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The role of secondary bile acids in breast and pancreatic cancer

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Introduction

Breast cancer

Breast cancer is the third most common cancer in the world and the number one cancer in women. The incidence of breast cancer varies significantly between different geographical and economic regions, with a higher incidence observed in developed countries. In Europe in 2012, the age-adjusted annual incidence of breast cancer per 100,000 population was 94,2, associated with a mortality rate of 23,1 per 100,000. According to the National Cancer Registry, 8250 new cases of breast cancer were reported in Hungary in 2019. Although the number of breast cancer cases is steadily increasing, the mortality rate is decreasing, mainly due to modern screening programs introduced in developed countries, improved medical, surgical, and radiological procedures, and newer molecular biology techniques. Thanks to these improvements, the five-year survival rate of breast cancer patients is now over 80%. Several risk factors can increase the risk of developing breast cancer, such as age, hormone replacement therapy, use of hormonal contraceptives, early menarche, late menopause, and dense breast tissue. Familial cases of breast cancer are often associated with mutations in the BRCA1 and BRCA2 genes, as well as overexpression of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER2). The risk of developing breast cancer can be reduced by improving general health, exercising regularly, eating a balanced diet, and avoiding smoking and alcohol.

Adenocarcinoma of the pancreas

Malignant tumors of the pancreas account for more than 10% of all gastrointestinal cancers. The most common of these is pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 90% of all pancreatic cancers. Worldwide in 2018, 458,918 new cases of PDAC were registered and 432,242 deaths were associated with this disease. The survival rate for PDAC is quite low, with a five-year survival rate of less than 5%. Several risk factors increase the chance of PDAC, such as age, smoking, alcoholism, obesity, and chronic pancreatitis. Early detection is hampered by the fact that symptoms of the disease do not appear until late in the course of the disease, so diagnosis is only made when the disease is advanced and often has metastasized to distant sites. The difficulties in treating PDAC are both genetic and cellular. Mutations present in tumors lead to increased gene instability, which plays a significant role in tumor growth and the development of resistance to treatments. Multiple alterations in signaling pathways may contribute to the mechanisms of resistance. Although the biological background of PDAC is not yet fully understood, mutations in certain genes, such as

KRAS, CDKN2A/p16, TP53 and SMAD4, and activation of related signaling pathways play a fundamental role in the development of treatment resistance.

Relationship between the microbiome and tumors

The human body is home to a variety of microorganisms that are essential for host homeostasis. Bacteria, fungi, protozoa, viruses, and their collective genome make up the human microbiome. The amount and composition of the microbiome present in different areas of the body is highly diverse. The microbiota of the gastrointestinal system accounts for 90% of the total microbiome, with up to 10^{14} bacteria. The gut flora has many essential functions in the human body and plays a significant role in maintaining the homeostasis of the host. It is actively involved in the digestion and absorption of nutrients and influences energy use and storage. This microbial ecosystem plays an important regulatory role in the immune system, protecting against pathogenic microorganisms by producing antimicrobial substances and inhibiting the development of allergic reactions. The gut microbiota promotes the production of various vitamins and enzymes through its metabolic activity. In addition, it produces neurotransmitters that contribute to gut-brain communication, thereby affecting the mental and neurological functions of the host.

Dysbiosis is an abnormal alteration of the microbiome, characterized by an abnormal composition and function of microorganisms. The altered microbiome associated with cancer is called the oncobiome. Hanahan and Weinberg introduced the concept of "Hallmarks of cancer", which describes the biological processes that drive oncogenesis and support the uncontrolled proliferation of tumor cells. The oncobiome is directly or indirectly involved in the establishment and regulation of these biological processes. The oncobiome plays a key role in evading the immune response, enhancing tumor-promoting inflammation, inducing invasion and metastasis, stimulating angiogenesis, inducing genome instability and mutations, and is also involved in the deregulation of cellular energy regulation. Several studies have demonstrated that oncobiotic transformation promotes tumor cell proliferation, invasiveness, metastasis, and tumor vascularization by enhancing vascular endothelial growth factor (VEGF) expression.

Oxidative/nitrosative stress

Physiologically produced reactive oxygen and nitrogen forms (ROS/RNS) are essential participants in many physiological processes. They play important roles in various signal transduction processes and in the elimination of pathogenic agents. Under normal conditions, the functioning of the prooxidant and antioxidant systems is in balance, protecting the body from the damaging effects of free radicals. However, due to increased free radical production and decreased antioxidant activity,

this balance is upset, leading to oxidative and nitrosative stress. Accumulating free radicals can damage protein, lipid and DNA molecules in cells and thereby induce apoptotic or necrotic processes that may play a role in the pathomechanism of many diseases such as arteriosclerosis, ischaemia/reperfusion injury, heart failure, inflammatory and neurodegenerative diseases, autoimmune diseases, and diabetes. In addition, research has demonstrated that redox imbalance has been shown to be involved in a variety of cancers.

Cellular redox balance is a finely regulated process in which NRF2 (nuclear factor, erythroid 2-like 2) plays an important role. NRF2 is a transcription factor involved in regulating the expression of antioxidant genes and is therefore crucial in maintaining cellular redox balance. The main function of NRF2 is to protect cells from external and internal damage caused by oxidative stress, which can affect intracellular lipids, proteins, nucleic acids, and carbohydrates. The activity of NRF2 is regulated by KEAP1 (Kelch-like ECH-associated protein 1), a repressor protein found in the cytoplasm. NRF2 regulates the expression of over 1000 genes, including SOD, CAT and GPX. NRF2 may promote tumor cell growth, metastasis and contribute to the development of resistance to therapy through its antitumor and cytoprotective effects.

Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a cellular developmental process in which epithelial cells lose their characteristics and transform into mesenchymal cells. During this process, cells lose their cell-cell contacts, apical-basal polarity, and their connection to the basement membrane, and acquire a spindle-like morphology, while acquiring invasive properties. Although the full spectrum of EMT-inducing signaling pathways is not yet fully understood, several transcription factors have been identified in breast and pancreatic gland cancer that promote EMT and contribute to the stem cell-like state. These include the SNAIL (snail family transcription repressor-1), SLUG (snail family transcription repressor-2), ZEB1 (zinc finger E-box binding homeobox-1) and TWIST (twist family BHLH transcription factor 1) proteins. These transcription factors are able to act on the marker proteins β-CATENIN, ZO-1 (zona occludens 1), CLAUDIN-1 and E-CADHERIN through different signalling pathways and thereby regulate EMT processes.

