SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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Introduction

Human microbiome

The ecological system formed by the totality of microorganisms living on the surface and the in cavities of the human body (including the skin, mouth, nose, vagina, intestines, etc.) is the microbiome, termed by the Nobel laureate American molecular geneticist Joshua Lederberg. The human microbiome is build up of billions of microorganisms. The diversity and function of the bacterial community is a prerequisite for healthy metabolism, consequently, the disruption of the homeostatic balance can initiate pathogenic processes. The composition of microbiome is specific for locations or organs and is characterized by individual variability that leads to differences between individuals. In the recent decades, the role of the microbiome in maintaining health and in the progression of diseases, such as malignant tumors, has come into focus. Alternatively, due to its high variability, the microbiome may be a potential biomarker.

Dysbiosis – oncobiosis

The human gastrointestinal tract is colonized by numerous bacterial species and consists of approximately ~10^{14} bacteria. The MetaHit microbiome study described approximately 3.3 million microbial genes, and meta-analysis studies identified more than 9.7 million bacterial genes in the feces of healthy adults. The gut microbiota is characterized by a high degree of diversity, which is an important factor in maintaining the homeostatic balance. The intestinal flora regulates digestion, contributes to the metabolism of bile acids and sterols, modulates energy homeostasis, produces vitamins and enzymes, and affects the production of leptin by adipocytes, as well as, glucose in the liver. The microbiome is able to synthesize neurotransmitters and neurotransmitter-like substances and, hence, affect the function of the nervous system (termed the gut-brain axis). The composition of the microbiome influences the proliferation and differentiation of epithelial cells, as well as, provides immune functions and help to maintain the integrity of the intestinal barrier. The microbiota plays a crucial role in the maturation of immune cells, the prevention of allergic reactions and the protection against pathogens.

The gastrointestinal microbiome is modulated by both external and internal factors, the most important are age, stress, genetic factors, hygiene, and lifestyle, including diet, physical activity, and smoking. In addition, probiotic and antibiotic exposure and the invasion of pathogens can also affect microbial composition. Abnormal changes to the composition and stability of the gastrointestinal microbiome can lead to dysbiosis that can induce pathological processes and promote neurological diseases, metabolic, cardiovascular or gastrointestinal disorders by deregulating cellular metabolism and inducing chronic inflammation that causes DNA damage. Decrease in bacterial diversity is associated with neoplastic diseases. The transformed microbiome in neoplastic diseases is termed the oncobiome. Microbial dysbiosis has been described primarily in oncological diseases associated with organs in direct contact with the bacterial community. However, bacterial metabolites can also reach to distant organs where they can exert their effects.

A large number of neoplastic diseases, including the breast cancer, have been linked to the changes in the composition of the microbiome. These changes are mainly caused by a decrease in bacterial diversity in breast cancer patients, which correlates with the molecular subtype. The microbial composition of triple-negative breast cancer cases differs from other types, which also correlates with the grade and aggressiveness of the disease. These observations suggest that oncobiosis plays a major role in the pathomechanism of breast cancer.

Malignant neoplasms of the breast

In developed countries, the most prevalent malignant tumor is breast cancer, which is considered to be the second most common cause of death among females. Approximately one million patients are diagnosed each year, and the age-adjusted estimated annual mortality of 23.1 deaths per 100,000 persons in Europe. There is a significant geographical and economic distribution of the incidence of breast cancer, with a higher risk of occurrence observed in developed countries. However, due to the promotion of screening programs and consequently early diagnosis, the five-year survival rate of breast cancer may exceed 80% in the developed world.

Many risk factors for malignant breast tumors are known, nevertheless, the cause of a large proportion of the cases remains unclear. The primary risk factors of the disease

are gender, age, and genetic background. The incidence of breast cancer increases significantly with age, and the disease mainly affects postmenopausal women. Prolonged hormone exposure, such as hormone replacement therapy, and early menarche or late menopause, is also associated with the higher prevalence of breast cancer. Genetic and epigenetic factors, including mutations of BRCA1 and BRCA2 genes, higher epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER2) expression, and inheritance or other neoplastic lesions in the anamnesis may contribute to the development of breast cancer. Furthermore, obesity, regular smoking, and alcohol consumption have been identified as risk factors of breast cancer. In contrast, pregnancy, breast feeding, regular physical activity, and a healthy lifestyle can reduce the predisposition.

