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Selective Changes In Cholesterol Metabolite Levels In Plasma Of Breast Cancer Patients After Tumor Removal

Pavel Soucek[a,b], **David Vrana**[c], **Yune-Fang Ueng**[d,e,f], **Shouzou Wei**[g], **Renata Kozevnikovova**[h], and **Frederick Peter Guengerich**[i]

[a]Department of Toxicogenomics, National Institute of Public Health, Prague, Czech Republic

[b]Biomedical Centre, Medical School Pilsen, Charles University, Pilsen, Czech Republic

[c]Department of Oncology, Palacky University Medical School and Teaching Hospital, Olomouc, Czech Republic

[d]National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei, Taiwan

[e]Department of Pharmacology, National Yang-Ming University, Taipei, Taiwan, ROC

[f]Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan, ROC

[g]Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, United States

[h]Department of Oncosurgery, MEDICON, Prague, Czech Republic

[g]Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, United States

Abstract

Introduction—Oxygenated metabolites of cholesterol ("oxysterols") can influence carcinogenesis and contribute to resistance to endocrine therapy, an effect mostly described *in vitro*.

Objectives—We sought to establish a method for screening plasma levels of oxysterols in breast cancer patients, estimate their individual variability and detection limits, and provide basic information about their roles in tumor biology.

Method—Liquid-chromatography coupled with tandem mass spectrometry was used for determination of levels of 25-hydroxycholesterol, 27-hydroxycholesterol, 7α-hydroxycholesterol, and 7-ketocholesterol in plasma sample pairs from patients before and 12–24 months after surgical removal of tumors (n=24). Deuterated standards of all oxysterols were used for method validation.

Address for correspondence: Assoc. Prof. Pavel Souček, PhD, Department of Toxicogenomics, National Institute of Public Health, Srobarova 48, Prague, 100 42, Czech Republic, phone: +420 267082711; pavel.soucek@szu.cz; www.szu.cz.

Compliance with Ethical Requirements

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declare that they have no conflict of interests.

Result—All oxysterols were successfully detected in patient plasma samples. A significant increase in the level of 7-ketocholesterol was observed in the samples following tumor removal and the start of therapy compared to the sampling before (p=0.002). This increase was unrelated to personal characteristics of patients, expression of estrogen receptor, or to adjuvant therapy type.

Conclusion—This study shows, for the first time, that circulating levels of oxysterols, especially 7-ketocholesterol, may reflect the presence of tumor cells in patients.

Keywords

oxysterols; breast cancer; estrogen; plasma; therapy

Introduction

Breast cancer (OMIM: 114480) is the most frequently diagnosed cancer and one of the leading causes of cancer death among women in the western world (Siegel 2016). Early diagnosis and effective therapy are prerequisite for improvement of these facts. Currently, there is no available blood-based test for prediction of breast cancer risk, which would enable preventive measures to diagnose the cancer in localized stages. Moreover, due to the heterogeneity of the disease, a significant number of patients still experience disease relapse or side effects of the treatment, and markers predicting these events and new therapy targets are urgently needed for these patients.

Oxygenated metabolites of cholesterol belong to a large group of sterols called oxysterols (Mutemberezi 2016). Oxysterols are formed in the human body or ingested in the diet, e.g., phytosterols. Despite their low tissue levels, they play important roles in the organism and, e.g., through interaction with liver X receptors, oxysterol-binding proteins, or some drug transporters, oxysterols can influence both carcinogenesis and cancer progression (Kloudova 2017).

The role of cholesterol and its metabolites in breast cancer onset and therapy outcome currently receives increasing attention due to the fact that breast cancer patients have higher cholesterol levels than healthy controls (Nelson 2013), and enzymes of cholesterol biosynthesis and the synthesis of the oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol contribute to endocrine therapy (e.g., tamoxifen or aromatase inhibitors) resistance in breast cancer models *in vitro* (Simigdala 2016). Tamoxifen is a selective estrogen receptor modulator (SERM) and, together with its metabolites (mainly 4-hydroxytamoxifen), blocks transcriptional activation of estrogen-responsible genes in breast tumor cells (Wang 2004). In addition to its SERM activity, recent studies have shown that cholesterol metabolism is involved in the anticancer pharmacology of tamoxifen (see ref. Poirot 2012 and refs. cited therein, (Segala 2013)) and that 27-hydroxycholesterol acts as an endogenous SERM, thus possibly influencing the pathology of breast cancer (DuSell 2008). Congruently, 27-hydroxycholesterol promotes faster growth and proliferation of mouse mammary gland tumors (Nelson 2013).

