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Research Article

Effect of Grapefruit Juice Intake on Serum Level of the Endogenous CYP3A4 Metabolite 4β-Hydroxycholesterol—an Interaction Study in Healthy Volunteers

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Abstract. 4β-Hydroxycholesterol (4βOHC) is an endogenous CYP3A4 metabolite. However, it is unclear whether circulating levels of 4βOHC may reflect hepatic CYP3A4 activity or both hepatic and intestinal enzyme activity. The aim of this study was to investigate the effect of grapefruit juice, regarded to be a selective intestinal CYP3A4 inhibitor, on serum 4βOHC levels in healthy volunteers. The participants ($n = 22$) consumed grapefruit juice twice daily for 3 weeks followed by a 2-week washout period. Blood samples for measurements of 4βOHC and the non-CYP3A4-derived oxysterols 24-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC), as well as lathosterol and total cholesterol, were drawn on days 0, 7, 21, and 35. Median individual changes (ratios) in cholesterol-corrected 4βOHC levels from baseline to weeks 1, 3, and 5 were 0.94 ($P = 0.2$), 0.98 ($P = 0.3$), and 0.97 ($P = 0.9$), respectively. In comparison, median changes (ratios) in cholesterol-corrected levels of 24OHC at the same points were 1.01 ($P = 0.6$), 0.98 ($P = 0.3$), and 0.99 ($P = 0.5$), and of 27OHC 1.01 ($P = 0.8$), 0.97 ($P = 0.5$), and 0.99 ($P = 0.2$). Surprisingly, serum concentration of cholesterol was significantly reduced by approximately 5% after 1 week ($P = 0.03$), while median cholesterol-corrected levels of lathosterol increased significantly and persistently by approximately 15% during the whole 5-week period ($P < 0.04$). In conclusion, the present findings suggest that intestinal CYP3A4 is not relevant for the overall formation of 4βOHC in healthy volunteers. The fact that grapefruit juice altered cholesterol homeostasis should be further investigated.

KEY WORDS: 4β-hydroxycholesterol; oxysterols; CYP3A4; grapefruit juice.

INTRODUCTION

Cytochrome P450 3A4 (CYP3A4) plays a major role in the biotransformation of approximately 30% of all clinically used drugs making it the most important enzyme in drug metabolism (1). There is an extensive interindividual variation in CYP3A4 enzyme activity (2,3), with metabolic phenotypes ranging up to 40-fold in human studies (3). Thus, at the same dosing of CYP3A4 substrates, the differences in effective concentrations may be substantial.

A lot of research has been performed to identify factors that determine individual variability in CYP3A4 metabolizer phenotype. So far, sex seems to be a consistent factor of importance with females generally exhibiting higher CYP3A4 activity than males (4,5). In addition, increased weight (BMI) and inflammation status have been associated with a reduction in CYP3A4 activity (6,7). However, unlike CYP2D6 and CYP2C19, there is no clear association between *CYP3A4* genotype and phenotype (8). Genotyping is therefore an inappropriate tool for determination of an individual's CYP3A4 phenotype. Instead, phenotyping is the only satisfactory approach to determine individual CYP3A4 metabolism.

Exogenous substrates of CYP3A4, like midazolam, have been used as biomarkers for determination of CYP3A4-mediated drug metabolism *in vivo* (9). Midazolam is validated as CYP3A4 biomarker, but a practical disadvantage is the necessity of standardized dose administration and blood sampling times after intake (10). It has therefore been increasing interest in using endogenous substrates of CYP3A4 as biomarkers for determination of CYP3A4 phenotype (11). One of the most studied endogenous biomarkers of CYP3A4 is 4β-hydroxycholesterol (4βOHC)

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(12–14), which is formed from cholesterol by CYP3A4 and to a lesser extent by CYP3A5 (15–17). However, a limitation of 4βOHC as an endogenous biomarker is that multiple enzymes are involved in the formation or elimination, hence reducing its specificity in reflecting CYP3A4 phenotype. Still, 4βOHC level has been shown to be sensitive towards administration of both CYP3A inducers (15,18–20) and inhibitors (21), but it is more suitable for detecting enzyme induction than inhibition. Based on this, 4βOHC may act as a CYP3A4 interaction biomarker to be applied during the early clinical phase of drug development.