Oxidative and nitrosative stress

Reactive oxygen and nitrogen species are also produced under physiological conditions by various biochemical reactions that perform important physiological functions, including the suppression of pathogenic flora and signal transduction. Therefore, the goal of cells is not a complete elimination but to maintain the optimal oxidant - reductant balance. Increased free radical and reactive metabolite production, as well as, decreased antioxidant capacity, can disrupt the redox homeostasis, which can induce oxidative stress. During this process, important biomolecules (proteins, lipids, nucleic acids) and cells can be damaged, in serious cases apoptosis or necrosis can occur. Oxidative stress has been associated with the development of numerous pathologies, including neurological disorders, atherosclerosis, hypertension, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, asthma, and neoplastic diseases. Chronic inflammation and the consequent oxidative stress can cause DNA damage and genomic instability, mutations can accumulate and lead to carcinogenesis. Therefore, in some malignancies, including breast cancer, the cytostatic effects of increased oxidative stress has been demonstrated.

Transcription factor erythroid nuclear factor 2 (NRF2) is vital in maintaining redox balance and plays a crucial role in regulating the expression of antioxidant genes. NRF2 protects cells against from the effects induced by exogenous and endogenous

oxidative stress, thus preventing intracellular lipid peroxidation, damage to proteins, nucleic acids, and carbohydrates. More than a thousand NRF2 target genes have been identified, most of them are important components of the antioxidant defense system, such as SOD, CAT, or GPX. Evolutionarily conserved cytoprotective NRF2 is generally pro-neoplastic, its activation promotes cell survival by stimulating antiapoptotic signals, induces metabolic changes, and creates a favorable environment for tumor cell proliferation and metastasis by supporting the migration and invasion of breast cancer cells.

Epithelial-mesenchymal transition and breast cancer

Morphological and molecular changes of cells during the tumor progression play a crucial role in the development of resistance to apoptosis and invasive phenotype. A key step in the process is that epithelial cells lose their apical-basal polarity, as well as, their intercellular and basement membrane connection and become migratory mesenchymal cells with a characteristic gene expression pattern. The phenotypic conversion of epithelial cells is due to their molecular reprogramming, a process called epithelial-mesenchymal transition (EMT). During EMT, the cytoskeletal system of cells rearranges, the cytokeratin intermediate filament network, characteristic of epithelial cells, is replaced by vimentin (Vim), and actin stress fibers that promote cell motility. The cells acquire an elongated, fibroblast-like morphology, and the close cell connections (zonula occludens, tight junction) between the cells are disrupted and eliminated. Furthermore, the expression of E-cadherin, involved in cell adhesion, decreases, whereas the expression of the mesenchymal marker, neural cadherin (N-cadherin), increases. EMT plays a critical role in physiological processes (embryogenesis, wound healing), however, the phenomenon of oncogenic EMT characteristic of neoplastic processes has also been identified in breast tumors, which through the bloodstream allows the migration of primary tumor cells and may lead to distant metastases. During the formation of metastases, the reverse process of EMT, the mesenchymal-epithelial transition (MET), often takes place in mesenchymal cells, which resets the epithelial character of neoplastic cells.

Several transcription factors and molecular events regulate EMT in breast tumors. Various DNA mutations, such as loss of E-cadherin, which provides epithelial

integrity, and release of matrix metalloproteinases (MMPs) stimulate cell invasion and metastasis by promoting tumorigenesis. Many transcription factors are involved in EMT as Snail (Snai1), Slug (Snai2), Twist1, ZEB1, and ZEB2, which induce EMT through repression of E-cadherin. These transcription factors are important to maintain the mesenchymal character as well as, the presence of the stem cell-like phenotype. These transcription factors promote metastasis formation and therapy resistance, which is associated with an unfavorable prognosis.

Metabolism of breast cancer

The metabolism of breast cancer cells shows characteristic pathological changes. Decreased mitochondrial oxidation, as well as, elevated flux through glycolysis and the pentose phosphate shunt characterize breast cancer cells, which is referred to as the Warburg effect. Metabolic changes that support tumor progression include the activation of mTOR (mammalian target of rapamycin) and the inhibition of forkhead box O1 protein (FOXO1), adenosine monophosphate activated protein kinase (AMPK), and peroxisome proliferator-activated receptor gamma coactivator-1α and β (PGC1α and PGC1β) leading to complex reprogramming. Transcription factor FOXO1 mainly induces cell cycle arrest or decreases the proliferative capacity of the cells, through the inhibition of mTORC1. In addition, AMPK downregulation has been observed in breast cancer patients inhibiting mitochondrial biogenesis by regulating PGC1α and PGC1β. Therapeutic methods that re-establish Warburg's metabolic changes identified in breast cancer, have antiproliferative effects.