Additionally, B-ring oxysterols (e.g., 7-ketocholesterol) are suspected to influence hormonal treatment efficacy because of cell death induction via interaction with the antiestrogen

binding site (AEBS) (De Medina 2009) and through their binding of estrogen receptors and AEBS in tumor cells (Segala 2013, de Weille 2013).

The aim of this study was to monitor plasma levels of selected oxysterols in paired plasma samples of breast cancer patients collected before surgical removal of the tumor and in the period 12–24 months after surgery. This approach provides basic information about the suitability of the method, individual variability, and detection limits of oxysterols in circulation of patients before a larger detailed study can be performed. The study also shows the first indication of differences that may be attributed to the tumor biology.

Materials and methods

Chemicals

7-Ketocholesterol (ChemSpider ID: 82599), 7α-hydroxycholesterol (96891), 25-hydroxycholesterol (58604), and 27-hydroxycholesterol (110495) and their deuterated standards (7-ketocholesterol-d_7, 7α-hydroxycholesterol-d_7, 25-hydroxycholesterol-d_6, and 27-hydroxycholesterol-d_6) were purchased from Steraloids (Newport, RI). Methanol, isopropanol, and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene) were purchased from Sigma-Aldrich (St. Louis, MO), and HPLC-grade solvents were products of Fisher Scientific (Pittsburgh, PA).

Patients

A total of 24 primary breast carcinoma patients diagnosed in the Department of Oncosurgery, Medicon and surgically treated in the Institute for the Care for Mother and Child (both in Prague, Czech Republic) during 2013 were included in the study. Blood samples were collected from each patient one day before surgical tumor removal (first sampling) and then from the same patient in the period 12–24 months after surgery (second sampling). As oxysterol levels are impacted by circadian and feeding rhythm, patients were asked to refrain from eating after 8 p.m. before the day of sampling, which was performed between 8–9 AM on the next day. There was no radiologically proven disease relapse at the time of second sample collection in any of the enrolled patients.

Blood samples were collected to one vacutainer tube with anticoagulant potassium EDTA for plasma separation. The separation was performed immediately after the blood withdrawal by centrifugation at 2,200 × *g* for 10 minutes at 4 °C. Then samples were aliquoted to three separate aliquots, snap frozen, and stored at −80 °C. Diagnosis of all patients was confirmed histologically according to standard diagnostic procedures (Tavassoli 2003). Hormonal receptor expression was evaluated based on a 1% cut off. Immunohistochemistry was utilized for ERBB2 (erb-b2 receptor tyrosine kinase 2) testing, 3+ scores were considered positive and 1+ as negative. In the case of 2+ scores, fluorescent in situ hybridization (FISH) was used for status confirmation. Refusal of the informed consent of the patient, preoperative chemotherapy or endocrine therapy, and lack of histological diagnosis were exclusion criteria for the study. Personal and clinical characteristics of the patients including adjuvant therapy are summarized in Tables 1 and 2.

All patients were informed about the study and those who agreed and signed an informed consent participated in the study. The study protocol was approved by the Ethical Commission of the National Institute of Public Health in Prague. The methods were carried out in accordance with guidelines approved by the above Ethical Commission.

Extraction of oxysterols from plasma samples

The method described by Helmschrodt (2013) was adopted for extraction of oxysterols from plasma. Briefly, thawed plasma samples were centrifuged at 3,200 × *g* for 5 minutes and 80 μl of plasma was added to vials containing 80 μl of a methanolic solution of a mixture of internal standards (400 pg of the deuterated version of each oxysterol) or blank (methanol without oxysterols). Then 1.44 ml of a methanol/isopropanol (1:1, v/v) mixture was added, and the samples were thoroughly mixed using a vortex device for 1 min and centrifuged for 10 min at 12,500 × *g*. Supernatants were transferred into amber glass vials and evaporated to dryness at room temperature under a stream of nitrogen in the dark. Vials with dried extracts were filled with argon gas, sealed, and stored at −80 °C until analysis. On the day of analysis, extracts were reconstituted with 80 μl of a methanol/water mixture (3:1, v/v) and centrifuged prior to analysis for 5 min at 3,220 × *g*. All solutions contained 0.05% (v/v) butylated hydroxytoluene to prevent autoxidation (Helmschrodt 2013).