Whether 4βOHC level may have a potential as biomarker for dose individualization of drugs metabolized by CYP3A(4) has been questioned (22), mainly due to its limited specificity and correlation with midazolam clearance (23), which is regarded as a gold standard for measuring CYP3A4 activity. However, there are some studies reporting significant correlations between 4βOHC level and exposure of CYP3A4 substrates (12,24–26) so this endogenous biomarker may have a potential usefulness as a factor in algorithms or models for estimating individual dose requirements. For 4βOHC to be successful as a potential biomarker in such models, it is essential that its level reflects both hepatic and intestinal CYP3A4 activity.

It has been suggested that 4βOHC mainly reflects hepatic CYP3A4 activity (13,27), possibly because a substantial part of its precursor cholesterol is synthesized in the liver (28). However, dietary cholesterol is also absorbed in the intestine where CYP3A4 is abundantly expressed (7,29). In line with this, two recent studies have provided a basis for speculations of a possible involvement of intestinal CYP3A4 in 4βOHC formation (12,18), but no studies have provided direct evidence that 4βOHC is formed by intestinal CYP3A4.

In order to clarify if 4βOHC is formed by intestinal CYP3A4, we conducted a study where 4βOHC level was measured in healthy volunteers before, during, and after intake of grapefruit juice (GFJ), which is regarded to selectively inhibit intestinal CYP3A4 metabolism (30). In addition, we measured the non-CYP3A4-derived oxysterols 24-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC), total cholesterol, and lathosterol, an endogenous marker for cholesterol synthesis.

METHODS

Subjects

Twenty-three healthy volunteer students recruited from School of Pharmacy at University of Oslo, Norway, were included in the study. All subjects abstained from consumption of GFJ at least 4 weeks before initiation of the study and none were treated with medication known to affect CYP3A4 enzyme activity. Prior to initiation of the study, all participants joined a meeting where the protocol was presented and the importance of the compliance with the GFJ intake was clearly communicated.

The study was approved by the Regional Committee for Medical and Health Research Ethics and the Hospital's Research Committee, and written informed consent was obtained from all participants.

Experimental Design

The study took place during a 5-week period, where blood samples were drawn before, during, and after GFJ intake (Fig. 1). Each subject consumed 200 mL GFJ twice a day (morning and evening) for 3 weeks followed by a 2-week wash out period. The decision behind the 3-week period of GFJ intake was based on the assumption of a new steady-state concentration of 4βOHC to be achieved within the timeframe. This assumption was founded on (i) a reported elimination half-life of approximately 60 h for 4βOHC after injection of a deuterium-labeled dose (16) and (ii) a reported degradation (inactivation) half-life of approximately 20–30 h for intestinal CYP3A4 (31). The consumed juice was prepared from yellow/white grapefruit concentrate (Cyprina, New Sevegep LTD, Nicosia-Cyprus), which is generally known to contain higher amounts of CYP3A4-inhibiting compounds than juice prepared from red/pink juice (32).

Blood samples were drawn from the included subjects the first day of the study (before administration of GFJ, day 0), and after 7 and 21 days of GFJ consumption. Two weeks after the GFJ consumption was terminated (day 35), a final blood sample was collected. Blood samples were drawn on standard BD Vacutainer® tubes (Becton-Dickinson Company, Plymouth, UK) for serum formation by certified health professionals in line with accredited procedures. Serum samples were stored at −20°C until analyses of the various oxysterols/lipids.