Breast cancer stem cells and oxidative stress

There is a special subtype of stem cells - termed to cancer stem cells - that are capable of self-renewal and further differentiation. The metabolism of cancer stem cells is characterized by increased mitochondrial oxidation and remarkable metabolic flexibility, which promotes tumor initiation and progression, as well as, contributes to the development of chemoresistance. There are two forms of breast cancer stem cells, the mesenchymal-like stem cells express the CD44, while epithelial phenotypes show aldehyde dehydrogenase-1 (ALDH1) positivity. Breast cancer stem cells and early

progenitors are characterized by lower ROS levels than cells without tumorigenic potential, which has been shown to be critical in maintaining the stem cell function. Increased oxidative stress reduces the clonogenicity of stem cells and results radiosensitization. Neoplastic cells are more sensitive to ROS accumulation than non-transformed cells, which can be used during therapy. Consequently, induction of oxidative stress appears to be a promising approach for the selective elimination of cancer cells, including cancer stem cells, with minimal damage of normal cell function.

Metabolism of indolepropionic acid and indoxylsulfate

Tryptophan is an essential amino acid. Approximately 4-6% of tryptophan undergoes bacterial transformation and serves as a precursor for many bioactive indole-containing metabolites. Gut bacteria catalyze the synthesis of indole from tryptophan through tryptophanase (TnaA) enzyme, which hydrolyzes tryptophan to form indole, pyruvate, and ammonia. The most widely studied is the tryptophanase operon (tnaCAB) from *Escherichia coli*, which is composed of two major structural genes. The TnaA gene is responsible for the enzyme activity, while TnaB encodes a low-affinity, high-capacity tryptophan-specific transporter. Other bacterial species have been identified that do not inducibly, but constitutively produce the enzyme.

The enzyme TnaA has been isolated from several Gram-positive and Gram-negative bacterial species, which is modulating the formation of indolepropionic acid (IPA) and indoxylsulfate (IS) bacterial metabolites, among others. The serum concentration of IPA is 0.291–1.095 μM in healthy adults. IPA regulates the expression of pro- and anti-inflammatory genes in the epithelial cells of the intestine, significantly enhances the production of anti-inflammatory interleukin IL-10, and reduces the release of inflammatory cytokines, tumor necrosis factor-α (TNF-α) and IFN-γ. The expression of IPA is observed not only in the gastrointestinal tract but also in the central nervous system, where it plays an important role in neuronal proliferation, differentiation, and has a neuroprotective effect.

IS is a bioactive indole derivative of tryptophan. Tryptophan is converted to indole via the indole-3-pyruvate intermediate and then enters to the liver through the circulation. In the liver, the indole-3-pyruvate is hydroxylated by Cyp2E1 and conjugated

with sulfate by the SULT1 and SULT2 sulfotransferases. The formation of IS is connected to the commensal bacterial flora. The reference serum concentration of IS in healthy volunteers is around 2 μM. At pathological concentrations IS may have cytotoxic effects. IS has also been associated with chronic renal failure as well as, vascular disease, and the inhibition of endothelial proliferation and migration has been observed. However, the exact pathomechanism is still unclear. It has been reported that IS can induce ROS production, which has a potential effect on breast cancer progression.

Indole derivatives, including IPA and IS are Aryl-Hydrocarbon Receptor (AHR) ligands, and also bind to Pregnane X-Receptor (PXR). AHR is a ligand-activated transcription factor that plays a critical role in xenobiotic metabolism and vascularization. AHR activation regulates the function of immune system and the mucosal immunity. The functional significance of the receptor in carcinogenesis is still unclear, however, in some studies, AHR agonists have been shown to be effective in reducing cell proliferation and migration potential.

An important member of the nuclear receptor superfamily is PXR. Its activation can be triggered by a number of endogenous and exogenous ligands, including steroids, antibiotics, and bile acids. Thus, it has an important role in the regulation of detoxification, inflammatory immune response, cell proliferation, glucose, cholesterol, and lipid metabolism, and the maintenance of endocrine homeostasis. PXR is a transcription factor with tumor-suppressor function, however, the exact molecular background of its antitumor role is not known. In breast cancer cells, the activated PXR induced apoptosis and cell cycle arrest through the stimulation of inducible nitric oxide synthase (iNOS).

Objectives

Several bacterial species live with the host in close connection in the human body. External and internal factors (diet, hygiene, genetic factors, immune system antibiotics and xenobiotics, etc.) can affect the composition of the microbiome. Dysbiosis of the gut microbiome can be associated with a number of diseases, including the breast cancer. Our hypothesis is that the gastrointestinal bacterial metabolites of tryptophan, such as IPA and IS may alter the signaling pathways of cancer cells, thereby, affecting the function of neoplastic cells. Moreover, we hypothesized that the biosynthesis of indole metabolites is altered in breast cancer patients.