Measurement of oxysterols

Dissolved extracts were subjected to the positive-ion APCI LC/MS/MS analysis as previously described (Shinkyo 2011). Briefly, oxysterols were resolved by UPLC (50 °C) on an Acquity BEH octadecylsilane (C18) column (1.7 μm; 1 mm × 100 mm) (Waters) using solvent mixtures of $H_2O/CH_3OH/CH_3CN$ (30:3.5:66.5, v/v/v), holding for 1 min, then programmed to CH_3OH/CH_3CN (5:95, v/v) over 4 min with a linear gradient at a flow rate of 0.16 ml/min and quantitated by positive-ion APCI/MS/MS-SRM monitoring of product ions: *m/z* 367.2 (25-hydroxycholesterol), *m/z* 367.2 (27-hydroxycholesterol), *m/z* 367.2 (7α-hydroxycholesterol), *m/z* 383.2 (7-ketocholesterol), *m/z* 385.2 (25-hydroxycholesterol), *m/z* 385.2 (27-hydroxycholesterol), *m/z* 385.2 (7α-hydroxycholesterol), *m/z* 401.2 (7-ketocholesterol), respectively, and *m/z* 373.2 (d_6-25-hydroxycholesterol), *m/z* 373.2 (d_6-27-hydroxycholesterol), *m/z* 374.2 (d_7-7α-hydroxycholesterol), *m/z* 390.2 (d_7-7-ketocholesterol), *m/z* 391.2 (d_6-25-hydroxycholesterol), *m/z* 391.2 (d_6-27-hydroxycholesterol), *m/z* 392.2 (d_7-7α-hydroxycholesterol), and *m/z* 408.2 (d_7-7-ketocholesterol). The injection volume (onto the column) was 20 μl. Quantitation was done using an isotope ratio method. The limit of quantitation (LOQ) for oxysterols in human plasma was 0.05 ng per sample based on calibration curves for each oxysterol.

Data analysis

Raw data were first processed in order to identify outliers and deviations from normal distribution by the Shapiro-Wilk test. Differences between the compared groups of patients were evaluated by the paired Wilcoxon test and the independent Mann-Whitney or Spearman test. Comparisons including and excluding outliers have been performed. A *p*-value of less than 0.05 was considered statistically significant. Analyses were conducted by the statistical program SPSS v16.0 (SPSS, Chicago, IL).

Results

Characteristics of patients

A total of 24 patients with both samplings and complete clinical follow up data were included in the study. Another 24 patients were collected for method establishment and individual variability estimate, but paired samples or complete data follow up were not available (data not shown). Eight patients were pre-menopausal and the rest were peri- or post-menopausal. Patients from the study set were mostly diagnosed with ductal adenocarcinoma histology at locally advanced stage I or II. One patient had an *in situ* tumor, and in five patients the tumor had spread into axillary lymph nodes. The majority of patients (17/24) received adjuvant therapy containing tamoxifen and eight patients had only tamoxifen before the day of second sample collection. All personal and clinical data are summarized in Tables 1 and 2.

Levels of oxysterols in breast cancer patients–comparison of paired samples before and after tumor removal

Oxysterols were analyzed in plasma samples of the 24 patients with both samplings available. The individual and mean ± S.D. plasma levels of oxysterols in these patients are presented in Table 3. Data were further processed by non-parametric tests due to the diversion of majority of data from the normal distribution ($p<0.05$ by the Shapiro-Wilk test). Analysis by the independent Mann-Whitney test showed that the level of 27-hydroxycholesterol was significantly lower and that the level of 7-ketocholesterol was higher in the second sampling compared to the first sampling ($p=0.034$ and 0.013, respectively, Table 4). Analysis by the paired Wilcoxon test showed non-significant results for both oxysterols as well as for others (Table 4).

When analyzing individual values, several extreme values (outliers) were noted. Thus, separate analyses without these outliers were performed as well (for value distribution see Fig. 1). One outlier with 5.5 ng/ml (closest value 1.6) of 25-hydroxycholesterol from the first sampling, one with 2.2 ng/ml (14.6) of 27-hydroxycholesterol from the second sampling, one with 8.4 ng/ml of 7α-hydroxycholesterol (6.7 from the first sampling) and one with 10.9 ng/ml (7.7 from the second sampling), two with 27.6 and 22.5 ng/ml 7-ketocholesterol (14.6 from the first sampling) and one with 16.1 ng/ml (12.1 from the second sampling) were removed from the analysis. The samples to these outliers were not further processed in the paired analyses. Sample pairs with oxysterol levels below the limit of quantitation in one or both samplings were also removed from subsequent paired analyses. Analysis by the independent Mann-Whitney test again showed that samples from the second sampling contained significantly higher 7-ketocholesterol levels compared to the corresponding first samplings ($p=0.007$). However, the previously observed difference for 27-hydroxycholesterol levels was non-significant ($p=0.053$), as well as differences for 25-hydroxycholesterol and 7α-hydroxycholesterol. These results were also confirmed by analyses using a paired Wilcoxon test (Table 4).