It was calculated that 20 subjects would be sufficient to detect a 30% reduction in 4βOHC levels after GFJ consumption, with a power of 80% ($\alpha = 0.05$). Twenty-three subjects were recruited to account for possible dropouts from the study.

Analysis of 4βOHC

The assay used for quantification of 4βOHC in serum is previously described in detail (12). Briefly, the sample preparation consisted of a liquid-liquid extraction with hexane as organic phase after de-esterification with ethanolic sodium hydroxide. Extracts, solved in methanol, were injected onto an Acquity ultra-performance liquid chromatograph (UPLC) coupled to a Micromass Quattro Premier tandem MS detector (Waters, Milford, MA, USA). For chromatographic separation, an UPLC BEH C18 column RP-shield (1.7 μm, 1 × 100 mm) from Waters was used, with gradient elution with a mix of water and methanol (85–95%), at a flow rate of 0.150 mL/min, runtime per sample of 10 min, and a temperature of 40°C. Atmospheric pressure chemical ionization (APCI) was used for ionization and detection was obtained with multiple reaction monitoring (MRM) at the transitions *m/z* 385.25 -> 367.45 (4βOHC) and *m/z* 392.30 -> 374.50 (4βOHC-D7; IS). At the lowest validated concentration (25 nmol/L), the intra- and interday precision and accuracy was < 15% while the intra- and interday precision and accuracy at the highest validated concentration (1600 nmol/L) was < 4%. The calibration curve was prepared in methanol and shown to be linear in the validated concentration range ($r^2 = 0.998$). Chromatographic separation between 4βOHC and 4αOHC was shown by injecting reference standards solved in methanol. All four

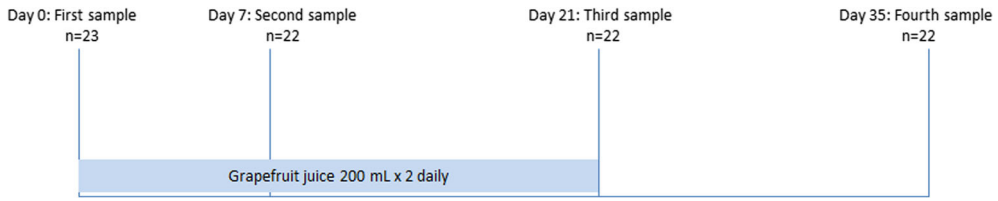

Fig. 1. Study design

samples from each participant collected during the study were prepared and analyzed at the same day to eliminate potential inter-day variation.

Analysis of Total Cholesterol, 24OHC, 27OHC, and Lathosterol

Total serum cholesterol was determined by a standard enzymatic method based on hydrolysis of cholesterol esters to free cholesterol (Roche Diagnostics GmbH, Mannheim, Germany). The non-CYP3A4-derived oxysterols 24OHC and 27OHC were determined by isotope-dilution gas chromatography-mass spectrometry, using deuterium-labeled internal standards as described before (33). Lathosterol was determined by gas chromatography-mass spectrometry using $^{2}H_{4}$-latholsterol as internal standard as described in (34).

Endpoints and Statistical Analyses

The primary endpoints were changes in the oxysterol levels, corrected for total cholesterol (precursor) concentration, during the study period. The changes in all lipids were expressed as median values of the participants' after-*vs*.-before ratios of measured concentrations at each time point, i.e., measurement day 7, 21, or 35/measurement day 0. While 4βOHC concentrations were normally distributed in the study population, this was not the case for the other oxysterols. Thus, the non-parametric Wilcoxon signed-rank tests were applied in the statistical analyses when comparing the ratios of relative cholesterol-corrected oxysterol changes. In addition to comparing the cholesterol-corrected oxysterol levels during the study period, we also assessed the time profiles of the absolute, uncorrected levels of each oxysterol and total cholesterol.

GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical calculations and graphical illustrations. P values < 0.05 were considered statistically significant.