The main objectives of our research were the following:

- Investigation of the biological and cytostatic effects of IPA and IS to *in vivo* mouse model grafted with breast cancer cells.
- Determination of the processes through which indole derivatives exert their antitumor activity on breast carcinoma cell lines.
- Identification of the associations between the survival of breast cancer patients and the expression of receptors that mediate antitumor effects and the expression of enzymes that regulate IS biosynthesis by *in silico* analyzes.
- To characterize the intratumoral expression of receptors responsible for the effects of indole derivatives in tissue blocks of breast cancer patients.
- Determination of the amount of bacteria and bacterial enzymes involved in indole biosynthesis in human breast cancer samples.

Results

Characterization of indolepropionic acid with antitumor activity in breast cancers

Indolepropionic acid supplementation reduces the progression of breast cancer *in vivo*

As the first step, we studied the effects of IPA treatment on the growth of tumors in a BALB/c mouse grafted with 4T1 breast cancer cells (10^5 cells/mouse). Half of the mice (n=10) received sterilized tap water "*per os*" as a control, while another 10 animals received IPA treatment (1 nmol/kg, p.o.). IPA had no effect on the formation of primary tumors, however, it significantly reduced the invasiveness of primary tumors and infiltration to the surrounding tissues, and reduced the number and total weight of metastases. Histological analysis of primary tumors showed an increased number of lymphocytes, and the mitosis score of the cancer cells was decreased in IPA-treated mice.

Indolepropionic acid treatment inhibits the proliferation of breast tumor cell lines

In our *in vitro* experiments, we examined the effects of IPA on the proliferation of mouse breast (4T1) and human breast cancer (SKBR-3) cell lines. IPA in concentration corresponding to the reference concentrations (0.1-0.8 μM) markedly reduced the colony-forming capacity of 4T1 cells, at the same time, neither the proportion of propidium iodide-positive necrotic cells nor the proportion of AnnexinV-FITC-propidium iodide double-positive apoptotic cells did not change. These results were also confirmed on SKBR-3 cells. Moreover, the IPA treatment did not affect the proliferation of non-transformed human fibroblast cells, similarly to other cytostatic metabolites.

Indolepropionic acid induces oxidative stress by reducing NRF2 expression

We assessed whether IPA can modulate processes characteristic of neoplastic cells. Although the role of oxidative stress in breast cancer is contested, several publications have pointed out that an increase in oxidative stress is responsible for inducing cytostasis or cell death. IPA treatment induced lipid peroxidation, as indicated by the

formation of thiobarbituric acid reactive substances (TBARS) and increased the production of 4-hydroxynonenal (4HNE), from which we conclude an enhanced oxidative stress. In parallel, the expression of NRF2, a main regulator of antioxidant enzymes, decreased, and we observed an increase of the expression of iNOS, which promotes nitrosative stress. IPA increased the expression of cellular energy stress markers (pACC, ACC, FOXO1, and PGC-1β) in cancer cells, while decreasing the proportion of breast cancer stem cells.

Indolepropionic acid reverses epithelial-mesenchymal transition (EMT)

Next, we observed morphological changes induced by IPA treatment in 4T1 breast tumor cells, as well as, we examined the expression levels of EMT marker genes. IPA stimulated the appearance of epithelial morphological features, suggesting induced MET. In good agreement with that, we measured increased resistance, indicative of stronger cell-cell connections compared to the control group. Our results were verified by a markedly decreased expression of mesenchymal markers (Vim, Fgfbpl, Snail, and β-catenin), while the expression of the epithelial E-cadherin was increased.

The antineoplastic effects of indolepropionic acid are mediated by the production of reactive species

The EMT and the characteristics of cancer stem cells are modulated by reactive oxygen and nitrogen species. To get an insight on whether IPA-induced cytostatic effects on breast cancer cells may be driven by oxidative stress, we examined the antitumor effects of IPA in the presence of thiol reductants glutathione (GSH) and N-acetyl-cysteine (NAC), as well as, the mitochondria-targeted antioxidant MitoTEMPO. As expected, thiol reductants reduced TBARS formation and protected against the IPA-mediated decrease in the proportion of ALDH1-positive cancer stem cells, suggesting an inhibition of IPA-induced effects. Interestingly, MitoTEMPO blocked the IPA-mediated cytostasis. Based on these results, we can conclude that IPA-induced production of reactive species determines the widespread antitumor effects of IPA.

Indolepropionic acid exerts its effects through AHR and PXR

Among the IPA receptors, we examined the effects of the metabolite via PXR and AHR receptors in detail in our study. Pharmacological inhibitors of AHR (CH223191) and PXR (ketoconazole) were used to confirm that these receptors were involved in modulating IPA-induced effects. The inhibition of AHR and PXR abolished several IPA-induced changes, namely MET, activation of AMPK through ACC phosphorylation, and the reduction of NRF2 expression.