In analyses stratified according to personal data of patients (see Table 1), significantly higher levels of 7-ketocholesterol in the first sampling of patients with post or perimenopausal

status compared to premenopausal ones were found, but no such association was seen in the second sampling (Table 5A). Similarly, associations of age, body mass index, or smoking status with level of individual oxysterols observed in one of the samplings were not consistent with the other sampling. Age significantly correlated with body mass index of patients (p=0.001, r^2=0.776). Interestingly, the 27-hydroxycholesterol level was significantly higher in the first sampling of patients with positive personal history of hypercholesterolemia, hypothyroidism, hysterectomy, or hormonal replacement therapy compared to patients without such conditions. On the other hand, in the second sampling of these patients opposite association was found (i.e., higher level in patients with negative history) (Table 5A).

Further analyses based on clinical data (Table 2) have been conducted. Patients treated with tamoxifen had significantly higher plasma levels of 7-ketocholesterol in the second sampling compared to the corresponding first sampling (n=15/16, first/second sampling without outliers, p=0.034 by the Mann-Whitney test). The rest of oxysterols did not differ between samplings of tamoxifen-treated patients. Analysis of a group of patients expressing the estrogen receptor also showed significantly higher 7-ketocholesterol levels in the second sampling compared to the corresponding first sampling (n=16/17, p=0.022 by the Mann-Whitney test).

We then performed analysis of differences in levels of oxysterols between groups of patients stratified by estrogen receptor expression and tamoxifen-based therapy. Patients with tumors expressing estrogen receptor had significantly higher levels of 7-ketocholesterol and lower levels of 27-hydroxycholesterol within the first sampling than those without expression (p=0.020 and 0.006, respectively, Table 5B). Patients treated with tamoxifen had higher levels of both 7-ketocholesterol and 7α-hydroxycholesterol in the first sampling than those treated by other regimens (p=0.035 and 0.024, respectively). These differences were non-significant in analyses of the second sampling (Table 5B).

Discussion

The lack of reliable predictive and prognostic biomarkers for prevention, diagnosis, and therapy of human cancer and the need for non-invasive diagnostics has emerged in a number of studies addressing this problem using circulating levels of various biomarkers, e.g., cell-free DNA, microRNA, exosomes, peptides, and metabolites. Except numerous studies using genomic and proteomic tools, lipidomics also represents a highly promising approach. The present exploratory study followed the question of feasibility of detection of selected oxysterols in plasma of breast cancer patients. We also asked whether tumor removal causes detectable perturbations in levels of oxysterols that could then be used, e.g., for estimation of the disease risk or monitoring of its recurrence.

Some oxysterols are considered to be tumor promoters (Nelson 2013, Silvente-Poirot 2014) and estrogen receptor agonists (see ref. Griffiths 2016 for review) and thus their role in breast cancer is currently attracting considerable interest. Although human plasma levels of oxysterols have already been assessed by various authors (reviewed, e.g., in Mutemberezi 2016), there is currently only one very recent study reporting differences in levels of various

oxysterols in plasma of breast cancer patients (Dalenc 2016). Thus, our study adds novel information essential for this evolving area of research.

The above cited study (Dalenc 2016) has shown that levels of oxysterols—including 25-hydroxycholesterol, 27-hydroxycholesterol, 7α-hydroxycholesterol, and 7-ketocholesterol—can be reliably detected in patients plasma by high-performance gas chromatography coupled to mass spectrometry (GC–MS). The present study used a different technique for oxysterol level detection, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The assay method may be the source of variations in oxysterols between the studies, e.g., the observed median of 27-hydroxycholesterol was between 55.6–58.7 ng/ml in the previous study and 27.9–31.7 in the present one. On the other hand, the mean levels of the rest of oxysterols we analyzed are comparable to other published data in humans (Mutemberezi 2016).

Our study was designed to compare levels of oxysterols before and after tumor removal. Hence, we provide the first evidence showing a significant rise of 7-ketocholesterol levels in the plasma of breast cancer patients after surgical removal of tumors. This change was found both in all patients and in a subgroup with estrogen receptor-expressing tumors treated with tamoxifen, suggesting the general nature of this phenomenon. Dalenc (2016) compared patients on day 0 and 28 (n=27) after the beginning of endocrine therapy with tamoxifen or aromatase inhibitors and observed a number of significant changes between both samplings, but none for 7-ketocholesterol and 27-hydroxycholesterol.

The present study differs from the previous one also in the fact that we followed breast cancer patients in the early stage I or II of the disease while Dalenc (2016) observed significantly higher levels of 25-hydroxycholesterol and 6-oxo-cholestan-3β,5α-diol in metastatic patients compared with adjuvant-treated (i.e., clinically considered as tumor-free) patients. Together with our data, these results indirectly support the view that levels of oxysterols may vary not just as a result of the treatment but also reflect the presence of tumor cells in the patients' bodies. It remains to be determined whether such variations provide a driving force for the tumor growth and its metastatic spread or are a consequence of tumorigenesis.

