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Cadaverine and lithocholic acid, metabolites of the microbiome reduce breast cancer aggressiveness

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Cadaverine and lithocholic acid, the metabolites of the microbiome reduce breast cancer aggressiveness

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The Examination takes place at the library of the Department of Physiology, Faculty of Medicine, University of Debrecen at 11:00 am, 13th of June, 2019.

Head of the **Defense Committee**:	László Csernoch, DSc
Reviewers:	Péter Nagy, DSc
	Tibor Pankotai, PhD
Members of the Defense Committee:…	András Mádi, PhD
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 13th of June, 2019.

INTRODUCTION

Breast cancer (BC) incidence worldwide

The incidence of BC has risen to unprecedented levels in recent decades, making it the third most common disease worldwide. Nowadays BC is the major cancer type of women and the fifth cause of death worldwide. Between 2008 and 2012 the incidence of BC enhanced by 20%, and mortality by 14%. Moreover, the incidence of BC increases with 0.5-1% in every year, its occurrence shows geographical and economic distribution.

The origin of almost 70 % of all BC cases remain unclear, still according to various studies, the principal identified risk factors are sex, age (higher risk between age 35 and 65) and inheritance. Genetic, epigenetic and environmental determinants are well-established risk factors, such as inherited loss of breast cancer type 1/2 (BRCA1/2) gene, high Insulin-like Growth Factor-1 (IGF-1) expression, the existence of *in situ* carcinomas, high fat diet, alcohol, radiation or obesity. Main risk factors are life exposure to hormones, associated with hormonal contraceptives, hormone therapy, puberty, pregnancy and menopause. In contrast early (<age 18) child-birth, breast feeding, regular physical activity, avoiding harmful passions and consuming fiber rich food reduces the risk of developing BC.

Introduction of the gut microbiota and its role in BC

A large number of diverse microbial species (~10^{14} bacteria) colonize the human gastrointestinal (GI) tract. Their collective bacterial genome harbors 150-fold more genes than the human genome. A complete study identified 9.9 million microbial genes across the fecal microbiome. The human gut microbiome serves a good model for investigating host-microbiome-diseases interactions.

The human gut microbiota is helpful in digestion, metabolism, and plays important role in human health. It plays a critical role in maturation, immune response, provide protection against pathogen overgrowth, helps to maintain intestinal barrier function, influence host-cell proliferation and vascularization, regulate intestinal endocrine functions, neurologic signaling, bone density, provide a source of energy biogenesis, biosynthesize vitamins, neurotransmitters, metabolize bile salts, react or modify drugs and eliminate exogenous toxins.

In contrast, several internal and external factors influence the gut microbiota, including age, diet, host genetic features, maternal colonization, hygiene, xenobiotic and antibiotic exposure, stress or travelling. The gut microbiota dysbiosis – imbalances in the function and composition of the intestinal microbes – related to numerous diseases. However, the number of directly tumorigenic bacteria is extremely low (some 10 bacterial species), dysbiosis is associated with cancers of the skin, colon, urinary tract, cervix, lung, lymphoma, prostate and breast. It is still unclear whether imbalances of gut microbial communities are a consequence or a cause of chronic diseases, but studies show, that discrete presence of some bacterial species or changes in the abundance of certain microorganisms can exert pathogenic effects that facilitate disease development.

The link between BC and GI microbiome dysbiosis has been investigated in control-case studies, however, the studies targeting this relationship are quite limited so far. Some papers confirm that in BC, beside of the weakness of immune system, a huge decrement occurs in the metabolic ability of the microbiome. A control-case study comparing the fecal microbiota between control and BC patients shows significantly altered microbiome composition and less diverse gut bacteria among BC patients. Otherwise, reduced microbiota diversity can be found in the case of obese patients, which is a well-known risk of BC. Differences in the absolute number of total bacteria and changes in the composition of the microbiota exist not only between control and BC patients, but between certain BC stages as well.

Epithelial to Mesenchymal Transition (EMT)

In general, EMT is a cellular developmental process, that allows epithelial cells to become mesenchymal or mesenchymal-like cells, loose their apical-basal polarity, cell-cell contacts and gain invasive features. EMT influence motility through mediating cell-cell adhesion-, and cell-ECM adhesion specific molecules, upon EMT the epithelial cytokeratin-based intermediate filament network is replaced with vimentin (VIM), actin stress fibers are formed and cells yield a more spindle-like shape. In EMT the expression of the mesenchymal N-cadherin increased, while an important epithelial feature (CDH-1) is down-regulated.

Oncogenic EMT is observed in carcinoma cells, is associated with tumor progression and metastasis, as it allows cancer cells to invade through the basement

membrane and intravasate into the circulatory system. Oncogenic EMT is observed in many BC models, and plays an important role in invasion and metastasis. Several EMT regulators (gene mutations, signaling molecules related to the tumor microenvironment, transcription factors) are able to induce metastasis to distant organs - mostly to the liver, brain, bones and lungs - and results poor disease outcomes. Distant metastases are generally characterized as having epithelial type morphology, and sometimes metastatic tumors have a greater degree of cellular differentiation as compared to the primary tumor, suggesting, that after EMT, the reverse mechanism (MET) occurred at some points.