Cancer stem cells

Tumor tissues are composed of heterogeneous cell populations. Two main cell populations can be distinguished in a tumor: a larger group of differentiated cells with limited proliferative capacity, and a smaller population of cancer stem cells capable of continuous division. Cancer stem cells are responsible for tumor development, metastasis, resistance to therapy and tumor recurrence. The

metabolism of tumor cells is characterised by increased mitochondrial phosphorylation and metabolic flexibility, which facilitates tumor progression and supports the development of drug resistance. Identification of cancer stem cells is often based on specific markers. One of the most used markers for breast and pancreatic cancer is the enzyme aldehyde dehydrogenase-1 (ALDH1). In cancer stem cells, the expression of antioxidant genes is elevated, resulting in much lower levels of free radicals. This is because, unlike normal cells, cancer stem cells are much more sensitive to high levels of ROS. ALDH1 activity contributes to tumor cell survival by reducing the amount of ROS. Hence, the enhancement of oxidative stress processes in tumor cells may be capable of selectively killing tumor cells.

Secondary bile acids

Primary bile acids are mainly synthesized from cholesterol in hepatocyte cells of the liver. These bile acids are conjugated by the liver to glycine or taurine and then secreted into the gallbladder with bile. After meals, they flow to the duodenum where they play an important role in the emulsification and absorption of lipids and lipid-soluble nutrients and in the regulation of cholesterol levels. The bile acids are then reabsorbed via the enterocytes of the distal ileum and re-enter the liver via the portal circulation for reuse. This is called the enterohepatic circulation. Although the reabsorption of bile salts is highly efficient, 400-800 mg of bile acids per day still leave the circulation, which then become substrates for microbiota in the large intestine and are converted into secondary bile acids. The enzymes responsible for the conversion of bile acids are bacterial bile acid hydrolysis (BSH) and hydroxysteroid dehydrogenase (HSDH). In humans, the most important secondary bile acids are lithocholic acid (LCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA). 7α-dehydroxylation of CA results in the formation of DCA, whereas 7α-dehydroxylation of CDCA results in the formation of LCA. The enzymes responsible for the conversion of bile acids are clustered in the highly conserved bile acid-induced (bai) operon complex gene organization system.

Bile acids exert their effects through several receptors, including the core receptors vitamin D receptor (VDR), farnesoid X receptor (FXR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), liver X receptor (LXR), and the Takeda G-protein coupled receptor (TGR5) membrane receptor. Peripherally expressed secondary bile acids can exert hormone-like effects through these receptors. Among other things, they can influence cellular redox status, immune responses, gastrointestinal mucosal barrier function, and tumor development and progression.

Objectives

Bacterial metabolites, produced by the gut microbiota, play an important role in the maintenance of host homeostasis and the regulation of many life processes. Secondary bile acids, LCA and DCA, synthesized by the gut microbiota, are able to reach distant tumor cells via the circulatory system and influence tumor cell behavior. In the studies on which my dissertation is based, we aimed to highlight the close relationship between the microbiome and breast cancers and between the microbiome and pancreatic adenocarcinoma.

The main objectives of our studies were to:

Study the effects of lithocholic acid in breast cancers.

- Identify the tumor cell-specific processes through which LCA exerts its effects.
- Investigate whether the effect of LCA can be demonstrated in a mouse model of tumor.
- Identify the receptors through which LCA exerts its effects on tumor cells.
- Identify associations between disease progression, patient survival and bile acid-induced effects.

Study the effects of deoxycholic acid in pancreatic cancers.

- Determine how DCA affects the levels of oxidative and nitrosative stress in cells.
- Investigate how DCA affects EMT processes.
- Determine the effect of DCA on the stem cell function of tumor cells.
- Determine how DCA affects cellular energy balance.

Materials and methods

Cell lines

Cell cultures were grown in a thermostat containing 5% carbon dioxide at 37°C. We used 4T1 mouse breast cancer, MCF7 human breast cancer, SKBR human breast cancer, CAPAN-2 human pancreatic adenocarcinoma, BxPC-3 human pancreatic adenocarcinoma and human primary fibroblast. The composition of the culture media is shown in Table 1.

Table.1 Composition of cell culture media.

Cell line	Medium	Distributor	FBS	Penicillin/ Streptomycin	L-glutamine	Pyruvate	HEPES
4T1	RPMI-1640	Sigma-Aldrich, R5886	10%	1%	2 mM	1%	-
BxPC-3	RPMI-1640	Sigma-Aldrich, R5886	10%	1%	2 mM	-	-
MCF7	MEM	Sigma-Aldrich, R8042	10%	1%	2 mM	-	-
CAPAN-2	MEM	Sigma-Aldrich, R8042	10%	1%	2 mM	-	-
Primer fibroblaszt	DMEM low glucose	Sigma-Aldrich, D5523	10%	1%	2 mM	-	-
SKBR	DMEM low glucose	Sigma-Aldrich, D5523	20%	1%	2 mM	-	10 mM

Materials

The substances used for our experiments and their concentrations for the treatments are listed in Table 2.

Table 2. List of materials used for treatments.

Material	Cat. number	Distributor	Concentration
CA	C1129	Sigma-Aldrich	0,01-10 μM
CDCA	C9377	Sigma-Aldrich	0,01-10 μM
CINPA1	HY-110249	MedChemExpress	5 μM
DCA	D2510	Sigma-Aldrich	0,7 μM
DY268	HY-110267	MedChemExpress	5 μM
GSH	G4251	Sigma-Aldrich	5 mM
GSK2033	HY-108688	Sigma-Aldrich	5 μM
LCA	L6250	Sigma-Aldrich	0,1-1 μM
MG-132	474790	Calbiochem	50, 100 mM
NAC	A7250	Sigma-Aldrich	5 mM
NF449	480420	Sigma-Aldrich	5 μM
RA839	5707	Tocris Bioscience	5, 10 μM
siCAR	#1 s19369 #2 s19370 #3 s19368	Thermo Fisher Scientific	30 nM
siNEG	4390843	Thermo Fisher Scientific	30 nM
siNRF2	#1 s9493 #2 s9492 #3 s9491	Thermo Fisher Scientific	30 nM
siPXR	s16910	Thermo Fisher Scientific	30 nM
siTGR5	#1 s195791 #2 s45559 #3 s45558	Thermo Fisher Scientific	30 nM
siVDR	s14777	Thermo Fisher Scientific	30 nM
tBHQ	112941	Sigma-Aldrich	5, 10 μM

Cell proliferation assay

For cell proliferation assay, sulforhodamine B (SRB; Sigma-Aldrich, 230162) staining was used. Cells were plated in 96-well culture dishes (4T1-1500 cells/well) overnight. Cells were then treated for 48 h and fixed with trichloroacetic acid (TCA; Sigma-Aldrich, T6399) at 10% final concentration for 1 h at 4°C. The wells were then washed five times with distilled water and stained with 0.4% (w/v) SRB stain dissolved in 1% acetic acid for 10 min. The cells were then washed five times in succession with 1% acetic acid to remove unbound dye. The bound dye was dissolved in 10 mM Tris-BASE solution. The absorbance was recorded using a spectrophotometer (Thermo Labsystems Multiskan MS, Walthman, MA, USA) at 540 nm wavelength.