Furthermore, we observed that 7-ketocholesterol levels were significantly higher in the second sampling compared to the first sampling of the subgroup of patients with tumors expressing estrogen receptor, as well as in patients treated with tamoxifen-based regimens. On the other hand, although levels of some oxysterols varied according to estrogen receptor expression or tamoxifen-based therapy in the first sampling, they did not differ in the second sampling again, suggesting that the tumor presence plays a key role.

Taken together, the rise of 7-ketocholesterol levels after tumor removal, which is not relevant to estrogen expression or tamoxifen therapy, suggests that it may be connected with tumor growth, independent of estrogenic activity. 7-Ketocholesterol was recently suggested as a potential diagnostic and predictive biomarker of Niemann-Pick disease (Boenzi 2016). It was also shown to be a cytotoxic and sensitizing agent in multidrug resistant chronic

myeloid leukemia cell lines *in vitro* (Rosa Fernandes 2017), but there is no report about its role in human cancer onset or progression *in vivo*.

From a mechanistic point of view, 7-ketocholesterol (together with other 7-oxysterols) is a ligand of nuclear liver X receptors (LXRA and LXRB) (Janowski 1999, Aye 2011) and an agonist of RAR-related orphan receptors (RORA and RORC) (Wang 2010), potentially modulating the mammalian clock. LXRs are considered to exert an anti-proliferative role through reduction of cellular cholesterol levels needed for tumor cell growth, and some studies suggest their roles in both cancer development (reviewed in (Kuzu 2016)) and potential as therapeutic targets. Very little is known about the role of RORs in human cancer, except that high RORC expression correlated with longer progression-free survival of breast cancer patients (Cadenas 2014) and RORA was shown to enhance proliferation through up-regulation of the cytochrome P450 19A1 aromatase in an estrogen receptor-expressing model of human breast cancer, MCF7 cells (Odawara 2009). These contradictory roles of RORs, together with the pleiotropic effects of LXRs, need to be further studied and explained.

Recently 25- and 27-hydroxycholesterol have been reported to increase the expression of estrogen receptor-controlled trefoil factor 1 (TFF1) gene in a panel of breast cancer model cell lines *in vitro*, suggesting that these oxysterols may be involved in endocrine therapy resistance (Simigdala 2016). Although our study did not find significant variations in levels of these oxysterols in patients treated by endocrine therapy in their second sampling (*in vivo*), we admit that samplings over a much longer period and evaluation of survival of patients are necessary for rigorous assessment of the utility of these oxysterols for monitoring of resistance.

Bearing in mind the potential influence of personal characteristics as age, body mass index, smoking status, or history of steroid homeostasis-related conditions on circulating levels of oxysterols, we also addressed the role of these factors. We have not observed consistent trend towards modulation of 7-ketocholesterol levels by these confounders. Interestingly, the level of 27-hydroxycholesterol was modulated by body mass index of patients in both samplings and thus, it should be further considered as a potential confounder in future studies.

Taken together, the present study shows that the assessment of 7-ketocholesterol, 7α-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol in plasma samples of breast cancer patients is feasible using liquid-chromatograpraphy-mass spectrometry. Most importantly, the above data suggest that 7-ketocholesterol levels rise after tumor removal. Further studies are necessary to assess the reproducibility of this observation and justify a more detailed search for the mechanism behind this phenomenon before clinical utilization, e.g., estimation of disease risk, monitoring of disease recurrence, or employment of therapeutic interventions can be considered.