Breast cancer stem cells (BCSCs) play a major role in cancer growth and the formation of metastases (EMT), treatment resistance and disease recurrence. The proportion of BCSCs vary between cell models of BC. Usually, the proportion of BCSCs is small (e.g. 4T1 mouse BC), while certain cell lines contain high percentage up to 25% (e.g. SKBR-3 human BC). BCSCs show an invasive gene signature which correlates with enhanced metastasis and poor overall survival. BCSC are capable of self-renewal, and either divide symmetrically, producing further stem cells, or asymmetrically, to generate progenitor cells, that can differentiate into various cell types within the tumor. Furthermore, BCSCs have increased proliferation rate and resistance to apoptosis, thereby, BCSCs may hamper the effectiveness of surgery, radiotherapy or chemotherapy. Recent investigations suggest, that BCSCs can perform various biochemical changes in response to different microenvironmental conditions. In contrast with the “aerob glycolysis” (Warburg mechanism), BCSCs can utilize OXPHOS, fatty acid oxidation and glutaminolysis, if necessary.

In summary, EMT can facilitate tumor progression through different ways: A) EMT can increase invasive and anti-apoptotic programs that drive cancer metastasis, B) EMT can generate structural end biochemical alterations of the tumor microenvironment, and C) EMT mediators can lead to the increment of CSC phenotype.

Microbial metabolites of the GI tract

Although, dozens of cancers affect organs that are in direct contact with microbes, recent data suggest that bacteria can affect organs in compartments distant to the microbiome through bacterial metabolites. Metabolites, produced by the microbiome, can

be absorbed and hence enter the circulation and exert their biological effect at distant sites in the body.

Deconjugated estrogen derivatives, lipopolysaccharide, secondary bile acids (BAs) and short chain fatty acids (SCFAs) were suggested to participate in regulating cancer cell proliferation or transformation, thought, the molecular mechanism through the bacterial metabolites expound their effect are largely unknown. Numerous bacterial metabolites have been identified as the microbes' own metabolites (e.g. SCFA) or modified products of the host (e.g. secondary BAs and amino acid derivatives). In some cases, both the host and the microbiota can produce certain metabolites (e.g. biologically active amines). We assume, that these bacterial metabolites – produced in the GI tract – through the circulatory system reach tumor cells located in distant parts of the body. These biologically active metabolites can modulate gene expression or signal transduction pathways of cancer cells, thereby influence different tumor hallmarks.

The role of cadaverine in BC

Biologically active amines are the degradation products of amino acids. Polyamines, such as cadaverine, putrescine, spermine and spermidine are belonging to this group. Between species, polyamine concentration and composition differ, for example, cadaverine is well-characterized in plats and bacteria, while show low abundance in most other species, in contrast with spermine, spermidine or putrescine. Intracellular polyamine levels are strictly regulated through biosynthesis, transport and catabolism. They are mainly synthetized from the amino acid methionine, ornithine, arginine and lysine by decarboxylase enzymes.

A less common biogenic amine, cadaverine, is formed through the direct decarboxylation of L-lysine catalyzed by lysine decarboxylase (LDC). The reference serum concentration of cadaverine is 0.1 - 0.8 μM as measured in healthy volunteers. Cadaverine plays significant role in cell survival at acidic pH, cell signaling, stress response and is also related to animal growth and development, cell proliferation and tumorigenesis. Moreover, cadaverine is an essential component of the cell wall, and plays an important role in the structural linkage between the outer membrane and peptidoglycan in bacteria.

LDC have been identified is widely distributed among prokaryotes and eukaryotes. In *E. coli* two types of LDC were discovered: the constitutive LdcC and the acid inducible CadA. They show high sequence conservation with 69% identity, however, optimal pH, stability and decarboxylation activity differ. The optimal concentration of cadaverine is maintained in *E. coli by the* cadaverine regulatory system that has two main components: the cadBA operon – that coding for lysine decarboxylase (CadA) and lysine/cadaverine antiporter (CadB) – and the regulatory inner membrane protein (CadC), what is responsible for pH sensing.

Recent studies discovered trace amine-associated receptors (TAARs) as recognition receptors of biogenic amines. First, TAAR13c was identified to specifically recognize cadaverine in zebrafish. All TAARs except TAAR1 functions as olfactory receptors in rodent, primate and fish, and they share a strong evolutionary relationship with biogenic amine G-protein coupled receptors (GPCRs). In mammals, TAAR13c is absent and other TAARs take over its role. In these cases, other TAARs were sequentially identified to detect the carrion odor cadaverine. TAAR13c has two main characteristics that allows cadaverine binding: a negatively charged $Asp_{3.32}$ – that supports amine recognition – and a second aspartate at position 5.42 or 5.43. Based on these molecular features, structural studies found similar TAARs in humans (TAAR6 and TAAR8), mice (TAAR6 and TAAR8b) and rats (TAAR6 and TAAR8a), that can be responsible for responding to cadaverine. In humans TAAR1 is mostly expressed in the brain, and - together with TAAR2 - is important in mediating amine-induced leukocyte functions. TAAR3 and TAAR4 are encoded by pseudogenes, TAAR5 are highly sensitive olfactory receptors, TAAR6 is associated with schizophrenia and bipolar disorders, TAAR7 is not expressed in humans, while TAAR8 often uses TAAR1 agonists and downstream signaling pathway. TAAR9 is a functional receptor in most of the human population, although, current understanding of TAAR-mediated functions and signaling is ill-characterized. However, TAAR family remain largely understudied as compared to other GPCRs, a recent study highlights the association between TAAR1 overexpression and positive survival rate among BC patient. These results suggest, that the family of TAARs may have important role in BC pathology.