Characterization of cytostatic effects of indoxylsulfate in breast tumors

Indoxylsulfate treatment reduces breast cancer aggression in a mouse model

We investigated the *in vivo* cytostatic effects of IS in a female BALB/c mice grafted with 4T1 breast cancer cells. Half of the mice (10 animals) received "*per os*" IS (2 nmol/kg bodyweight), while the control experimental group (10 animals) received sterilized tap water. The IS dose corresponded to the reference serum concentration in humans (4 μM). IS treatment did not affect the number and size of primary tumors, but significantly reduced the infiltration rate to the surrounding tissues. Moreover, IS supplementation markedly decreased both the number and total weight of metastases.

Indoxylsulfate treatment reduces the proliferation of breast cancer cells

IS, similar to other cytostatic metabolites, inhibited the proliferation of several breast cancer cell lines (4T1, MCF7, SKBR-3, MDA-MB-231, ZR75-1) in SRB assays. The IS concentration that we used corresponded to the human normal serum concentration of IS (0.25-4 μM). Antiproliferative effects of IS were also confirmed by measuring colony formation of 4T1 cells. However, the proportions of apoptotic and necrotic cells did not change. Moreover, IS treatment did not affect the non-transformed human fibroblast cells.

Indoxylsulfate-mediated oxidative and nitrosative stress

First, we characterized the main oxidative/nitrosative stress markers in the regulation of tumor progression to obtain a more complex picture of the antineoplastic function of IS. In 4T1 tumor cells both the amount of TBARS and the expression of 4HNE showed a significant increase, which are important markers of lipid oxidative damage.

Then we found induced expression of nitrotyrosine (NTyr), which characterizes protein damage and nitrosative stress. Based on these results, we hypothesized that, IS treatment also stimulates the production of reactive species similar to IPA. These changes correlated with increased iNOS and decreased NRF2 expression. Due to the downregulation of NRF2, the expression of antioxidant enzymes (GPX1, GPX3, SOD3, and CAT) also decreased.

Effect of indoxylsulfate on the epithelial-mesenchymal transition (EMT)

We investigated the EMT, which promotes the migration and invasion of tumor cells. IS treatment reverted EMT in a dose-dependent manner, coinciding with enhanced resistance in ECIS measurements, suggesting better cell-to-cell and cell-to-surface binding. In order to understand the molecular mechanism of IS-induced MET, we determined the EMT markers. The expression of mesenchymal markers was markedly reduced, while genes specific for epithelial cells showed increased expression after IS treatment. The migration of breast cancer cells was significantly reduced in the Boyden-chamber experiments.

Indoxylsulfate induced metabolic changes

To explore the metabolic changes of IS, we assessed the glycolysis and the mitochondrial oxidation of the 4T1 cells with Seahorse XF96 oximeter and then we characterized the expression of genes regulating mitochondrial biogenesis. IS treatment reduced extracellular acidification rate (ECAR). In hypometabolic cancer cells, the central regulators of the energy sensors were induced (AMPK, ACC, FOXO1) in response to IS, and the proportion of ALDH1-positive breast cancer stem cells was decreased, a feature that was connected to changes in cellular metabolism.

Indoxylsulfate exerts its effects via AHR and PXR receptors

The effects of indole derivatives are regulated by the AHR and the PXR signaling pathways. To verify it, we used pharmacological inhibitors where both CH223191 and ketoconazole blocked the IS-induced morphological changes (MET). AHR and PXR antagonists inhibited IS-mediated lipid peroxidation and the expression of E-cadherin was

blocked by CH223191 inhibitor, whereas ketoconazole had no effect on it. In contrast, phosphorylation of ACC and AMPK was prevented by both inhibitors.

Human studies

Expression of AHR and PXR was positively correlated with the survival of breast cancer patients

The effects of both IPA and IS are mediated by AHR and PXR receptors. Thus, we used a Kaplan-Meier plotter database (kmplot.com) to assess whether the expression of these receptors is associated with the survival of human breast cancer patients. We found that higher expression of AHR and PXR correlated with significantly longer survival. This protective effect disappeared in cases of triple-negative breast cancer (TNBC).

Expression of SULT and Cyp2E1 correlates with survival in breast cancer patients

Next, we studied how the expression of human IS biosynthesis enzymes affects the survival of breast cancer patients using the kmplot.com database. Higher expression of the Cyp2E1 as well as, the SULT1A1 and SULT1A2 enzyme isoforms provided better survival in breast cancer patient. While there was no effect on survival in TNBCs.