SDS-PAGE and Western blot

The protein isolation process was performed on ice throughout to preserve protein quality. After each treatment, cells were washed with 1x PBS solution. Cells were digested in cold RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% TritonX 100, 0.5% sodium deoxycholate, 1 mM EDTA, 1

mM Na3VO4, 1 mM PMSF, 1 mM NaF, protease inhibitor cocktail), during which the samples were sonicated on ice for three 10 s runs on setting 2 (Qsonica Q125 Sonicator, Newtown, Connecticut, USA), cooled for proper digestion. Protein concentration of samples was measured using a PierceTM BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA, 23225). Samples were then added to 5x SDS sample buffer (50% glycerol, 10% SDS, 310 mM Tris-HCl, pH 6.8, 100 mM DTT, 0.01% bromophenol blue) and β-mercaptoethanol.

Protein samples (20 µg) were separated by molecular weight on SDS polyacrylamide gels (8 or 10%). The separated samples were then transferred onto nitrocellulose membrane. The aspecific binding sites on the nitrocellulose membrane were blocked with BSA dissolved in 5% 1x TBS-Tween buffer for 1 h at room temperature. Primary antibodies were diluted in 2.5% BSA (prepared in 1x TBS-Tween) and the membrane was incubated overnight. The membrane was then washed three times for 10 minutes in 1x TBS-Tween buffer. The peroxidase-labelled secondary antibodies were left on the membrane for 1 hour diluted in 2,5 % BSA (prepared in 1 TBS-Tween). The membranes were then washed three times for 10 min in 1x TBS-Tween buffer. Antibody-labeled protein bands were visualized by chemiluminescence reaction (SuperSignal West Pico Solutions, Thermo Fisher Scientific, 35060). Detection was performed using ChemiDoc Touch Imaging gel documentation system (Bio-Rad, Hercules, CA, USA). Image Lab 6.1 software (Bio-Rad) was used for band densitometry. The antibodies used for the assays are listed in Table 3.

Table 3. Antibodies used for the Western blot method.

Antibody	Dilution	Distributor/Cat. number
4HNE	1: 1000	Abcam (ab46545)
ALDH1	1: 1000	Abcam (ab227948)
Anti-mouse IgG, Peroxidase linked	1: 2000	Sigma-Aldrich (A9044)
CAR	1: 1000	Abcam (ab186869)
CLAUDIN-1	1: 1000	Cell Signaling (13255)
E-CADHERIN	1: 1000	Cell Signaling (3195)
GPX3	1: 1000	Abcam (ab104448)
iNOS	1: 1000	Novus (NB300-605)
KEAP1	1: 1000	Cell Signaling (8047)
Nitrotirozin	1: 1000	Millipore (06-284)
Nitrotirozin	1: 1000	Thermo Fisher (A21285)
NRF2	1: 1000	Abcam (ab31163)
NRF2	1: 1000	Novus (NBP1-32822)
SLUG	1: 1000	Cell Signaling (9585)
SNAIL	1: 1000	Cell Signaling (3879)
TGR5/GPBAR1	1: 1000	Novus (NBP2-23669)
ZO-1	1: 1000	Cell Signaling (8193)
β-actin	1:20000	Sigma-Aldrich (A3854)
β-CATENIN	1: 1000	Cell Signaling (8480)

RNA isolation, reverse transcription and RT-qPCR

The RNA isolation process was performed on ice throughout to preserve nucleic acid quality. Cells were cultured in 6-well culture dishes (CAPAN-2 - 10,000 cells/well, 4T1 - 5,000 cells/well) overnight. Treatments were continued for 48 h, and total RNA was isolated from the cells using TRIzol reagent (Invitrogen Corporation, 15596026) according to the manufacturer's instructions. Tissue samples from primary tumors removed during animal experiments were disrupted in TRIzol reagent using a ball homogenizer (Qiagen Tissuelyser II; Qiagen, Mexico City, Mexico) with continuous cooling. DNA contamination was removed from RNA samples using DNase treatment (Sigma-Aldrich, 10104159001). Sample concentration and quality were determined using a NanoDrop1000 instrument (Thermo Labsystems Multiskan MS, Walthman, MA, USA). For reverse transcription, 2 µg of RNA samples were transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA, 4368814) according to the manufacturer's instructions. The qPCR reaction was performed in a final volume of 10 µl. The reaction mixture contained 0.5 µM of primers, 20 ng of cDNA sample and qPCRBIO SyGreen Lo-ROX Supermix (PCR Biosystems Ltd., London, UK, PB20.11-01). Real-time quantitative PCR was measured on a LightCycler 480 II (Roche Applied Science, Basel, Switzerland). For normalization, a normalization factor calculated from the geometric mean of the Cp values of the 36B4 and cyclophilin housekeeping

genes was used. The relative expression of the genes tested in the samples was $2^{-\Delta\Delta CP}$ method. Sequences of primers used for real-time quantitative PCR are listed in Table 4.

Table 4. Sequences of primers used for real-time qPCR.