Bile salt biotransformation and lithocholic acid (LCA)

BAs are synthesized from cholesterol in hepatocytes. Two main primary BAs – cholic acid (CA) and chenodeoxycholic acid (CDCA) – are produced in the human liver. Primary BAs are subsequently conjugated to glycine or taurine. These fully ionized BA salts are carried to the gallbladder to concentrate. Bile salts are highly effective detergents, after a meal they get secreted to the intestine, where BAs promote the emulsification, absorption and digestion of lipids and lipid-soluble nutrients. More than 95% of the BAs are reabsorbed in the distal ileum and return to the liver for reuse. This process is called “enterohepatic circulation” and occur four to twelve cycles a day. However, the bile salt absorption is highly effective (~95%), still 200-600 mg of bile salts leave the enterohepatic circulation daily, and become substrates for bacterial transformation in the colon. The transformation of bile salts occurs with the contribution of bile salt hydrolase (BSH) and hydroxysteroid dehydrogenase (HSDH) enzymes, expressed by intestinal bacteria. After taurine and glycine-conjugated BAs are deconjugated, bacterial 7α/β-dehydroxylation (7α/β-HSDH) of CA and CDCA occur, causing formation of the secondary BAs deoxycholic acid (DCA) or LCA.

The most important bacterial bile salt biotransformation is 7α-dehydroxylation in the human GI tract, although, some intestinal anaerobic bacteria are capable of both 7α- and β-dehydroxylating activities. LCA is a hydrophobic secondary BA that is primarily formed by the intestinal bacteria by 7α-dehydroxylation of CDCA or by 7β-dehydroxylation of ursodeoxycholic acid (UDCA). Most of deconjugated LCA is lost in feces, therefore, it dominates in human fecal samples.

The BA-inducible (bai) operon is a highly conserved and complex gene organization system, in which baiH codes the enzyme (3-dehydro-4-7β-oxidoreductase), that is responsible for LCA production. LCA is recognized to exert its effect through farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor (VDR) or G protein-coupled BA receptor (TGR5) signaling. Through these receptors, LCA affects a wider range of biological activities than initially highlighted. They can activate different signaling pathways (cell death or survival), involve in energy metabolism, inflammatory response and can protect the intestinal mucosa from bacterial invasion (antimicrobial property).

Secondary BAs, that produced by the intestinal flora, appear in peripheral tissues, like liver, kidney, heart, where they exert hormone-like effects. It seems like they are in connection with various kinds of carcinomas, gallstones and chronic inflammatory diseases, based on recent studies. The reference serum concentration of LCA is 30-50 nM, however, in the breast tissue higher LCA concentrations (up to 1 μM) was reported.

AIMS

The bacterial metabolites produced by the gut microbiota (among LCA and cadaverine) play significant role in regulating the metabolism and energy homeostasis of the host, while, through numerous internal and external factors (diet, hygiene, immune system etc.) the host can modulate the microbiome. These metabolites – through the circulatory system – can reach and affect tumor cells in distant locations in the human body. In this study we wanted to highlight the strong connection between microbiome, host and breast cancer.

We planned to:

- Characterize the effect of LCA and cadaverine on different type of breast cancer cell lines.
- Identify the molecular mechanisms through which these bacterial metabolites exert cytostatic activity.
- Investigate the biological effects of the above metabolites in a mouse model. Subsequently, measure the changes of tumor hallmarks in mouse model.
- Examine the level of LCA in breast cancer.
- Measure the amount of bacteria/enzymes producing these metabolites in breast cancer patients.

RESULTS

THE EFFECTS OF CADAVERINE IN BC

Cadaverine treatment reduces metastasis formation in 4T1-grafetd mice

As first step we investigated the effects of cadaverine supplementation to mice homotopically grafted with 4T1 breast cancer cells (200000 cells/mouse). Cadaverine supplementation (500 nmol/ kg p.o. q.d.) did not alter the number of primary tumors that grew from the grafted mice, but there was a trend towards tumor with lower mass**.** In line with that, the number of metastases decreased and, as with the primary tumors, there was a trend for smaller tumors in the cadaverine-treated mice.

Importantly, cadaverine treatment decreased the invasivity of the primary tumors. Histological examination of the primary tumors revealed that cadaverine treatment decreased the rate of mitosis and the heterogeneity of nuclear morphology.

Cadaverine administration does not impair BC proliferation

We investigated whether cadaverine administration could influence the proliferation of cultured BC cell lines. We used five different established BC cell lines of which four were of human (MD-MBA-231, SKBR3, ZR-75-1 and MCF7), while one was of murine origin (4T1). The cadaverine concentration that we used corresponded to the reference concentration of cadaverine in human serum (0.1-0.8 μM). Cadaverine slowed proliferation of 4T1, MDA-MB-231 and SKBR-3 cells as measured in SRB assay or in colony forming assays, although the changes were not statistically significant. Importantly, the same concentrations of cadaverine did not hinder the proliferation of non-transformed primary human skin fibroblasts. We assessed whether slower proliferation could be due to the toxicity of cadaverine to cells. The proportion of the PI positive cells did not increase upon cadaverine treatment, nor did the apoptotic fraction in 4T1 cells.