AHR and PXR expression in tissue samples from breast cancer patients

These results of our *in silico* studies were supplemented by the characterization of intratumoral expression of AHR and PXR in tissue microarray (TMA) consisting of 88 breast cancer patients archived tissue blocks. Expression of the nuclear AHR - represents the AHR activity - decreased with the progression of disease. In line with that, lower AHR activity was observed in not otherwise specified tumors (NST) compared to lobular tumors. As the Nottingham grade of patients, expression of PXR increased. We found lower PXR expression in highly proliferating and less differentiated tumors.

Bacterial indole biosynthesis is repressed in early stages of breast tumors

To assess the relationship between bacterial biosynthesis of indole metabolites and breast cancers, we investigated the capacity of gut microbiome to synthesize indole derivatives. Bacterial species encoding the bacterial TnaA operon were identified using

available databases. We designed primers for the TnaA-coding regions of the bacteria, then the abundance of DNA encoding the enzyme in human fecal samples was determined by qPCR. When comparing samples from breast cancer patients with healthy volunteers, the abundance of TnaA was markedly decreased in *Alistipes shahii* and a similar trend was observed in *Bacteroides xylanisolvens*. Then we correlated the abundance of TnaA with the stage of the disease, and we observed the most pronounced decrease in clinical stage 0 (*in situ* carcinoma) patients.

Fecal protein expression of *E. coli* TnaA correlates with the number of tumor infiltrating lymphocytes

To complete our knowledge, we examined protein-level changes to bacterial indole biosynthetic enzymes. We determined the expression of *E. coli* TnaA in the feces of breast cancer patients and found that the proportion of TIL increased with higher TnaA expression. In good agreement with the results, we showed an association between *E. coli* TnaA expression and TIL in breast cancer patients by analyzing linear regression.

Discussion

Based on the literature, dysbiosis of the gastrointestinal microbiome induces pathogenic processes, contributing to the formation of neoplastic lesions. However, changes in the composition of the microbiome are not only observed in malignant disease, but can often be exacerbated by tumor progression. Thus, the maintenance of eubiotic balance plays a central role in the prevention of breast cancer. Although the causal link between the microbiome and breast tumors, as well as, the exact pathomechanisms are less known, in many cases an association has been shown between them. Goedert and colleagues reported a decrease in the diversity of the bacterial community in a study of the microbiome of breast cancer patients. Oncobiosis in malignant breast tumors has also been described in the breast's own microbiome. The majority of the studies show suppressed diversity of the microbiome in breast cancers, resulting in limited biosynthetic capacity, which is probably explained by a reduced production and availability of bacterial metabolites. Several microbial metabolites, such as short-chain fatty acids, lithocholic acid, or cadaverine have been shown to have cytostatic effects on breast tumors. The functional relationship between microbiome and breast cancer is supported by the observation that regular antibiotic exposure can decrease bacterial diversity and increase the incidence of breast cancer, while probiotic exposure has a preventive effect. These findings confirm our hypothesis that there is an association between the decreased number of bacterial species and the incidence of breast cancer.

Cytostatic effect of indolepropionic acid and indoxylsulfate in breast cancer

Bioactive indole-containing metabolites can affect the physiological function of the human body mainly through the activation of AHR and PXR. They play a key role in maintaining the gut mucosal homeostasis, promoting resistance to pathogenic invasion, and influencing gastrointestinal immunity by regulating the expression of pro- and anti-inflammatory cytokines. Decreased levels of bacterial tryptophan metabolites may be associated with inflammatory bowel disease, accordingly and a protective effect of increased IPA biosynthesis was found in mice with ulcerative colitis. Changes in

tryptophan metabolism have been associated with various neoplasms. In the serum of patients with hematologic diseases and with colorectal-, ovarian-, skin-, and lung cancers markedly decreased tryptophan concentrations were detected. In contrast, other studies highlighted the tumor-promoting effects of tryptophan metabolites, as increased expression of tryptophan-degrading enzymes (IDO and TDO) was observed in 58% of human tumors, which may contribute to tumor cell migration and metastasis.

In this study, we characterized how IPA can modulate the behavior of different breast cancer cells. We determined the IPA-mediated effects in cellular and murine models, our results showed that IPA had antineoplastic properties when administered in the human reference range. IPA supplementation in mice reduced the invasiveness and infiltration capacity to the surrounding tissues of breast tumor cells, thereby, decreasing the number of metastases. IPA treatment inhibited the proliferation and migration of breast cancer cells, while it enhanced oxidative stress, induced an anti-tumor immune response and MET, and altered the metabolic activity of tumor cells via the AHR and PXR signaling pathways. Similar to other antitumor metabolites (e.g., lithocholic acid and cadaverine), IPA treatment did not exert a cytostatic effect on the non-transformed fibroblast cells.