Mouse primers		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
36B4	AGATTCGGGATATGCTGTTGG	AAAGCCTGGAAGAAGGAGGTC
CAT	CCTTCAAGTTGGTTAATGCAGA	CAAGTTTTTGATGCCCTGGT
GCLC	GATTCGGGATGGGCAACT	AAAGGTATCTTGCCTCAGATATGC
GPX2	GTTCTCGGCTTCCCTTGC	TTCAGGATCTCCTCGTTCTGA
GPX3	GGCTTCCCTTCCAACCAA	CCCACCTGGTCGAACATACT
HMOX1	AGGCTAAGACCGCCTTCCT	TGTGTTCCTCTGTCAGCATCA
iNOS	GAAGTGCAAAGTCTCAGACATGG	GATTCTGGAACATTCTGTGCTGTC
NOX4	GCAGATTTACTCTGTGTGTTGCAT	TCCCATCTGTTTGACTGAGGT
NQO1	AGCGTTCGGTATTACGATCC	AGTACAATCAGGGCTCTTCTCG
NRF2	CATCAGGCCCAGTCCCTCAAT	CAGCGGTAGTATACAGCCAGCT
SOD1	CCATCAGTATGGGGACAATACA	GGTCTCCAACATGCCTCTCT
SOD2	TGCTCTAATCAGGACCCATTG	GTAGTAAGCGTGCTCCCACAC
SOD3	CTCTTGGGAGAGCCTGACA	GCCAGTAGCAAGCCGTAGAA

Human primers		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
36B4	CCA TTG AAA TCC TGA GTG ATGTG	GTC GAA CAC CTG CTG GAT GAC
CYCLOPHILIN	GTC TCC TTT GAG CTG TTT GCA GAC	CTT GCC ACC AGT GCC ATT ATG
SNAIL	GCT GCA GGA CTC TAA TCC AGA	ATC TCC GGA GGT GGG ATG
TCF7L2	ACG TAC AGC AAT GAA CAC TTCAC	GGC GAT AGT GGG TAA TAC GG
TWIST1	GGG CCG GAG ACC TAG ATG	TTT CCA AGA AAA TCT TTG GCATA
WNT5B	CGG GAG CGA GAG AAG AAC T	CGT CTG CCA TCT TAT ACA CAGC
β-CATENIN	TGT TAA ATT CTT GGC TAT TACGACA	CCA CCA CTA GCC AGT ATG ATGA

Investigating cell invasion

Cell invasion assays were performed using a Corning BioCoat Matrigel Invasion Chamber (Corning, NY, USA 354480, 354481) equipped with an 8 μm PET membrane. In the upper chamber, CAPAN-2 cells (20 000 cells/well) were cultured overnight in serum-free medium. The next day, cells in the upper chamber were treated with DCA (0.7 μM) in serum-free medium, while the lower chambers were filled with medium containing 10% FBS, DCA (0.7 μM) and stromal cell-derived factor-1 (SDF1-a) chemoattractant (final concentration: 100 ng/ml, Sigma-Aldrich; SRP4388). After

48 h, the non-migrated cells remaining on the upper part of the membrane were removed by washing with cotton wool and repeated washes with PBS. Cells that had migrated to the lower part of the membrane were fixed with 100% methanol. After drying, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) stain and the membrane was mounted on a slide. Migrating nuclei were counted using the Opera Phoenix High Content Screening System and images were analyzed using Harmony 4.6 software. The invasion index was calculated as the ratio of cells migrating across the matrigel-containing membrane to cells migrating across the control membrane as follows:

- **Invasion %**

= (mean of cells migrating through matrigel membrane / mean of cells migrating through control membrane) *100

- **Invasion index**

= Invasion % treated cells / Invasion % control (untreated) cells

Lipid peroxidation test

The accumulation of reactive radicals (ROS/ RNI) in cells due to increased oxidative and nitrosative stress induces the peroxidation of polyunsaturated lipids, resulting in the formation of reactive electrophilic aldehydes such as 4-hydroxyquinonenal (4HNE) or malondialdehyde (MDA). These reactive molecules can form stable covalent bonds with the lysine, cysteine and histidine side chains of proteins and thus serve as good biomarkers for the detection of oxidative processes in cells. In our studies, the extent of lipid peroxidation was determined by Western blot analysis of 4HNE-protein adducts and by determination of thiobarbituric acid reactive substances (TBARS) levels. MDA reacts with thiobarbituric acid (TBA) to give a colored reaction product which can be used to assess the extent of lipid peroxidation. For TBARS assay, 4T1 cells were grown in T150 culture flasks overnight. After 48 h of treatment, cells were washed with 1x PBS and collected in Eppendorf tubes. After centrifugation, 8.1% SDS, 20% acetic acid, 0.8% TBA (Sigma-Aldrich, T5500) and distilled water were added to the cell pellet and incubated at 96°C for 1 hour. After centrifugation, the absorbance of the supernatant was measured with a spectrophotometer (Thermo Labsystems Multiskan MS) at 540 nm wavelength.

Aldehyde dehydrogenase positivity test

The amount of aldehyde dehydrogenase-1 (ALDH1) positive cells was measured in response to treatments using Aldefluor stem cell kit (StemCell Technologies, Vancouver, Canada, 01700). CAPAN-2 cells were cultured overnight in 6-well culture dishes (100 000 cells/well) and then treated with DCA (0.7 μM) for 48 h. After treatment, the cells were incubated in Aldefluor assay buffer

containing 0.5 ml ALDH1 substrate (5 µl/ml) for 45 min at 37 °C. Cells used as negative controls were treated with diethylaminobenzaldehyde (DEAB; 50 mmol/l), known as an inhibitor of ALDH1. The percentage of ALDH1 positive cells in the samples was determined by flow cytometry. Results were analyzed using Flowing Software 2.5.1.

Determination of mitochondrial oxygen consumption and extracellular acidification

The oxygen consumption rate (OCR) (mitochondrial oxidation rate) and the rate of pH change, extracellular acidification rate (ECAR) (glycolytic flux rate) of CAPAN-2 cells were determined using a Seahorse XF96 oximeter (Agilent Technologies, Santa Clara, CA, USA). CAPAN-2 cells were cultured overnight in 96-well Seahorse culture vessels (5000 cells/well) and treated with DCA (0.7 µM) for 48 h. Following treatment, cells were incubated in pre-warmed XF test medium for 1 hour at 37°C, CO_2-free incubator. Baseline OCR was recorded five times for 5 min. Then, etomoxir (50 µM), oligomycin (10 µM) and antimycin (10 µM) were added to the cells. OCR was measured 5 times for 5 min after each addition. Finally, data were analyzed normalized to protein content. SRB staining was used to measure protein content as previously described.