Cadaverine revert EMT and suppress invasion ability

We assessed whether cadaverine treatment can revert mesenchymal-like cancer cells to epithelial-like cells. First, we performed a measurement in the ECIS system in which 0.1 µM cadaverine increased total resistance, suggesting better adherence of cells. To verify these findings, we stained cells with Phalloidin-Texas Red to visualize the arrangement of the actin cytoskeleton. Cadaverine treatment changed the fibroblast-like morphology of the 4T1 cells to a rather cobblestone-like morphology, that is characteristic for epithelial cells. Treatment of MDA-MB-231 breast cancer cell line with cadaverine led to similar morphological changes.

To gain insight into the molecular mechanism through which MET takes place we performed an RT-qPCR screen on EMT genes. The assay revealed differential expression of 11 genes after cadaverine treatment most were suppressed. MMP2, MMP3 and MMP9 support movement; Tgfb3, FgFbp1, Erbb3 and Er1 support proliferation; while Krt14, Notch1, CDH1, IgFbp4 and Spp1 support cell adhesion. In line with these observations, cadaverine-treated cells were slower in migrating to open areas in scratch assays and also performed worse in Boyden-chamber transmigration tests. These data were further supported by the observation that MMP9 expression was suppressed by cadaverine treatment in 4T1 cells, as well as in MDA-MB-231 and SKBR-3 breast cancer cell lines.

We assessed metabolic changes evoked by cadaverine administration using the Seahorse metabolic flux analyzer. Cadaverine treatment reduced glycolytic flux, that is a characteristic of breast cancer stromal cells. Therefore, we assessed the “stem-ness” of 4T1 cells using the aldefluor assay and found a mild reduction in cancer cell “stem-ness”. We found similar reductions in cancer cell “stem-ness” in MDA-MB-231 cells upon cadaverine treatment.

Cadaverine exert its beneficial effects through TAARs

The trace amine-associated receptor family serve as receptors for cadaverine. Although, most studies on TAAR focused on olfaction, a study linked TAAR1 to breast

cancer. Indeed, higher expression of TAAR1, TAAR2, TAAR5, TAAR8 (in ER- cases) and TAAR9 provided better survival in breast cancer.

First, as TAAR receptors are G protein-dependent receptors, we assessed their involvement by treating 4T1 cells with NF449, a Gsα-subunit-selective G-protein antagonist, a treatment that abolished the anti-EMT effect of cadaverine. Next, we silenced TAAR1, TAAR8 and TAAR9 in MDA-MB-231 cells. The silencing of TAAR1, TAAR8 and TAAR9 prevented the cadaverine-elicited mesenchymal-to-epithelial transition and the silencing of TAAR8 and TAAR9 prevented the cadaverine-induced decrease in MMP9 expression.

We assessed the available databases to collect data on TAAR1, TAAR8 and TAAR9. TAAR1 and TAAR9 expression did not show any major association with carcinogenesis or breast cancer subtypes in contrast to TAAR8. TAAR8 expression decreased in pre-cancerous lesions, such as hyperplastic enlarged lobular units (HELUs) as compared to normal terminal ductal lobular units (TDLUs). In line with that, TAAR8 expression decreased in breast cancer. Decreases in TAAR8 expression was different between the histological subtypes of breast cancer. TAAR8 expression was lower in triple negative breast cancers as compared to non-triple negative breast cancers. There was a trend for lower TAAR8 expression in DCIS when compared to healthy tissue, TAAR8 expression was lower in ductal invasive breast cancer as compared to healthy ducts and there was a trend for lower TAAR8 expression in lobular invasive breast cancer as compared to healthy lobes. Taken together, apparently, in early stage of breast cancer TAAR8 expression decreases that is more pronounced in triple negative breast cancers.

Cadaverine biosynthesis is suppressed in breast cancer

To get an insight whether intestinal cadaverine biosynthesis is modified in breast cancer patients, we assessed the abundance of the DNA coding for LdcC and CadA in human fecal DNA from the experimental cohort 1. When comparing healthy individuals and BC patients we observed trends for lower abundance of *E.coli* CadA and also *E. coli*, *Enterobacter cloacae* and *Hafnia alvei* LdcC DNA in BC patients, although, these changes were not significant. Reduced CadA and LdcC abundance was more pronounced in clinical stage 0 patients as compared to the pool of all patients.

Subsequently, we assessed the protein levels of *E. coli* LdcC protein in feces by Western blotting. In the feces of stage 1 patients LdcC protein levels were markedly lower than the levels in the feces of healthy subjects, in line with the lower fecal DNA abundances.

We assessed the GEO database to study LDC expression in human breast cancer. There was no difference in LDC mRNA expression between control and breast cancer cases or in LDC expression of the normal breast epithelium and cancer epithelium in patients. Rather as an exception, LDC expression was lower in basal-like breast cancer as compared to control (normal) breast epithelium of non-diseased individuals.

Finally, we assessed how expression of LDC in humans affects the outcome of breast cancer using the kmplot.com database. Although, differences in LDC expression did affect overall survival of the patients, in grade 1 patients higher expression of LDC was associated with significantly longer survival than lower expression of LDC. Interestingly, while LDC expression did not affect survival in ER^-PR^- patients, higher LDC expression correlated with better survival in ER^+ PR^+ patients.