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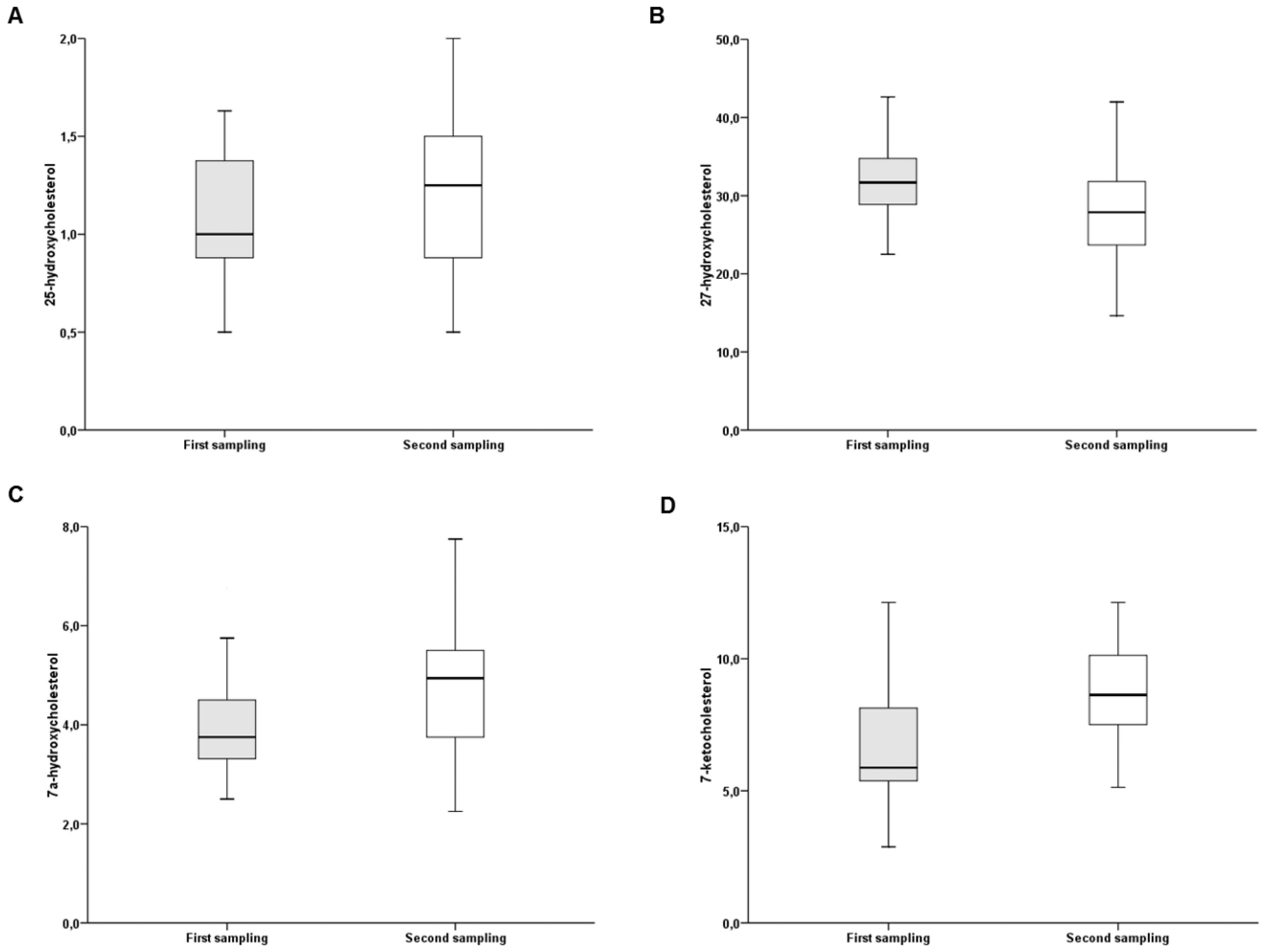

Fig. 1.

Distribution of levels of oxysterols in patients before and after tumor removal with all outliers removed

A, 25-hydroxycholesterol; B, 27-hydroxycholesterol; C, 7α-hydroxycholesterol; D, 7-ketocholesterol. All levels are expressed as ng/ml of plasma.

Table 1

Personal data of patients

Sample ID	Age at diagnosis	Menopausal status	Body mass index	Smoking	Personal history related to cholesterol and hormones
433	75	post	25	current smoker	hysterectomy
434	60	post	26	never smoker	hysterectomy
435	48	pre	23	never smoker	hypercholesterolemia
438	54	pre	22	never smoker	none
440	58	post	25	current smoker	none
443	75	post	27	never smoker	hypercholesterolemia, hormonal replacement therapy
445	43	pre	22	never smoker	none
452	62	post	24	never smoker	hormonal replacement therapy
453	56	peri	23	ex-smoker	none
456	54	pre	19	current smoker	none
463	55	post	23	current smoker	none
473	41	pre	20	ex-smoker	hormonal replacement therapy
476	60	post	22	never smoker	hypercholesterolemia, hypothyroidism, hysterectomy
500	71	post	28	never smoker	none
504	61	post	26	N/A	hypercholesterolemia, hypothyroidism
505	57	post	22	never smoker	none
523	39	pre	19	ex-smoker	hypothyroidism
535	60	post	31	never smoker	none
590	36	pre	22	never smoker	none
599	82	post	26	never smoker	none
600	59	post	25	current smoker	none
606	37	pre	21	N/A	none
611	57	post	26	never smoker	none
614	70	post	24	never smoker	hypercholesterolemia, hysterectomy