Animal experiments

The animal experiments were performed under a permit (1/2015/DEMÁB) approved and registered by the University of Debrecen Workplace Animal Experimentation Committee. Total RNA was isolated from primary tumors from our previous experiments according to the 3R principle. In this experiment, we inoculated 4T1 breast cancer cells into female Balb/c mice and examined the effect of LCA on the growth and behavior of the resulting tumor. The mice were cultured in the "SPF" (specific pathogen free) section of the Experimental Animal Facility of the Centre for Life Sciences, University of Debrecen. Our treatments were performed in the "MD" (minimal disease) section of the same animal house. Mice were housed in standard size cages (65 × 207 × 140 mm, 530 cm2 area; Techniplast, 1284 L Eurostandard Type II. L) according to the specifications, in Lignocel Select Fine (J. Rettenmaier und Söhne) litter. A maximum of six animals per cage was used. Mice were allowed unrestricted access to food and drinking water (sterilized tap water). Light and dark cycles were alternated every 12 hours. The animal house temperature was 22 ± 1 °C. The animals were provided with clean cages and bedding weekly. All animals received humane treatment according to the guidelines and were cared for by qualified staff under veterinary supervision. A total of 10 animals were used for the study. Female Balb/c mice 8-10 weeks old (20-25 g) were used for our experiments.

For xenograft formation, 4T1 cells were suspended in cold PBS (2×10^6/ml), followed by a 1:1 suspension of the cell suspension and cold Matrigel (Sigma-Aldrich; E1270). Mice were injected with

50 µl (10^5 cells/nipple) of this suspension under their second inguinal breast bud using a 50 µl needle. The condition of the mice and the growth rate of the tumors were monitored daily. LCA for treatment was stored in 96% ethanol at a concentration of 7.5 mM at -20°C. The treatment volume and the substrate used as a control sample were diluted 1x each day in PBS to the appropriate concentration (200 µl/30 ttg). Mice were treated "per os" between 8:00 and 10:00 h each day. On day 18 after inoculation, mice were killed by cervical dislocation and primary tumors and possible metastases were removed.

Determination of total antioxidant capacity

One method for the determination of total antioxidant capacity is based on the oxidation of the compound 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS). The green-colored ABTS cation radical, prepared from ABTS with potassium persulphate, is colored by reaction with the antioxidants in the sample. The degree of discoloration is directly proportional to the number of radicals reacted, and thus the antioxidant content of the sample can be determined. For the preparation of ABTS radicals, a 10% solution of potassium persulphate (24,5 mM) was added to the ABTS solution (7,4 mM) the night before the test. Before the experiment, the absorbance of the ABTS solution was adjusted to 1,2 with 50 mM Gly-HCl solution, pH = 4,5. 50 µl of ABTS solution was added to 5 µl of the sample to be tested. The mixture was then incubated for 30 min at room temperature. The absorbance of the samples was recorded at 405 nm using a Tecan Spark multi-label reader. Antioxidant activity was expressed as a percentage of control samples. Ascorbic acid concentration series was used as a positive control.

Tissue microarray and immunohistochemistry

To investigate the expression of 4HNE, iNOS and TGR5 observed in tumor tissue, we performed tissue microarray (TMA) using archived tissue blocks from 88 breast cancer patients, on which immunohistochemistry was performed. After surgical removal of breast cancer tissues, they were fixed in formalin and embedded in paraffin. These tissue microblots were used for the study. Three technical duplicates per tissue block were prepared for the study. The Leica Bond Max™ protocol was used for immunohistochemistry. The antibodies and conditions used for immunohistochemistry are summarized in Table 5. Sections were scored after immunohistochemical staining using an "H-score" scoring system that considers the intensity and percentage of staining.

Table 5. Antibodies used for immunohistochemistry.

Antibody	Distributor	Antigen retrieval	Dilution	Detecting
iNOS	Thermo Fisher Scientific (PA5-16855)	Ventana BenchMark ULTRA/Roche Cell Conditioning 1 (CC1) 20 min, 95°C	1:100	UltraView Universal DAB Detection kit/Roche
4HNE	Abcam (ab46545)	Ventana BenchMark ULTRA/Roche Cell Conditioning 1 (CC1) 20 min, 95°C	1:1000	UltraView Universal DAB Detection kit/Roche
TGR5	GeneTEX (GTX100026)	pressure cooker (Avair) 1m citrate puffer, pH=6	1:1000	EnVision Flex (K8000, Dako, Santa Clara, CA, USA)

In silico tests

The correlation between survival time in breast cancer patients and the expression of the genes of interest (CAR, TGR5, NRF2, KEAP1, iNOS, nNOS(a), nNOS(b), NOX4) was analyzed using the Kaplan-Meier plotter database (https://kmplot.com/analysis/).

Statistical analysis

All experiments were performed at least three independent times. GraphPad Prism 8.0.1 was used for statistical evaluation of the data. To achieve a normal distribution, fold change data were log2 transformed. Student's t-test was used to compare two groups. For comparisons of multiple groups, we used one-way ANOVA statistical test followed by Tukey's posthoc test or Dunnett's posthoc test. Data are presented as mean ± SEM. In our statistical analyses

p <0,05 was considered as a significant difference. Correlation tests were performed using Pearson correlation test and linear regression. Before analysis, the mitotic index was log2 transformed. Calculations were performed using R project (version 3.5.2).

Results

Effect of lithocholic acid in breast cancer

The antiproliferative effect of lithocholic acid is mediated through inhibition of NRF2

In our experiments, we first investigated whether LCA affects the expression of key elements of the NRF2/ KEAP1 antioxidant pathway. The concentrations of LCA used in our experiments corresponded to normal human LCA concentrations (0.1-1 μM) measured in human breast tissue. We showed that in 4T1 mouse breast cancer cells, LCA treatment decreased the level of NRF2 protein and in parallel increased the expression of KEAP1 protein, which inhibits NRF2. In our previous study, we showed that LCA reduces breast cancer cell proliferation. In parallel, we found that CA and CDCA primary bile acids do not affect breast cancer cell proliferation.

We wondered whether the inhibitory effect of LCA on proliferation could be reversed by pharmacological activation of NRF2. We treated 4T1 cells with LCA in combination with an NRF2 activator, RA839, and found that activation of NRF2 abolished the antiproliferative effect of LCA. The efficacy of RA839 was verified by measuring mRNA expression of NRF2 target genes. RA839 treatment increased the mRNA expression of the NAD(P)H quinone dehydrogenase 1 (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC), catalase (CAT) and hemoxygenase 1 (HMOX1) genes.