THE EFFECTS OF LCA IN BC

LCA attenuates the aggressiveness of breast cancer in mice

We tested the effects of LCA in mice that were grafted with 4T1 cells and were treated with LCA (15 nmol LCA p.o. q.d.) or vehicle for 18 days. At the time of the sacrifice the infiltration capacity of the primary tumor to the surrounding tissues markedly decreased upon LCA treatment. Furthermore, the number of the metastases was also lower in the LCA-treated group.

LCA Biosynthesis is suppressed in early phases of human breast cancer

We next investigated how BA and LCA metabolism relates to BC in humans. LCA is produced through the deconjugation of chenodeoxycholic acid (CDCA) conjugates, followed by a dihydroxylation on carbon 7 by the action of the enzyme 7α/β hydroxysteroid dehydrogenase (7-HSDH) that is the rate-limiting step of LCA formation. The enzymes

involved in the 7-dehydroxylation of BAs are organized into one operon called the BA-inducible (*bai*) operon wherein the *baiH* ORF codes for 7-HSDH in most bacterial species.

Total bile acid, CDCA and LCA levels were reduced in serum from breast cancer patients as compared to age and sex matched healthy individuals, and we observed a similar trend in all other bile acids we examined. Since both primary and secondary bile acid levels were lower in breast cancer patients, we assessed the ratio between CDCA (the substrate for LCA synthesis) and LCA in human serum. We found a decrease in the LCA/CDCA ratio in breast cancer patients compared to healthy individuals, and this decrease that was more marked when only stage 1 patients were assessed. At later stages LCA/CDCA ratio normalized and even increased above the ratio of healthy individuals in stage 3 patients.

To clarify how intestinal LCA biosynthesis is modified in BC, we assessed the abundance of the *baiH* (responsible for LCA production) ORF in human fecal DNA samples from the experimental *cohort 1*. In our experiments we identified the baiH ORF of anaerobic, Gram-positive and Gram-negative species and measured the abundance of the baiH ORF using qPCR assays. When all patients were compared to healthy controls, the abundance of baiH of *Clostridium sordelli, Staphylococcus haemolyticus, E. coli* and *Pseudomonas putida* was lower in BC patients, and the change was significant in the case of *Pseudomonas Putida*. These results correlate with the lower LCA levels and LCA/CDCA ratio of BC patients. A more pronounced decrease in the abundance of the baiH of *Bacteroides thetaiotaomicron, Clostridium sordelli, Staphylococcus haemolyticus, E. coli* and *Pseudomonas putida* were observed in stage 0 and stage 1 patients than in the pool of all patients.

DISCUSSION

To date no direct, casual relationship had been shown between BC and the microbiota, although several control-case studies highlight interconnections. Goedert and co-workers - comparing the fecal microbiota of BC patients with closely matched control women - found significantly altered microbiome composition and less diverse gut bacteria. In addition, milk ducts in the breast are colonized by bacteria, and changes in the microbiome of BC tissue has also been observed. These finding suggest, that LCA and cadaverine may be produced by the breast's own microbiota, not only by the microbiome of the GI tract, however, share of the sources remain unclear. The relationship between BC and the microbiota is strengthened by the positive correlation between BC incidence/recurrence and antibiotic treatment, moreover, this association was also found in men. These observations support our hypothesis of the relationship between microbial functions and BC.

The effects of cadaverine in BC

In contrast to other biogenic amines (spermine, spermidine, putrescine), cadaverine is ill-characterized. Based on the literature, serum level of diamines are higher in cancer patients than in healthy individuals and surgical removal of tumors normalize serum diamine levels. Increased putrescine levels are in positive correlation with carcinogenesis, however, polyamine involvement is not proven in cancer development. Cadaverine levels are more variable: some studies report increases, others show trends for decrement in different cancer diseases, nevertheless, BC patients still have not been evaluated.

In this study we characterized how cadaverine affect the behavior of BC cells. We characterized the cadaverine-driven effects in different (mouse and human) BC cell lines. Our data highlights that cadaverine has tumor suppressor role in BC in concentrations corresponding to the human reference serum concentrations. Cadaverine significantly inhibited EMT by modifying the expression of genes participating in proliferation, adhesion and cell movement. Cadaverine also suppressed the ability of cells for invasion, and the metastatic potential of 4T1 cells in vitro and in vivo (BALB/c mice).

We also found significant changes in the OCR of cadaverine-treated cells, thereby, cadaverine may shift the metabolic profile of BC cells toward “reverse Warburg effect” by reducing stem-like characteristic of the cells.

Our results indicating the possible involvement of TAARs (TAAR1, TAAR2, TAAR5, TAAR8 and TAAR9) in the anticancer effect of cadaverine, in line with the study about TAAR1 and better BC survival. Elevated expression of these receptors correlates with better BC survival. To validate these findings, we tested TAAR1, TAAR8 and TAAR9 involvement in cadaverine-evoked effects. All three measured receptors seem to participate in regulating MET, however, only TAAR8 and TAAR9 influenced MMP9 protein expression. Available metadata highlights that TAAR8 is reduced in precancerous stages and downregulated together with worsened disease. Due to the limited availability of reagents, we cannot exclude that – besides the ones we examined - other TAARs could be equally involved in regulating cadaverine effects.