RESULTS

Of the 23 subjects included in the study, one withdrew consent in the early phase due to mouth itching after drinking GFJ. Of the remaining 22 subjects fulfilling the study, 17 (77%) were females. Median age and body mass index of the participants were 24 years (range; 19, 48) and 22 kg/m^2 (range; 17.6, 29.4), respectively. For one subject, there was not sufficient serum volume to analyze 24OHC, 27OHC, and lathosterol in addition to 4βOHC and cholesterol.

In Table I, baseline levels of the oxysterols and other lipids, as well as BMI, and age are shown and compared in relation to sex. 4βOHC levels at baseline ranged from 22 to 104 nmol/L (median 50.0 nmol/L), where females had significantly higher levels of 4βOHC (median 57.9 nmol/L) than males (40.8 nmol/L, $P = 0.03$). Contrary, females had significantly lower median levels of lathosterol compared to males (1350 ng/mL *vs*. 2032 ng/mL, $P = 0.04$).

The median relative changes (ratios after-*vs*.-before) in cholesterol-corrected 4βOHC, 24OHC, 27OHC, and lathosterol concentrations at days 7, 21, and 35 compared to baseline are presented in Table II. While uncorrected 4βOHC levels decreased significantly from baseline to week 1 of GFJ intake by 11% ($P = 0.04$, data not shown), the reduction was not significant when correcting for cholesterol (−6%; $P = 0.2$, Table II). The observed cholesterol-corrected 4βOHC levels (ratios) were 4% and 3% lower at week 3 and week 5, respectively, as compared with baseline ($P > 0.3$).

Compared with baseline, the lathosterol serum concentration was increased by 11% after 1 week of GFJ consumption ($P = 0.07$), 14% after 3 weeks ($P = 0.04$), and 15% after 5 weeks ($P = 0.04$). The total serum cholesterol concentration was significantly reduced by approximately 5% after 1 week of GFJ consumption ($P = 0.03$), but was not significantly lower than baseline after 3 weeks of GFJ intake or following washout ($P > 0.09$, Table II). The observed cholesterol-corrected serum concentrations of 24OHC and 27OHC were more or less at the same level during the whole study period (Table II).

In Fig. 2, the time profiles of cholesterol and all oxysterols relative to baseline values are shown both as unadjusted (Fig. 2b) and cholesterol-adjusted concentrations (Fig. 2a), while Fig. 3 shows the time profiles of their absolute levels during the study period.

DISCUSSION

In the present study, we measured levels of the endogenous CYP3A4 metabolite 4βOHC in healthy volunteers before, during, and after consumption of GFJ, which is a selective intestinal CYP3A4 inhibitor (30). Overall, only a small, insignificant decrease in cholesterol-corrected 4βOHC level was observed during GFJ intake. Thus, the study provides evidence that intestinal CYP3A4 most likely is not important for the systemic concentration of 4βOHC, which suggests a limited usefulness of 4βOHC as a biomarker for dose requirements of orally administered CYP3A4 substrates.

The monitoring of potential 4βOHC changes during GFJ intake was complicated by a significantly and persistently reduced level of total cholesterol during GFJ consumption. The reason for the reduced cholesterol level after GFJ intake is unclear, but the rapid effect may indicate that GFJ reduced intestinal cholesterol absorption. Actually, Mulvihill *et al.* have reported that naringenin, an antioxidant found in high amounts in grapefruit, reduces cholesterol in LDL receptor-

Table I. Measured Median Serum Concentration of Lipids and Oxysterols, Body Mass Index (BMI), and Age Presented by Gender. Italicized *P* Values Illustrate Statistical Significance