Lithocholic acid induces oxidative stress via inhibition of NRF2, leading to cytostasis

In the antioxidant and prooxidant assays, we observed that the protein level of another antioxidant, glutathione peroxidase 3 (GPX3), was also decreased, while the mRNA expression of the prooxidant NADPH oxidase 4 (NOX4) and the inducible NO synthase (iNOS) protein level were increased by LCA treatment. In addition, LCA enhanced lipid peroxidation in 4T1 cells, as indicated by elevated levels of thiobarbituric acid reactive substances (TBARS) and 4HNE adducts. Furthermore, we showed that nitrotyrosine levels were elevated in LCA-treated 4T1 cells, indicating increased nitrosative stress. We wondered whether LCA-induced oxidative-nitrosative stress is NRF2-dependent. We showed that pharmacological activation of NRF2 prevented the increase of both TBARS, 4HNE, and iNOS protein levels in LCA combined treatment. We treated 4T1 cells with thiol antioxidants in the presence of LCA to determine whether LCA-induced oxidative stress plays a role in the antiproliferative effect of LCA. We found that both glutathione (GSH) and N-acetylcysteine (NAC) inhibited the LCA-induced antiproliferative effect. Furthermore, we also showed that LCA alone had no rooting effect at the concentration used in our study (0.1-1 μM), nor at higher

concentrations (up to 300 μM) where ascorbic acid acted as an antioxidant. Our results were also confirmed in MCF7 and SKBR-3 human breast carcinoma cells. We observed that LCA had no effect in untransformed human fibroblast cells, indicating that the effects of LCA are breast cancer specific. From these results, we concluded that LCA is able to enhance oxidative-nitrosative stress processes through inhibition of NRF2, which induces cytostasis in breast cancer cells.

Oxidative stress induced by lytocholic acid is mediated by TGR5 and CAR receptors

In the following, we wanted to identify the receptors through which LCA can exert its effects. We have used pharmacological agents to inhibit these receptors, as well as siRNA depletion experiments in treatments combined with LCA. Our results showed that the LCA-induced decrease in NRF2 protein levels was prevented by NF449 inhibitors of the TGR5 pathway and CINPA1 inhibitors of CAR, whereas the LXR inhibitor GSK2033 and FXR inhibitor DY268 inhibitors were ineffective. To obtain a more comprehensive picture of TGR5, CAR, VDR and PXR receptors, we silenced them using siRNA technique in MCF7 human breast cancer cell line. As with pharmacological agents, silencing of TGR5 and CAR receptors effectively blocked the LCA-induced decrease in NRF2 protein levels, and silencing of both TGR5 and CAR receptors blunted LCA-induced elevated iNOS protein expression. These results indicate that LCA exerts its effects on oxidative stress in breast cancer cells through TGR5 and CAR receptors.

LCA inhibited antioxidant defense in a mouse model of breast cancer

Next, we investigated whether LCA affects the redox status of tumors *in vivo* environment. Balb/c female mice inoculated with 4T1 mouse breast cancer cells, were orally treated with 15 nmol LCA daily, and at the end of the study, the mice were sacrificed, and the tumors were harvested. We measured mRNA expression of anti- and pro-oxidant genes in tumors from control and LCA-treated mice. LCA significantly decreased mRNA levels of the antioxidant genes NRF2, CAT, HMOX1, SOD1 and SOD2. Furthermore, the expression of the prooxidant iNOS and NOX4 was increased in LCA-treated mice, but this increase was not significant. These results suggest that LCA can also affect the redox balance of tumor cells, which may have a beneficial effect on disease outcome.

Elements of the lytocholic acid-induced antitumor pathway showed correlation with disease stage, grade and receptor status

Tissue microarray (TMA) was used to investigate the expression of LCA-induced oxidative/nitrosative stress markers in tumor samples from 88 breast cancer patients and *in silico* examined the impact of gene expression in the LCA-TGR5 pathway on patient survival. In our studies,

we found that iNOS and 4HNE levels were decreased in stage II and III patients compared to stage I patients, and further decreased in stage IV patients. Then, when we classified patients according to pathological grade of disease, we observed that 4HNE expression was significantly decreased in stage 2 and 3 patients compared to stage 1 patients. Consistent with this, high expression of KEAP1 correlated with better survival in patients with grade 2, while high expression of CAR correlated with better survival in patients with grade 3.

Based on immunohistochemical classification of patients, we found that the expression of TGR5, iNOS and 4HNE was decreased in TNBC cases compared to ER+ cases. Consistent with this *in silico* studies, we found that high expression of CAR, KEAP1, iNOS, nNOS, NOX4 and low expression of NRF2 were associated with better patient survival when all patients or ER+ positive cases were considered. This was not true for TNBC cases. Finally, patients were also classified according to the cell division index (mitosis score). Staining for 4HNE, a direct indicator of tissue oxidative damage, decreased with increasing cell division index, and 4HNE staining showed a strong negative correlation with mitotic index. Overall, overexpression of elements of the LCA-TGR5 oxidative stress pathway conferred better survival in breast cancer patients. The LCA-TGR5-oxidative stress pathway may have a protective function in breast cancer, loss of genes in this pathway is associated with poor clinical prognosis.

Effect of deoxycholic acid on pancreatic adenocarcinoma cells

Deoxycholic acid decreases the expression of genes involved in the EMT process and the invasion ability of cells

To investigate the effects of DCA, we first examined the expression of epithelial and mesenchymal marker genes involved in the EMT process at the mRNA and protein levels upon DCA treatment. DCA treatment decreased the mRNA expression of mesenchymal genes TCF7L2, WNT5B, B-CATENIN, TWIST1 and SNAIL. DCA significantly decreased SLUG protein levels in the human pancreatic adenocarcinoma cell line BxPC-3. Examination of epithelial markers revealed that DCA treatment significantly increased the levels of ZO1 and E-CADHERIN protein in BxPC-3 cells, and CLAUDIN-1 epithelial adhesion protein, whose decreased expression is associated with improved survival, showed decreased expression upon DCA treatment in CAPAN-2 cells.

To detect effects on cell invasion, a matrigel invasion chamber was used. DCA treatment significantly reduced the invasion ability of CAPAN-2 cells compared to the control sample. These results indicate that DCA may inhibit the EMT process in pancreatic adenocarcinoma cells through reducing the expression of genes key in the EMT process and the invasion ability of cells.

