.38	0.03 (0.01, 0.06)	0.01	115	0.41	0.01 (−0.01, 0.04)	0.4	103	0.01	0.03 (−0.01, 0.07)	0.17
16-Pathway												
E3	120	1.46	0.32 (0.1, 0.54)	0.004	118	1.55	0.07 (−0.15, 0.29)	0.53	106	0.06	0.28 (−0.1, 0.67)	0.15
16aOHE1	115	0.44	0.06 (−0.17, 0.29)	0.61	113	0.45	0.17 (−0.06, 0.4)	0.14	102	0.2	−0.13 (−0.55, 0.29)	0.55
16ketoE2	121	0.45	0.07 (0.01, 0.13)	0.02	119	0.49	0 (−0.06, 0.06)	0.99	108	0	0.08 (−0.03, 0.2)	0.17
2-Pathway												
2OHE1	114	2.78	0.26 (−0.05, 0.56)	0.1	111	2.94	−0.07 (−0.38, 0.24)	0.65	100	−0.11	0.45 (0.03, 0.86)	0.04
2OHE2	114	0.36	0.03 (0, 0.07)	0.09	109	0.34	0.03 (−0.01, 0.07)	0.15	100	0.04	0 (−0.07, 0.07)	1
2MeOE1	112	0.7	0 (−0.11, 0.12)	0.95	106	0.76	0.01 (−0.11, 0.13)	0.9	98	0.01	−0.02 (−0.22, 0.19)	0.86
4-Pathway												
4OHE1	107	0.22	0.04 (−0.01, 0.09)	0.09	109	0.24	0.02 (−0.02, 0.07)	0.32	103	0.03	0.01 (−0.07, 0.09)	0.79
Ratio												
2OHE1/16aOHE1	111	10.91	2.09 (−0.37, 4.54)	0.1	109	9.99	1.24 (−1.24, 3.73)	0.33	98	0.65	2.05 (−2.09, 6.2)	0.33
Progesterone												
PdG	94	432.17	7.25 (−22.35, 36.85)	0.63	94	420.19	−10.62 (−40.22, 18.98)	0.48	94	−4.63	5.89 (−36.06, 47.84)	0.78

[1] N indicates the number of participants providing measurements, including replicate measures provided by some participants. Individuals with measurements below the detection level of the analyte were excluded from the respective analysis. Individuals with fluctuating baseline values (defined as the difference between the 2 baselines exceeding the SD of the first baseline) of a specific analyte were also excluded from the respective analysis.

[2] Baseline values represent study population means of urinary sex steroid hormone excretion before intervention.

[3] Baseline values represent study population mean differences in urinary sex steroid hormone excretion after whole milk consumption compared with its respective baseline.

[4] The beta value indicates the difference between the study population mean value of the analyte post intervention minus the study population mean value at baseline. A linear mixed effects model was used, where repeated measurement corresponding to the crossover design was taken into account by a random intercept term.

[5] The beta value indicates the difference between the study population mean difference in the analyte post semiskimmed milk consumption minus the study population mean value at its respective baseline and the study population mean difference in the analyte post whole milk consumption minus the study population mean value at its respective baseline. A linear mixed effects model was used, where repeated measurement corresponding to the crossover design was taken into account by a random intercept term.

16aOHE1, 16a-hydroxyestrone; 16ketoE2, 16-ketoestradiol; 2OHE1, 2-hydroxyestrone; 2OHE2, 2-hydroxyestradiol; 2MeOE1, 2-methoxyestrone; 4OHE1, 4-hydroxyestrone; E1, estrone; E2, estradiol; E3, estriol; PdG, pregnanediol glucuronide.

crea; 95% CI: −0.05, 0.56) although not statistically significant. The ratio of 2OHE1/16aOHE1 remained unchanged.