Baseline	Whole population	Females ($n = 17$)	Range	Males ($n = 5$)	Range	*P* value
4βOHC (nmol/L)	50.0	57.9	26.7, 104.0	40.8	22.0, 55.0	*0.031*
24OHC (ng/mL)	84.5	90.0	62.0, 144.0	68.0	61.0, 151.0	0.183
27OHC (ng/mL)	130.5	126	102.0, 163.0	152.0	109.0, 253.0	0.06
Lathosterol (ng/mL)	1611.0	1350.0	698.0, 2371.0	2032.0	1657.0, 3143.0	*0.038*
Cholesterol (mmol/L)	4.6	4.6	3.4, 5.8	4.5	4.0, 6.8	0.969
BMI (kg/m^2)	22.0	21.0	*	23.2	*	0.055
Age (years)	23.5	22.0	19, 48	26.0	22, 39	0.114

*Not given due to personally identifiable data

null mice (35). Thus, it would be relevant to investigate how and to which extent naringenin may lower cholesterol in further human studies. In parallel with total cholesterol, serum level of lathosterol was increased after 1 week of GFJ consumption and remained elevated during the whole study period. This finding likely reflects an increased cholesterol biosynthesis compensating for the possibly reduced absorption.

Since 4βOHC is transported within lipoproteins (15), a change in lipoprotein level will affect the concentration of 4βOHC in the circulation. Bodin *et al.* have also shown that the oxysterol concentration is proportional to the concentration of cholesterol (15), and since we found a reduction in cholesterol concentration, it was considered necessary to account for the changes in cholesterol levels when studying changes in 4βOHC. In the present study, we also included analyses of the non-CYP3A4-derived oxysterols 24OHC and 27OHC in addition to 4βOHC. All three oxysterols are transported in lipoproteins in the circulation and are distributed in lipoproteins in the same manner as cholesterol (15). Thus, reduced levels of circulating lipoproteins, due to lowered cholesterol concentration, are likely an underlying mechanism of the reduction in all three oxysterols. However, the insignificant observation of a larger and more persistent drop in cholesterol-corrected level of 4βOHC than 24OHC and 27OHC may suggest that GFJ to some extent reduce the CYP3A-mediated 4βOHC formation as well.

There are several issues of relevance for the sensitivity of detecting a change in 4βOHC level during inhibition of intestinal CYP3A4 in the study. One aspect is whether dietary cholesterol actually is exposed to CYP3A4 enzymes in the enterocytes. Absorption of dietary cholesterol is thought to involve incorporation of cholesterol into bile salt micelles (36), which possibly could restrict the contact of free cholesterol with CYP3A4 during absorption. However, recent studies have shown that cholesterol is absorbed *via* transporter-facilitated mechanisms (37) supporting that CYP3A4 enzymes in the enterocytes have access to cholesterol for oxidative metabolism.

Another relevant aspect of the study is the time needed to detect a potential change in 4βOHC after starting GFJ intake, where both the elimination half-life of 4βOHC and degradation (inactivation) half-life of intestinal CYP3A4 are important to consider. While the half-life of 4βOHC is reported to be ~17 days based on time to reach a new steady-state concentration after termination of the potent CYP3A4 inducer rifampicin (38), Bodin *et al.* estimated the half-life to be approximately 60 h after injection of deuterium-labeled 4βOHC in healthy volunteers (16). Regarding the degradation/inactivation of CYP3A4, a study on hepatic enzymes has estimated a half-life of approximately 30 h (39), while intestinal half-life of CYP3A4 is estimated between 10 and 33 h (31). Thus, if the abovementioned data is used as a basis, the 3-week timeframe of GFJ intake in the present study should be sufficient to detect a possible change in intestinal CYP3A4-mediated formation of 4βOHC. However, these underlying assumptions are associated with substantial uncertainty of relevance in whether a new steady-state concentration of 4βOHC could be achieved within the 3-week period of grapefruit juice consumption.