Deoxycholic acid induces oxidative-nitrosative stress in pancreatic adenocarcinoma cells

To study the redox status of cells, we monitored the expression of markers of oxidative-nitrosative stress. The level of iNOS protein, responsible for NO production, was increased in CAPAN-2 and BxPC-3 cells after DCA treatment. In addition, elevated nitrotyrosine and 4HNE levels were observed after DCA treatment. Based on these results, we believe that DCA increases the levels of oxidative and nitrosative stress in pancreatic cancers, as indicated by elevated lipid peroxidation.

Deoxycholic acid increases mitochondrial activity in pancreatic adenocarcinoma cells

Seahorse analysis allowed us to simultaneously determine the extent of mitochondrial oxidation and glycolysis in CAPAN-2 cells. We showed that DCA treatment simultaneously enhanced mitochondrial oxidation (OCR) and glycolysis (ECAR). At the same time, the OCR/ECAR ratio was not altered, suggesting that cells become hypermetabolic following DCA treatment.

Deoxycholic acid reduces stem cell proliferation

ALDH1 is a stem cell marker in many tumors, including pancreatic adenocarcinoma. Following DCA treatment, we measured ALDH1 activity using Aldefluor assay and found that DCA significantly reduced the number of ALDH1 positive CAPAN-2 cells. Furthermore, we showed that DCA also reduced ALDH1 protein levels in cells. These results suggest that DCA may reduce the stemness of pancreatic adenocarcinoma cells.

Discussion

Now we have a large body of data showing that pathological processes during dysbiosis of the microbiome are associated with tumorigenesis and tumor progression. In addition to the direct immunogenic effect of the microbiome, the importance of the endocrine-like function of the microbiome has been described in several cancers, including breast and pancreatic adenocarcinoma. The microbiome, located in the gastrointestinal region, produces microbial metabolites during its metabolism, which can influence the properties of breast and pancreatic cancer cells. Several metabolites have been identified that are able to influence tumor cell properties by exerting pro- or anticarcinogenic effects. In breast cancers, several studies have shown that LCA, cadaverine, indoxyl sulphate, and indolepropionic acid, as bacterial metabolites, were able to induce antineoplastic effects. In addition, studies on the relationship between pancreatic adenocarcinoma and microbial metabolites demonstrated that secondary bile acids formed during microbiome metabolism, such as LCA, DCA, and UDCA, had cytostatic effects in pancreatic adenocarcinoma. These observations suggest that alterations in the diversity of the microbiome may be associated with the development and progression of breast and pancreatic cancers.

Role of lithocholic acid in breast cancers

Our studies have shown that LCA-induced oxidative stress is mediated through a decrease in NRF2 expression and an increase in the expression of various prooxidant proteins. In other words, the increase in oxidative and nitrosative stress induced by LCA results from a shift in the balance between prooxidant and antioxidant systems. The increased production of ROS and RNI has a primary role in slowing down the proliferation of breast cancer cells. In breast cancers, it has been described that LCA can inhibit the EMT process and influence tumor cell metabolism through oxidative stress-mediated pathways.

In our own experiments, we have demonstrated that LCA-induced elevated oxidative stress leads to inhibition of breast cancer cell growth, but we have not been able to demonstrate that elevated oxidative stress leads to EMT inhibition and metabolic rearrangement. One possible reason for this is that the increase in the amount of free radicals in the model we used was lower than in the study. We have shown that the oxidative stress responses induced by LCA are mediated by TGR5 and CAR receptors in breast cancers.

The role of oxidative stress in breast cancer is complex, as sustained increased oxidative stress is procarcinogenic, whereas moderate oxidative stress exerts a cytostatic effect. In our study, oxidative stress markers and higher expression of prooxidant genes were associated with low stage, low grade

and non-TNBC cases. However, later stage, higher grade and TNBC cases showed decreased expression of prooxidant genes and oxidative stress markers. These results suggest that the LCA-TGR5-oxidative stress pathway has a protective function in breast cancer, and loss of genes in this pathway is associated with poor clinical prognosis. Consistent with this observation, increased CAR expression was associated with better survival, which was not observed in TNBC cases. Our results support the beneficial cytostatic effect of oxidative stress. Our own results correlate well with those of another study, which found that increased oxidative stress due to DNA damage and the resulting accumulation of mutations is a risk factor for breast cancer, whereas increased lipid peroxidation correlates with longer survival. In breast cancer patients, high expression of NRF2 is an unfavorable prognostic factor for tumor recurrence and disease-free survival. There are nucleotide polymorphisms in the NRF2 and KEAP1 genes that may affect protein expression. Polymorphisms with higher NRF2 or lower KEAP1 expression are associated with worse clinical outcome, and low NRF2 expression levels have been shown to enhance the efficacy of chemotherapeutic agents.

Our analysis shows that signaling through estrogen and HER2 affects the activity of LCA-induced pathways. We observed higher expression levels of TGR5, iNOS and 4HNE in ER+/HER2 and HER 2 cases compared to TNBC cases. This trend is similar to the findings in tracer-amine-associated receptors (TAAR) identified as cadaverine receptors. Although the molecular mechanism for the enhanced efficacy of LCA in ER+/HER2 tumors has not been elucidated, other studies have supported the importance of the HER2 signaling pathway, for example, by finding that HER2 signaling induces iNOS expression and reduces cell proliferation.

The diversity of the gastrointestinal microbiome and, in parallel, the serum levels of cytostatic LCA are also reduced in breast cancer. Low serum concentrations of LCA correlate well with higher proliferation rates of tumor cells. In addition, we have shown that primary bile acids such as CA and CDCA were less efficient than LCA in inducing cytostasis in breast cancers. Based on the above data, it can be concluded that a decrease in the biomass of the microbiome leads to a decrease in the amount of LCA, thus reducing the efficiency of the cytostatic processes exerted by LCA.

In our previous study, we have shown that LCA reduces the aggressiveness of breast cancer cells via the TGR5 receptor. In the present study, we found that LCA is a physiological ligand of TGR5 in healthy individuals and that its expression and activation may be a protective factor in breast cancer patients. We also identified the CAR receptor as an alternative receptor mediating the effects of LCA. Similar to the high expression levels of TGR5, elevated expression of CAR was associated with better survival.

Our present results demonstrate that the microbiome metabolite LCA induces secondary bile acid oxidative and nitrosative stress by disrupting the balance between pro- and antioxidant systems in

breast cancer patients. LCA is capable of exerting hormone-like effects by affecting the properties of breast cancers distant from the site of production. We found that lower expression and activity of participants in the LCA-TGR5 signaling pathway is associated with poorer clinical outcome in breast cancer. These findings suggest that targeting the TGR5/CAR signaling pathway and oxidative stress may open new opportunities for the treatment of breast cancers.