We also calculated the difference in the effect of semiskimmed milk on sex steroid levels compared with the effect of whole milk (Table 3). The mean urinary excretion of the catechol estrogen 2OHE1 was 0.45 ng/mg crea higher after consumption of semiskimmed milk than after consumption of whole milk (95% CI: 0.03, 0.86). None of the other hormone metabolite levels differed significantly between the 2 types of cow milk.

Discussion

In this randomized crossover intervention trial, we found high concentrations of sex steroid hormones in cow milk with particularly high levels of progesterone and androgens. The concentrations of all hormones were generally higher in milk with higher fat content. Milk consumption resulted in a significant increase in urinary E1 concentrations, whereas E2, E3, and 16ketoE2 levels only increased after semiskimmed milk consumption. Urinary PdG levels were not significantly affected.

Although earlier studies using radioimmunoassays were inconsistent, newer studies using mass spectrometry have identified similar concentrations to those found in our study (18, 19). Hartmann and colleagues measured sex steroid hormones in a number of food items in Germany using gas chromatography–mass spectrometry (18). They found progesterone concentrations of 9.8 μg/kg in whole milk, 48.6 μg/kg in cream, and 141 μg/kg in butter. Progesterone levels in meat and other foods studied were substantially lower. Overall, cow milk and dairy products accounted for about 60–80% of estrogens and progesterone ingested with animal products. Ginther (20) and Hoffmann (21) found similar progesterone concentrations in milk samples. Courant et al., using also gas chromatography-mass spectrometry, measured E1 levels of 187 ng/L and 17β-E2 levels of 13.5 ng/L in whole milk; the majority of the estrogens were conjugated (17). Malekinejad and colleagues applied capillary liquid chromatography–tandem mass spectrometry to bovine milk and found nonconjugated E1 concentrations of 17.1 ng/L in milk containing 1.5% fat and 20 ng/L in milk containing 3.5% fat; the respective nonconjugated 17β-E2 concentrations were 13.9 and 20.6 ng/L (22), respectively. Between 85% and 92% of estrogens in commercial cow milk were found to be conjugated, including in organic milk. Farlow et al., also using capillary liquid chromatography–tandem mass spectrometry, identified high concentrations of estrogens and estrogen metabolites including catechol estrogens in milk (19).

Food processing, including fermentation and pasteurization, does not materially affect estrogen and progesterone concentrations in milk (2, 18, 19), whereas testosterone levels increase in cheese (18, 23). Fermenting bacteria or clotting enzymes may contribute to testosterone formation, possibly via the androgen metabolite estrone, which may serve as a precursor for testosterone (24, 25).

Sex steroid hormones differ substantially in their bioavailability. Although 17β-E2 is the estrogen with the highest biological activity, the bioavailability of free (unconjugated) 17β-E2 is only about 10% due to its first-pass effect, and the plasma half-life is 1 h (26–28). Conjugated estrogens, however, have a much higher bioavailability and longer half-life (29, 30). Hence, they are used in hormone replacement therapy. The high proportion of conjugated estrogens in milk found in this and prior studies suggests good bioavailability. Although conjugated estrogens are not biologically active, the conjugates can be cleaved in the human gut by bacterial sulfatases and glucuronidases producing free biologically active estrogens (22, 31).

Indeed, we observed a statistically significant increase in E1 concentrations after milk consumption. This can be explained by the substantial proportion of conjugated estrogens in our milk samples (84–96%, depending on the type of estrogen measured). Remarkably, despite no huge differences in total estrogen levels and percentage of conjugates between semiskimmed and whole milk, E1 levels were even higher after intake of semiskimmed milk, and E2, E3, and 16ketoE2 values significantly increased only after semiskimmed milk consumption. Perhaps a high fat intake inhibits enzymes needed for deconjugation similar to bioconversion of polyphenols, which are inhibited by fat in the diet (32).