Our experiments show that bacterial cadaverine biosynthesis is suppressed in early stage BC. This observation suggests that the simplification of gut microbiome in early stage BC leads to a decreased production of antiproliferative bacterial metabolites like cadaverine. Most drastic changes of the microbiota were associated with stage 1 carcinoma, in later stages cadaverine production restored and reached levels similar to levels of the controls. The in silico data we gathered for TAAR8 suggest that in parallel to the simplification or dysbiosis of the microbiome in tumors, the expression of cadaverine-sensing apparatus is probably also downregulated.

Bacteria on breast duct surfaces and human cells can also synthesize cadaverine. However, the share of these sources (breast tissue vs. tumor vs. gut vs. host) in cadaverine production is largely unknown, high human expression of LDC prolongs survival in early stage BC patients. These data further validate the potential anti-cancer properties of cadaverine.

The effects of LCA in BC

LCA, a metabolite of the microbiome, is synthesized in the gut and transferred to the breast through the circulatory system, where it might play an important role in bringing

about an anti-tumor microenvironment. LCA is known to induce cell death in neuroblastoma, prostate cancer and MCF7 BC cells. As we noted earlier, 1 μM LCA concentration, used in our in vivo study, stands close to the LCA concentrations reported in the breast. LCA may be produced by the breast's own microbiota and not only by the gut microbiome, nevertheless, the share of the two sources in not known. Our in vivo experiments demonstrated that LCA – similarly to cadaverine - significantly reduces the infiltration rate of primary tumors, as well as the metastatic potential of the tumor.

In my dissertation we also tried to partly answer the question: which bacteria species are important in LCA-mediated modulation of BC. Goedert and co-workers' found that compared with control patients, BC patients have significantly altered microbial composition (both α and β diversity change), while Ridlon and co-workers' research shows, that anaerobic microbes are important in the production of secondary BAs, among them LCA. Our study highlights decrement in both the aerobic and the anaerobic microbial populations with early stage BC. Our study confirmed, that both primary and secondary BA levels are suppressed in BC patients. We also measured the ratio between CDCA and LCA. LCA/CDCA ratio diminished in BC patients compared to control, and the decrement was the marked in stage 1, that normalized at later stages of BC. These data are in good correlation with Tang an co-workers' findings, that demonstrate reduced glycolithocholate sulphate levels in BC patients. These results suggest the involvement of both aerobic and anaerobic flora in secondary BA production, and propound widespread suppression of the microbiome in BC.

SUMMARY

There is a strong connection between the microbiome and the host. The bacteria – living in different cavities of the human body – produces numerous bacterial metabolites (among cadaverine and LCA), that can alter the metabolism and energy homeostasis of the host. Through internal and external factors (diet, personal hygiene, genetics, age) the host can also influence the composition and function of the microbiome. Alterations in the composition or function of the microbiome is associated with BC, among other diseases.

Cadaverine - a biologically active diamine - is produced by the enzyme LDC by the gut microbiota from the amino acid L-lysine. Through the bloodstream cadaverine reaches BC cells at distant locations of the body. Although, cadaverine slightly reduced the proliferation and colony formation of 4T1 cells, it could convert mesenchymal-like cells to epithelial-like cells. To gain epithelial-like features, cancer cells undergo MET by suppressing the expression of genes play important role in cell proliferation, movement and invasion. Cadaverine treatment also decreased the “stem cell-ness” of 4T1 cells. Moreover, cadaverine increased the cell-cell and cell-surface connections, thereby, obstructing migration. These data were further supported by the observation, that cadaverine supplementation to mice grafted with 4T1 BC cells resulted in reduced infiltration of primary tumors to surrounding tissues, reduced tumor mass as well as the number of metastases. Histological examination of primary tumors revealed that cadaverine supplementation decreased the rate of mitosis and the nuclear pleomorphism. Our results show that members of the TAAR family (TAAR1, TAAR8 and TAAR9) could be responsible for the cadaverine-evoked effects. Silencing of these receptors prevented the cadaverine-elicited EMT and the cadaverine-induced decrement in MMP9 expression. Metadata analysis shows that TAAR8 is reduced in early stage of BC and were further downregulated together as the disease progressed. We also assessed human LDC expression using kmpolt.com database and found, that in grade 1 patients higher LDC expression correlated with significantly longer survival. To verify these findings, we compared the abundance and expression of LDC in samples from healthy individuals and BC patients. Both the abundance of LDC DNA and the protein levels were suppressed in early stages of BC compared to the controls.

We also assessed how the level of LCA – another bacterial metabolite, a secondary BA – differ in the serum of control vs. BC patients, and which bacterial species play important role in LCA production. In contrast to other studies it seems that both aerobic and anaerobic flora participate in LCA production. The abundance of baiH ORF (responsible for LCA production) decreased significantly in early stage BC samples compared to matched control samples. BA composition of human serum samples highlight, that total BA levels, as well as LCA levels and LCA/CDCA ratio were reduced in serum from BC patients as compared to healthy individuals, the decrement was the more pronounced in stage 1 BC.