N/A: not available

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Table 2

Clinical data of patients

Sample ID	Surgery date (day/month/year)[a]	Histology	Grade	Lymph node metastasis	Stage	ER expression	PR expression	ERBB2 expression	Ki67 expression	Adjuvant therapy
433	30/01/2013 (21)	lobular	1	absent	IIA	positive	positive	negative	5%	TAM, AI
434	01/02/2013 (21)	ductal	3	absent	Tis	positive	positive	negative	20%	TAM, AI
435	01/02/2013 (21)	ductal	1	absent	IA	positive	positive	negative	10%	TAM, AI
438	06/02/2013 (25)	ductal	3	present	IIB	negative	positive	negative	30%	TAM
440	08/02/2013 (21)	tubular	1	absent	IA	positive	positive	negative	5%	AI
443	13/02/2013 (21)	ductal	3	absent	IA	negative	negative	negative	60%	none
445	13/02/2013 (23)	ductal	3	absent	IIA	negative	negative	negative	40%	AC, PCT
452	22/02/2013 (23)	tubular	1	absent	I	positive	positive	negative	10%	TAM
453	27/02/2013 (24)	ductal	1	absent	I	positive	positive	negative	5%	TAM
456	01/03/2013 (20)	ductal	1	absent	I	positive	positive	negative	20%	TAM
463	13/03/2013 (24)	ductal	1	present	IIA	positive	positive	negative	10%	EC, TAM
473	22/03/2013 (20)	ductal	3	absent	I	negative	negative	positive	30%	EC
476	22/03/2013 (20)	ductal	2	absent	I	positive	positive	negative	15%	TAM, AI
500	03/05/2013 (18)	ductal	3	absent	I	positive	positive	negative	30%	TAM, AI
504	10/05/2013 (20)	tubular	2	absent	I	positive	positive	negative	15%	TAM
505	15/05/2013 (20)	ductal	1	absent	I	positive	positive	negative	5%	TAM
523	23/06/2013 (19)	ductal	3	absent	I	negative	negative	negative	50%	EC
535	10/07/2013 (16)	ductal	2	absent	IIA	positive	positive	negative	20%	EC, TAM, AI
590	23/09/2013 (14)	ductal	3	present	IIA	positive	positive	positive	20%	EC, TXT, HER, TAM
599	25/10/2013 (13)	mucinous	1	absent	IIA	positive	positive	negative	15%	TAM
600	01/11/2013 (15)	ductal	3	present	IIA	positive	positive	negative	30%	EC, PCT, AI
606	20/11/2013 (12)	ductal	3	absent	IA	negative	negative	negative	60%	none
611	29/11/2013 (14)	ductal	2	present	IIA	positive	positive	negative	10%	EC, TAM
614	13/12/2013 (11)	ductal	1	present	IIA	positive	positive	negative	3%	TAM

[a]Sampling interval in months in parentheses

ER=estrogen receptor, PR=progesterone receptor, ERBB2=erb-b2 receptor tyrosine kinase 2, Ki67=proliferation-related Ki-67 antigen

Table 3

Individual levels of oxysterols in patients with two samplings

	25-hydroxycholesterol		27-hydroxycholesterol		7α-hydroxycholesterol		7-ketocholesterol	
Sample ID	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**
433	0.6	1.0	32.1	24.1	4.5	7.4	9.0	10.4
434	5.5	BLQ	36.2	27.0	8.4	5.0	12.1	10.2
435	BLQ	1.7	33.7	16.6	4.5	BLQ	5.9	8.4
438	BLQ	0.6	37.2	30.1	4.4	3.7	5.6	8.2
440	0.5	0.7	31.2	36.2	4.0	5.1	5.6	8.6
443	BLQ	1.4	34.9	24.9	3.7	4.9	5.9	8.7
445	0.9	0.9	39.7	21.9	2.5	4.6	5.1	6.5
452	1.5	1.4	33.6	27.9	3.4	4.5	8.5	7.7
453	1.1	BLQ	24.0	32.1	6.7	5.0	9.6	8.9
456	BLQ	1.0	28.0	35.0	4.4	7.7	6.1	11.1
463	1.2	0.6	34.6	24.4	3.7	3.0	6.9	6.2
473	0.9	BLQ	40.1	17.6	2.7	2.6	4.9	5.2
476	BLQ	1.2	30.4	30.4	2.5	2.2	10.9	5.1
500	1.0	1.5	29.7	14.6	6.7	10.9	27.6	16.1
504	BLQ	0.6	31.2	22.7	5.7	5.6	22.5	10.0
505	0.9	2.0	23.2	33.7	2.9	5.7	6.2	7.6
523	1.0	0.5	42.6	2.2	3.5	5.0	2.9	8.2
535	0.6	1.6	24.0	31.5	5.6	2.9	6.4	6.6
590	1.6	1.1	32.9	29.1	2.7	4.7	5.7	10.7
599	BLQ	1.2	22.9	25.0	4.5	5.4	5.4	10.5
600	1.5	1.5	31.9	42.0	3.5	6.0	8.1	9.2
606	BLQ	1.4	22.5	30.0	3.2	5.5	5.0	7.4
611	1.1	1.9	31.5	39.7	3.6	3.4	4.4	12.1
614	1.5	1.7	31.5	23.2	5.6	4.4	5.9	9.6
Mean	**1.3**	**1.2**	**31.7**	**26.8**	**4.3**	**5.0**	**8.2**	**8.9**
S.D.	**1.2**	**0.4**	**5.5**	**8.6**	**1.5**	**1.9**	**5.7**	**2.4**