Role of deoxycholic acid in pancreatic cancers

In the second part of my dissertation, we investigated how DCA, when administered at a reference concentration of serum (0.7 μM), can affect tumor cell characteristics in human pancreatic adenocarcinoma cells. In our experiments, we have shown that DCA treatment inhibits EMT processes, reduces tumor cell stem cell proliferation, increases oxidative and nitrosative stress levels, and renders tumor cells hypermetabolic in human pancreatic adenocarcinoma cells. These effects include both pro- and anticarcinogenic effects. During the Warburg-type metabolic rearrangement that characterises tumor cells, tumor cells become hypermetabolic and prefer glycolysis-based energy production even in the presence of sufficient oxygen. DCA induced mitochondrial oxidation, including all elements of cellular respiration such as oligomycin-sensitive and resistant respiration, etomoxir-sensitive and resistant respiration, and glycolytic flux. Nevertheless, the relative ratios of mitochondrial oxidation and glycolysis were not altered by DCA treatment, suggesting that cells became hypermetabolic, but neither mitochondrial oxidation nor glycolysis processes were dominant. Increased mitochondrial oxidation and glycolytic activity in pancreatic adenocarcinoma may be associated with the development of chemoresistance and tumor cell survival.

In our further studies, we found that DCA decreased the expression of markers of mesenchymal morphology and concomitantly increased the expression level of markers of epithelial morphology. In this context, we found that the tumor stem cell marker ALDH1 showed decreased expression following DCA treatment. From these results, we concluded that DCA is able to inhibit EMT processes and reduce stem cell proliferation in pancreatic adenocarcinoma cells.

Several microbial metabolites have been shown to exert antineoplastic effects through the induction of oxidative and nitrosative stress. In our experiments, we observed that DCA increased the production of 4HNE and increased nitrotyrosine, which suggest that oxidative and nitrosative stress levels in cells are elevated.

The results of our experiments show that DCA can induce both pro- and antineoplastic effects. Furthermore, we found that DCA retains its biological activity at sub-micromolar concentrations, although the effects are smaller compared to those of high micromolar concentrations shown by previous studies. Based on the foregoing, we believe that DCA, a metabolite of the microbiome, has

the potential to modulate tumor cell characteristics and thereby influence the progression of pancreatic adenocarcinoma, thus creating an opportunity for the development of new therapeutic approaches.

Summary

The human body contains many microorganisms, the genome of which is called the human microbiome. The diversity of the microbiome is strongly influenced by various external (nutrition, hygiene, stress) and internal (age, genetics) factors. At the same time, the microbiome synthesizes several metabolites during its metabolism, which can affect the energy balance of the host and the functioning of its immune system. Changes in the composition of the microbiome are associated with several pathological processes, including the development of neoplastic diseases.

Secondary bile acids formed by the gastrointestinal microbiome, as microbial metabolites, can reach distant sites in the body via the systemic circulation and modulate the properties of tumor cells through hormonal effects. In this way, secondary bile acids can establish a link between the microbiome and tumors.

By investigating the effects of lithocholic acid (LCA) secondary bile acid in breast tumor cells, we found that LCA can mediate its effects through TGR5 or CAR-mediated signaling pathways to modulate tumor cell effects. We showed that LCA enhanced oxidative nitrosative stress levels in breast tumor cells through inhibition of NRF2. In TMA assays, we observed that patients with early-stage breast tumors had increased expression of LCA-induced TGR5-NRF2 oxidative/nitrosative stress pathway participants compared to late-stage patients, which are associated with a worse prognosis.

Examining the secondary bile acid effects of deoxycholic acid (DCA) in pancreatic tumor cells, we found that DCA inhibited the EMT process by decreasing mesenchymal marker gene expression and increasing epithelial marker gene expression, and that DCA reduced the migratory ability of tumor cells. DCA also reduced the expression of the stem cell marker ALDH1 and induced hypermetabolism of pancreatic tumor cells.

Our observations so far have shown that different secondary bile acids act differently in different tumors. LCA exerts antineoplastic effects in mammary and pancreatic tumors, but the effect is mediated through different receptors. DCA has no effect in breast tumors, whereas a mixed effect in pancreatic tumors was detected.

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Candidate: Patrik Kovács
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. Schwarcz, S., **Kovács, P.**, Kovács, T., Ujlaki, G., Nyerges, P., Uray, K., Bai, P., Mikó, E.: The pro- and antineoplastic effects of deoxycholic acid in pancreatic adenocarcinoma cell models.
   Mol. Biol. Rep. 50 (6), 5273-5282, 2023.
   DOI: http://dx.doi.org/10.1007/s11033-023-08453-x
   IF: 2.6

2. **Kovács, P.**, Csonka, T., Kovács, T., Sári, Z., Ujlaki, G., Sipos, A., Karányi, Z., Szeőcs, D., Hegedűs, C., Uray, K., Jankó, L., Kiss, M., Kiss, B. K., Laoui, D., Virág, L., Méhes, G., Bai, P., Mikó, E.: Lithocholic acid, a metabolite of the microbiome, increases oxidative stress in breast cancer.
   Cancers (Basel). 11, 1-31, 2019.
   DOI: http://dx.doi.org/10.3390/cancers11091255
   IF: 6.126

List of other publications

3. Schwarcz, S., **Kovács, P.**, Nyerges, P., Ujlaki, G., Sipos, A., Uray, K., Bai, P., Mikó, E.: The bacterial metabolite, lithocholic acid, has antineoplastic effects in pancreatic adenocarcinoma.
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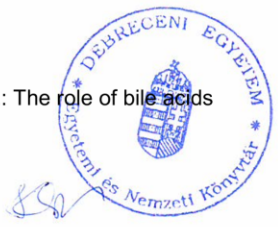

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5. Kovács, T., Mikó, E., Vida, A., Sebő, É., Tóth, J., Csonka, T., Boratkó, A., Ujlaki, G., Lente, G., **Kovács, P.**, Tóth, D., Árkosy, P., Kiss, B. K., Méhes, G., Goedert, J. J., Bai, P.: Cadaverine, a metabolite of the microbiome, reduces breast cancer aggressiveness through trace amino acid receptors.
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DOI: http://dx.doi.org/10.1016/j.bbabio.2018.04.002
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