The diversity of the bacterial community is critical to the normal antitumor immunity, and some bacterial metabolites, including tryptophan derivatives, may contribute to the regulation of immune response. The effects of tryptophan and its microbial catabolites are mediated by AHR and PXR receptors. Decreased indole production due to insufficient intake of tryptophan has an immunosuppressive effect in an AHR-dependent manner, which correlates with the elevated antitumor immune response that we observed. Accordingly, the number of TILs increases with the proportion of TnaA expression which is responsible for IPA biosynthesis.

Our observations suggest that IPA treatment induced oxidative-nitrosative stress in breast cancer cells by reducing NRF2 expression and activity, resulting a decrease in antioxidant capacity. In addition, increased iNOS expression and release of mitochondrial reactive derivatives were observed, which correlates with the effects of lithocholic acid. Although the function of increased oxidative-nitrosative stress is still unclear in breast tumors, recent studies have reported the importance of prooxidant-induced cytostatic effects. Our data suggest that increased production of reactive oxygen and nitrogen

derivatives is essential for IPA-mediated cytostasis. Furthermore, oxidative stress is a key regulator of tumor cell characteristics, and thus increased production of prooxidants may contribute to the loss of stem cell-like properties of breast cancer cells. In our experiments, IPA treatment reduced the proportion of breast cancer stem cells, while its effect was prevented in the presence of thiol reductants.

The regulatory function of oncobiotic transformation in EMT, as well as, in the formation of metastases has been already explained in several publications. In our study, IPA treatment induced MET in the same way as several microbial metabolites, which slowed the migration and invasion of cancer cell, and reduced the number of metastases in murine model grafted with breast tumor cells. In addition, the expression of mitochondrial biogenesis-stimulating transcription factors (AMPK, FOXO1, and PGC1β) was induced in an AHR/PXR-dependent manner.

In the second part of our study, we characterized the effects of IS on breast tumor cells. The antineoplastic effects of IS are regulated by AHR and PXR receptors similarly to IPA. However, in this experiments, AHR proved to be more dominant, while the cytostatic effect of IPA can be mediated by both AHR and PXR receptors. We observed that lower AHR and PXR expression correlated with the stage and the grade of the disease, as well as, the higher mitotic activity, which may be due to the loss of indole-mediated signaling.

In cellular and animal models, IS treatment reduced proliferative activity, and decreased the invasion of breast cancer cells and the formation of metastases, while did not affect the proliferation of non-transformed cells, which is a common feature of metabolites with cytostatic properties in breast tumors. As with other bacterial metabolites, the molecular mechanism of the tumor suppressive effect of IS is primarily based on the reverting EMT. Our results show that the inhibition of EMT slows cell migration, thereby, reducing metastases. IS decreased the expression of mesenchymal markers (Vim, Fgfbpl, Tgfb3, MMP9, Snail and β-catenin), while an increase in the expression of E-cadherin and ZO-1 involved in adherent cell connections was observed, suggesting a functional role of EMT in the progression of breast cancer.

In addition, IS treatment increased iNOS expression, as well as, decreased activation of NRF2 - similar to the abovementioned IPA - induced oxidative-nitrosative

stress, resulting in distraction of redox homeostasis. The changes induced by increased release of prooxidants play a major role in the breast cancer cells cytostasis and reduced the proportion of breast cancer stem cells, contributing to the IS-induced antitumor effect. In addition, significant metabolic changes were detected during IS treatment, which may also promote the suppression of breast cancer stem cell and result in tumor cell cytostasis.

From a broader perspective, tryptophan and indole metabolism are closely related to the survival of breast cancer, therefore, our finding contributes to complete our picture of the pathomechanism of breast cancer.

The relevance of our observations to human breast cancer

Tryptophan and indole metabolism are strongly correlated with the progression of breast cancer as well as, the survival. Auslander et al. associated a link between high extracellular tryptophan levels with poor survival in breast cancer patients. IS levels have been shown to be downregulated in both estrogen receptor positive (ER+) and negative (ER-) breast cancers, while the proliferation marker, Ki67 positivity is negatively correlated with the IS levels in malignant breast tumors. These meta-analysis data suggest that indole derivatives, including IS, promote the survival of breast cancer patients and that their levels decrease with the progression of the disease.

Supporting the literature, our results show that the IPA biosynthetic capacity of the microbiome is markedly reduced in patients with early stage of breast cancer. The most pronounced change was observed in women with *in situ* carcinoma, confirming associations with other bacterial metabolites, such as lithocholic acid and cadaverine. Our results are in good correlation with the intratumoral expression pattern and activity of AHR and PXR, respectively. Decreased expression of these receptors were observed in highly invasive and proliferating, as well as, undifferentiated breast tumors. Based on our observations and the available literature, we hypothesize that due to dysbiosis of the gastrointestinal microbiome, tryptophan metabolism is suppressed in breast cancer cases, and it seems to be the most profound in the early stage and *in situ* breast carcinoma. As a result of decreased IPA biosynthesis, breast cancer cells are released from cytostatic inhibition, leading to a more aggressive phenotype. In summary, oncobiosis seems to play a crucial role in the progression of breast cancer.