All levels in ng/ml of plasma. BLQ=below limit of quantification, S.D.=standard deviation, F=first sampling, S=second sampling

Table 4

Differences in plasma levels of oxysterols between both samplings

All data	**Independent Mann-Whitney test**		**Paired Wilcoxon test**	
	Number of patients F/S	***p*-value**	**Number of patients F/S**	***p*-value**
25-hydroxycholesterol	16/21	0.488	13/13	0.285
27-hydroxycholesterol	24/24	**0.034**	24/24	0.075
7α-hydroxycholesterol	24/23	0.105	23/23	0.104
7-ketocholesterol	24/24	**0.013**	24/24	0.086
Data without outliers				
25-hydroxycholesterol	15/21	0.287	13/13	0.285
27-hydroxycholesterol	24/23	0.053	23/23	0.127
7α-hydroxycholesterol	23/22	0.088	21/21	0.068
7-ketocholesterol	22/23	**0.002**	22/22	**0.007**

For individual variability and average levels see Table 3. F/S=first/second sampling. Significant results in bold.

Table 5

Differences in plasma levels of oxysterols in stratified analyses

Analyses stratified by personal characteristics of patients (Table 1)					
First sampling	**Characteristics**	**Numbers of patients**	**Oxysterol concentration[a]**		***p*-value**
			negative[d]	**positive[d]**	
25-hydroxycholesterol	None	-	-	-	-
27-hydroxycholesterol	History	14/10	29.5 ± 5.6	34.7 ± 4.0	**0.030**[b]
7α-hydroxycholesterol	Age	23	r^2=0.488		**0.018**[c]
	Body mass index	23	r^2=0.570		**0.004**[c]
7-ketocholesterol	Menopause	8/14	5.2 ± 1.0	7.5 ± 2.3	**0.009**[b]
	Age	22	r^2=0.497		**0.019**[c]
Second sampling					
25-hydroxycholesterol	Smoking	13/6	1.4 ± 0.4	0.9 ± 0.4	**0.022**[b]
27-hydroxycholesterol	History	14/9	30.4 ± 7.2	23.8 ± 4.5	**0.020**[b]
7α-hydroxycholesterol	None	-	-	-	-
7-ketocholesterol	None	-	-	-	-

Analyses stratified by clinical characteristics of patients (Table 2)					
First sampling	**Characteristics**	**Numbers of patients**	**Oxysterol concentration[a]**		***p*-value**
			ER+/TAM+	**ER−/TAM−**	
25-hydroxycholesterol	None	-	-	-	-
27-hydroxycholesterol	ER expression	18/6	30.2 ± 4.1	36.2 ± 7.2	**0.020**[b]
7α-hydroxycholesterol	TAM therapy	16/7	4.5 ± 1.3	3.3 ± 0.5	**0.035**[b]
7-ketocholesterol	ER expression	16/6	7.3 ± 2.2	4.9 ± 1.1	**0.006**[b]
	TAM therapy	15/7	7.2 ± 2.2	5.4 ± 1.6	**0.024**[b]
Second sampling					
All oxysterols	None	-	-	-	-

[a] Mean ± standard deviation in ng/ml of plasma

[b] Significance by the Mann-Whitney test

[c] Significance by the Spearman rho test

[d] Negative versus positive means groups of patients coded as never smokers versus current or ex smokers (smoking), premenopausal versus post or perimenopausal (menopausal status) and patients with negative versus positive personal history related to cholesterol and hormones. Personal history of hypercholesterolemia, hypothyroidism, hysterectomy or hormonal replacement therapy was coded as positive. Significant results in bold. Associations observed in both samplings are in grey.

ER=expression of estrogen receptor. TAM= therapy by tamoxifen

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