This study confirms that cadaverine and LCA can suppress hallmarks of BC cells, thereby, puts cadaverine and LCA on the list of bacterial metabolites that control carcinogenesis. These observations support the hypothesis that bacterial metabolites influence carcinogenesis in organs far from the gut, however, this field requires more research to understand the connection between microbes and distantly located tumors.

KEYWORDS

cadaverine, LCA, microbiome, breast cancer, stem cell, EMT, MET, invasion, metastasis, trace amine-associated receptor, lysine decarboxylase, baiH

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Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Kovács, T.**, Mikó, E., Vida, A., Sebő, É., Tóth, J., Csonka, T., Boratkó, A., Ujlaki, G., Lente, G., Kovács, P., Tóth, D., Árkossy, 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.
   Sci Rep. 9 (1), 1-14, 2019.
   DOI: http://dx.doi.org/10.1038/s41598-018-37664-7
   IF: 4.122 (2017)
2. Mikó, E., Vida, A., **Kovács, T.**, Ujlaki, G., Trencsényi, G., Márton, J., Sári, Z., Kovács, P., Boratkó, A., Hujber, Z., Csonka, T., Antal-Szalmás, P., Watanabe, M., Gombos, I., Csóka, B., Kiss, B. K., Vígh, L., Szabó, J., Méhes, G., Sebestyén, A., Goedert, J. J., Bai, P.: Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness.
   Biochim. Biophys. Acta Bioenerg. 1859 (9), 958-974, 2018.
   DOI: http://dx.doi.org/10.1016/j.bbabio.2018.04.002
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List of other publications

3. Vida, A., Kardos, G., **Kovács, T.**, Bodrogi, B., Bai, P.: Deletion of poly(ADP?ribose) polymerase-1 changes the composition of the microbiome in the gut.
   Mol. Med. Rep. 18 (5), 4335-4341, 2018.
   DOI: http://dx.doi.org/10.3892/mmr.2018.9474
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4. Trencsényi, G., Dénes, N., Nagy, G., Kis, A., Vida, A., Farkas, F., Péli-Szabó, J., **Kovács, T.**, Berényi, E., Garai, I., Bai, P., Hunyadi, J., Kertész, I.: Comparative preclinical evaluation of 68Ga-NODAGA and 68Ga-HBED-CC conjugated procainamide in melanoma imaging.
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   DOI: http://dx.doi.org/org/10.1016/j.jpba.2017.02.049
   IF: 2.831

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5. Kertész, I., Vida, A., Nagy, G., Emri, M., Farkas, A., Kis, A., Angyal, J., Dénes, N., Péli-Szabó, J., **Kovács, T.**, Bai, P., Trencsényi, G.: In Vivo Imaging of Experimental Melanoma Tumors Using The Novel Radiotracer 68Ga-NODAGA-Procainamide (PCA).
   J. Cancer. 8 (5), 774-785, 2017.
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   IF: 3.249
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   Methods Mol. Biol. 1608, 185-200, 2017.

Total IF of journals (all publications): 16,404
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

19 February, 2019

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

CONFERENCE PRESENTATIONS AND POSTERS

Conference presentations:

Mikó E, Vida A, **Kovács T**, Ujlaki G, Sebő É, Toth J, Trencsényi G, Márton J, Sári Z, Kovács P, Boratkó A, Hujber Z, Csonka T, Antal-Szalmás P, Watanabe M, Gombos I, Vígh L, Szabó J, Méhes G, Sebestyén S, Goedert JJ, Bai P (2018) *Interactions between the microbiome and human breast cancer.* FEBS3+ Siófok, 2018.

Mikó E, Vida A, **Kovács T**, Újlaki G, Sári Z, Bai P (2018) *How to throw a stone into a pond? Connections between breast cancer and the microbiome (Hogyan dobjunk követ a tóba? Az emlőrák és a mikrobiom kapcsolata).* Biannual Conference of the Pro Scientia Gold Medal Laureates, Budapest, Hungary

G Trencsenyi; G Nagy; A Vida; J Angyal; N Denes; JP Szabo; **T Kovacs**; I Garai; P Bai; I Kertesz *Investigation of experimental melanoma tumors using the novel melanin specific radiotracer Ga-68-NODAGA-Procainamide (PCA)* (2017) Annual Congress of the European-Association-of-Nuclear-Medicine. Barcelona, Spanyolország: 2016.10.15 - 2016.10.19, Megjelenés: Spanyolország

Mikó E, Vida A, **Kovács T**, Ujlaki G, Trencsényi G, Márton J, Sári Z, Boratkó A, Hujber Z, Csonka T, Watanabe M, Kiss M, Antal-Szalmás P, Kovács P, Lovas B, Goedert JJ, Szabó J, Méhes G, Sebestyén A, Bai P (2017) *Microbiome – tumor relations, a new aspect of tumorigenesis*. HunLifeSci Eger, Hungary

Mikó E, Vida A, **Kovács T**, Újlaki Gy, Trencsényi Gy, Márton J, Sári Zs, Kovács P, Boratkó A, Hujber Z, Csonka T, Antal-Szalmás P, Mitsuhiro W, Szabó J, Méhes G, Sebestyén A, Goedert JJ, Bai P (2017) *The role of lithocholic acid in the pathology of breast cancer (A litokólsav szerepe az emlőtumor patológiájában)*. Conference of the Hungarian Physiological Society, Debrecen, Hungary