In addition to the abovementioned aspects, the actual CYP3A4 inhibitory action in the healthy subjects during GFJ

Table II. Overview of Median Individual Change (Ratios) in Cholesterol-Corrected Serum Concentrations of the Various Oxysterols, Lathosterol, and Cholesterol Itself, at the Different Time Points Related to the Baseline Values. Italicized *P* Values Illustrate Statistical Significance

	Day 7			Day 21			Day 35		
	Ratio	Range	*P* value	Ratio	Range	*P* value	Ratio	Range	*P* value
4βOHC/C ($n = 22$)	0.94	0.76, 1.25	0.20	0.96	0.75, 1.32	0.25	0.97	0.77, 1.37	0.86
24OHC/C ($n = 21$)	1.01	0.84, 1.17	0.64	0.99	0.88, 1.17	0.34	0.97	0.86, 1.13	0.53
27OHC/C ($n = 21$)	1.01	0.91, 1.23	0.82	0.98	0.82, 1.16	0.53	0.98	0.82, 1.13	0.16
Lathosterol/C ($n = 21$)	1.11	0.66, 2.69	0.07	1.14	0.45, 1.95	*0.04*	1.15	0.63, 2.17	*0.04*
Cholesterol ($n = 22$)	0.95	0.77, 1.15	*0.05*	0.98	0.66, 1.13	0.23	0.96	0.72, 1.29	0.09

Measured units: 4βOHC in nmol/L; 24OHC, 27OHC, and lathosterol in ng/mL; and cholesterol in mmol/L

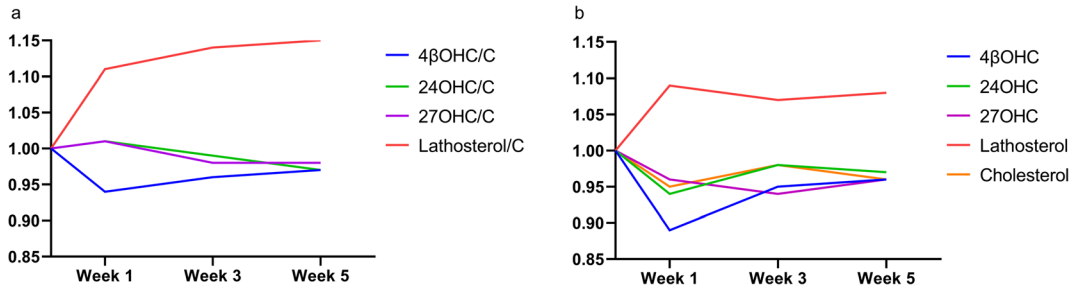

Fig. 2. Time profiles of cholesterol-corrected median changes (**a**) and uncorrected median changes (**b**) of the various oxysterols, lathosterol, and cholesterol compared to baseline. Ranges are not shown of visual reasons. Measured units: 4βOHC in nmol/L; 24OHC, 27OHC, and lathosterol in ng/mL; and cholesterol in mmol/L

intake is a relevant issue. First, it is essential that the participants were compliant with the protocol's strict requirement to consume 200 mL twice daily. The type of GFJ juice used was white (yellow), which has a characteristic untasteful bitterness that over time can be exhausting to drink in larger quantities. The participants filled a form at every visit where none reported non-compliance with the protocol's requirement on GFJ intake. However, at the debrief meeting after completion of the study, some of the participants informed orally about practical challenges with carrying the juice carton at "all kind of places" for intake of the "evening dose." Thus, the fact that the small reduction in 4βOHC level after 1 week was not substantiated after 3 weeks of twice daily GFJ intake might be biased by partial "non-compliance" occurring over time. Regarding the type of GFJ applied in the study, yellow/white GFJ has consistently been shown to reduce the presystemic metabolism of multiple CYP3A4 substrates (40–42). Despite that phenotyping with an exogenous CYP3A4 biomarker, such as midazolam, would have been valuable as a "positive control," we consider it likely that intestinal CYP3A4 was substantially and persistently inhibited by GFJ intake during the time frame of the study.

A final point regarding the potential of detecting an effect of GFJ intake on the 4βOHC level is that previous studies on administration of short-term CYP3A4-inhibiting drugs, such as ketoconazole and itraconazole, only found about 10–30% decreases in 4βOHC concentrations, which the power calculation of the current study was based upon. A weakness when investigating these rather modest concentration changes is that the estimates are fragile with respect to random variability.