Ingested (and endogenous) estrogens reaching the circulation are primarily metabolized in the liver along an oxidative pathway, and estrogen metabolites are excreted via urine. Although not significant, semiskimmed milk consumption slightly increased 4OHE1 levels. Although we did not determine catechol estrogens in the milk samples we used, in the study by Farlow et al. 4OHE1-levels in 2% and skim milk were found to be about one-third higher than in whole milk (19). The 4OH metabolites belonging to the group of catechol estrogens and the 16aOH metabolites have been found to be genotoxic (11, 33).

Although progesterone levels in our milk samples were high, particularly in semiskimmed milk, no significant urinary increase was observed after ingestion. Orally ingested progesterone has a low bioavailability unless micronized, a technique used in supplements such as hormone replacement therapy (34). Indeed, only 12% of progesterone was conjugated in our study, which may have resulted in low absorption and contributed to the lack of association observed. In addition, progesterone might have been hydroxylated quickly and thereby escaped our measurements because we did not monitor hydroxy-progesterones.

The effect of milk consumption on measurable sex steroid hormone levels has been investigated in a few other studies. In a study with 7 men and 7 children, levels of E1, E2, E3, and pregnanediol increased after intake of 600 mL/m^2 whole milk in spot urine samples (35). Carruba and colleagues observed that an intervention assigning 100 healthy postmenopausal women to a Mediterranean diet decreased urinary estrogen levels by more than 40% compared with a Western diet characterized by a high consumption of milk and dairy products, meat, and animal fat (36). Despite the low bioavailability of ingested progesterone, in a study including 17 healthy males, a 30–100% increase in progesterone levels in saliva was found after consumption of high-fat dairy products (37). Participants consumed 2 teaspoons (9.80 mL) of butter, 2 ounces (56 g) of cheese, and 1 quart (0.94 L) of ice cream in 1 day; saliva samples were collected before and after the intervention. Progesterone levels in saliva are indicative of the amount of bioavailable, free progesterone but show a much greater variation over a 24-h period than those of serum concentrations (38). Thus, multiple samples are required to obtain reliable information.

Limitations to our study include a possible lack of adherence to the study protocol. However, participants had been extensively instructed before the start of the study. Completeness of 24-h urine collection was checked by analyzing creatinine excretion (39). Furthermore, we do not know whether a longer intervention period would have yielded similar results, however, prior studies on this question detected changes in estrogen metabolite concentrations in urine levels within a few days (35) and in progesterone levels in saliva after a 1-day intervention (37). Although both enzyme immunoassay and LC-MS analysis are reliable, approved methods for the assessment of urinary PdG and estrogens, respectively, small effects of milk consumption on sex steroid levels are difficult to detect.

Conversely, to our knowledge, our study is the first to examine the effect of cow milk consumption on urinary levels of estrogens, estrogen metabolites, and PdG in postmenopausal women. We chose to measure hormone metabolites in urine rather than in blood because 24-h urine samples generate a more time-integrated value than a single blood sample. Moreover, with 5 biospecimen donations within a 20-d period, we expected compliance to be higher with a urinary protocol than with invasive blood collection. Indeed, our compliance was excellent, exceeding 99%. Other strengths of our study were the assessment of hormone levels in both in the milk samples and in the urine of the study participants.

In summary, in this randomized crossover intervention study we observed an increase in urinary estrogens and estrogen metabolites after ingestion of 1 L of cow milk daily for 4 d. Measurable levels of estrogens, androgens, and progesterone were identified in the cow milk selected for this study, in particular in high-fat milk. The increase in bioavailable estrogen levels—predominantly after intake of semiskimmed milk—is of concern in the context of epidemiologic studies linking regular milk consumption to hormone-sensitive cancers.

The authors' responsibilities were as follows—KBM and AO designed the study, developed the research plan, and obtained funding; AO conducted the research and collected the samples; FC, AAF, and AO performed the laboratory assays; NB performed the statistical analysis; KBM, NB, and AO interpreted the results; KBM wrote the manuscript; KBM and AO had primary responsibility for the final content of the manuscript; NB, FC, and AO provided a critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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