Mikó E, Vida A, **Kovács T**, Újlaki Gy, Kovács P, Lovas B, Sári Zs, Jankó L, Márton J, Nagy L, Bai P (2016) Diverse ideas around the mitochondria. Biotechnology days at the University of Debrecen

Mikó E, Vida A, Fodor T, **Kovács T**, Újlaki Gy, Kovács P, Lovas B, Hujber Z, Trencsényi Gy, Sebestyén A, Bai P (2016) *In search for natural small molecule modulators of breast cancer*. Annual meeting of the Hungarian Biochemical Society, Szeged, Hungary

Mikó E, Vida A, Fodor T, **Kovács T**, Újlaki Gy, Kovács P, Lovas B, Bai P (2016) *When mitochondria do the inverse.* Mebrane transport conference Sümeg, Hungary

Fodor T, Mikó E, Vida A, **Kovács T**, Abdul-Rahman O, Nagy L, Trencsényi Gy, Bai P (2015) *New ways to revert Warburg-type metabolism.* FEBS3+ meeting, Protoroz, Slovenia

Conference posters:

Kovács T, Mikó E, Vida A, Sebő É, Toth J, Csonka T, Boratkó A, Ujlaki Gy, Lente G, Kovács P, Tóth D, Árkosy P, Kiss B, Méhes G, Goedert JJ, Bai P (2018) Cadaverine, a microbial diamine, reduces breast cancer aggressiveness. EMBO Conference on the Microbiome, Heidelberg, Germany

Kovács T, Mikó E, Vida A, Sebő É, Toth J, Csonka T, Boratkó A, Ujlaki Gy, Lente G, Kovács P, Tóth D, Árkosy P, Kiss B, Méhes G, Goedert JJ, Bai P (2018) *Cadaverine reduces breast cancer aggressiveness*. Cell Symposia: Metabolites as Signalling Molecules, Seattle, USA

Mikó E, Vida A, **Kovács T**, Ujlaki Gy, Trencsényi Gy, Márton J, Sári Zs, Kovács P, Boratkó A, Hujber Z, Csonka T, Antal-Szalmás P, Watanabe M, Gombos I, Csoka B, Kiss B, Vígh L, Szabó J, Méhes G, Sebestyén A, Goedert JJ, Bai P (2018) *Lithocholic acid, a bacterial metabolite modulates the behavior of breast cancer*. EMBO Conference on the Microbiome, Heidelberg, Germany

Mikó E, Vida A, **Kovács T**, Ujlaki Gy, Trencsényi Gy, Márton J, Sári Zs, Kovács P, Boratkó A, Hujber Z, Csonka T, Antal-Szalmás P, Watanabe M, Gombos I, Csoka B, Kiss B, Vígh L, Szabó J, Méhes G, Sebestyén A, Goedert JJ, Bai P (2018) *Bioenergetic changes in breast cancer cells by lithocholic acid.* European Bioenergetics Conference, Budapest, Hungary

Kovács Tünde, Mikó Edit, Vida ANdrás, Boratkó Anita, James J.Goedert, Bay Péter (2017) *Biogén aminok hatása emlőtumor sejtvonalakra* MÉT-MFT Conference Debrecen

Mikó E, **Kovács T**, Vida A, Csonka T, Sebő É, Tóth J, Sebestyén A, Méhes G, Goedert JJ, Bai P (2018) *Cytostatic bacterial metabolites are reduced in early stage breast cancer - Bacterial metabolites in breast cancer initiation*. Cell Symposia: Metabolites as Signaling Molecules, Seattle, USA

Vida A, **Kovacs T**, Lente G, Kovacs P, Bai P (2017) *A Horizontal and Longitudinal Study of the Gastrointestinal Tract Microbiome in PARP1 Knockout Mice.* PARP2017, Budapest, Hungary

Mikó E, Vida A, **Kovács T**, Ujlaki Gy, Trencsényi Gy, Márton J, Sári Zs, Boratkó A, Hujber Z, Csonka T, Watanabe M, Kiss M, Antal-Szalmás P, Kovács P, Lovas B, Goedert JJ,

Szabó J, Méhes G, Sebestyén A, Bai P (2017) *Microbiome – tumor relations, identifying a novel set of tumor metabolites?* Oncometabolism, Figuera de la Foz, Portugal

Újlaki Gy, Mikó E, Vida A, **Kovács T**, Sári Zs, Kovács P, Bai P (2017) *The effects of secondary bile acids on the metabolism of A2780 ovarian carcinoma cells (A másodlagos epesavak hatása az A2780 ovárium karcinóma sejtek metabolizmusára).* Conference of the Hungarian Physiological Society, Debrecen, Hungary

Kovács T, Mikó E, Vida A, Boratkó A, Goedert JJ, Bai P (2017) *Effects of biogenic amines on breast cancer cells (Biogén aminok hatása emlőtumor sejtekre).* Conference of the Hungarian Physiological Society, Debrecen, Hungary

Kovács P, Mikó E, Vida A, **Kovács T**, Sári Zs, Újlaki Gy, Bai P (2017) *The effects of LCA on oxidative stress in breast cancer cells (Az LCA hatása az oxidatív stressz folyamataira emlőtumor sejtekben).* Conference of the Hungarian Physiological Society, Debrecen, Hungary

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