The potential usefulness of 4βOHC as a biomarker for individualized dosing of CYP3A4 substrates has been a subject of debate (22,43). The limited correlation between 4βOHC/cholesterol ratio and midazolam clearance (23), which might reflect that other non-CYP3A4 mechanisms also affect 4βOHC level, argues against its value as quantitatively robust and specific CYP3A4 biomarker (22). However, Vanhove *et al.* recently reported that 4βOHC/C showed slightly better correlation than midazolam clearance with the clearance of tacrolimus, a CYP3A4 substrate (24). Multiple factors are of importance for tacrolimus clearance, but despite the negative findings of the present study, the observations of Vanhove *et al.* indicate that 4βOHC may have role as a biomarker to be included in models for estimating individual dose requirements of orally administered CYP3A4 substrates.

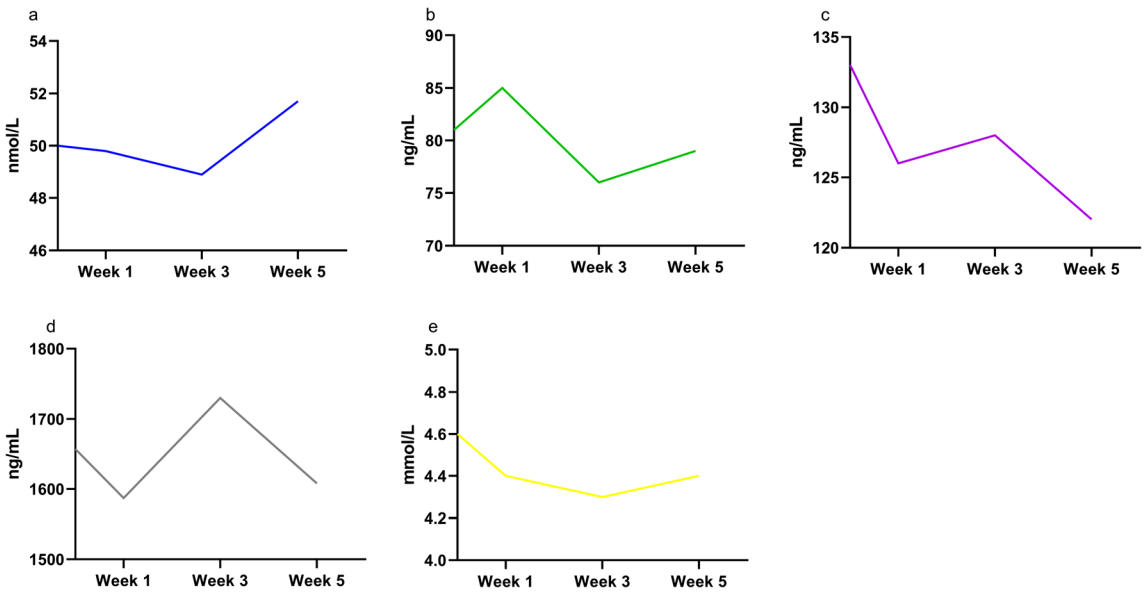

Fig. 3. Time profile of 4βOHC (**a**), 24OHC (**b**), 27OHC (**c**), lathosterol (**d**), and cholesterol (**e**), presented as median values

CONCLUSION

The present study shows that long-term grapefruit juice intake does not significantly reduce 4βOHC level. Thus, intestinal CYP3A4 is unlikely to play an important role in the formation of 4βOHC, which argues against its clinical potential as a simple dosing biomarker of orally administered CYP3A4 substrates. The fact that GFJ altered cholesterol homeostasis in the healthy study participants should be further investigated in human studies.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was approved by the Regional Committee for Medical and Health Research Ethics and the Hospital's Research Committee, and written informed consent was obtained from all participants.

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