These scientific results highlight the functional role of oncobiome in breast cancer, supported by human population-based studies, animal experiments, observations of bacterial metabolite supplementation, nutritional and pharmacological studies. These studies open a new perspective in the therapy of malignant breast tumors through identifying bacterial metabolites and oncobiome components with cytostatic effects.

Summary

Many bacterial species live on the surface and in the cavities of the human body in dynamic interaction with the host. The microbiome can influence the physiological processes of the host, as well as, the progression of pathological diseases through the biosynthesis of various bacterial metabolites (among IPA and IS). Both external and internal factors (age, diet, hygiene, genetic factors, immune system, use of antibiotics and xenobiotics etc.) can determine the microbial composition and function. Dysbiosis of the gut microbiota is associated with a number of diseases, including breast tumors.

From dietary tryptophan, in a bacterial tryptophanase enzyme-dependent manner IPA and IS can be formed. These metabolites can reach tumor cells in distant organs of the human body through the bloodstream. The effects of IPA and IS were characterized by both cellular and mouse models. Based on our results, these indole-containing bioactive metabolites have antineoplastic properties. In concentrations corresponding to the human reference serum concentration, both IPA and IS supplementation reduced the level of infiltration to the surrounding tissues, thereby, decreased the number and total mass of metastases. Histological analysis of primary tumors showed an elevated ratio of lymphocytes, suggesting an induction of antitumor immune response. In addition, IPA as well as IS treatments reduced the proliferative activity and migration of breast cancer cells, at the same time, they had no effect on non-transformed human fibroblast cells. We also observed increased oxidative and nitrosative stress due to the downregulation of NRF2 and increased expression of iNOS. The imbalance of redox homeostasis plays an important role in inducing cytostasis, as well as, contributes to reduce the proportion of cancer stem cells. Moreover, both IPA and IS treatments enhanced mesenchymal-epithelial transition (MET) and metabolic changes, resulting in tumor-suppressing effects.

The effects of indole metabolites are mediated by Aryl-Hydrocarbon Receptor (AHR) and Pregnane X-Receptor (PXR) signaling pathways. Based on the kmplot.com database analysis, our data show that higher expression of both AHR and PXR correlated with significantly longer survival of breast cancer patients. In line with these observations, the receptors were characterized by decreased expression in highly proliferating and

undifferentiated breast cancers, thus associated with poor prognosis. Furthermore, increased expression of liver enzymes (Cyp2E1, SULT1, and SULT2) involved in the synthesis of IS correlates with better overall survival. To verify the connection between bacterial biosynthesis of indole metabolites and breast cancer, we assessed the abundance of bacterial tryptophanase DNA in human fecal samples. Our results showed, that the capacity of the microbiome to biosynthesize indole derivatives was significantly decreased, that was more pronounced in early stage, *in situ* breast carcinoma.

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List of publications related to the dissertation

1. **Sári, Z.**, Mikó, E., Kovács, T., Jankó, L., Csonka, T., Lente, G., Sebő, É., Tóth, J., Tóth, D., Árkosy, P., Boratkó, A., Ujlaki, G., Török, M., Kovács, I., Szabó, J., Kiss, B., Méhes, G., Goedert, J. J., Bai, P.: Indolepropionic Acid, a Metabolite of the Microbiome, Has Cytostatic Properties in Breast Cancer by Activating AHR and PXR Receptors and Inducing Oxidative Stress.
Cancers (Basel). 12 (9), 1-27, 2020.
DOI: http://dx.doi.org/10.3390/cancers12092411
IF: 6.126 (2019)

2. **Sári, Z.**, Mikó, E., Kovács, T., Boratkó, A., Ujlaki, G., Jankó, L., Kiss, B. K., Uray, K., Bai, P.: Indoxylsulfate, a Metabolite of the Microbiome, Has Cytostatic Effects in Breast Cancer via Activation of AHR and PXR Receptors and Induction of Oxidative Stress.
Cancers (Basel). 12 (10), 1-23, 2020.
DOI: http://dx.doi.org/10.3390/cancers12102915
IF: 6.126 (2019)

List of other publications

3. **Sári, Z.**, Kovács, T., Csonka, T., Török, M., Sebő, É., Tóth, J., Tóth, D., Mikó, E., Kiss, B. K., Szeőcs, D., Uray, K., Karányi, Z., Kovács, I., Méhes, G., Árkosy, P., Bai, P.: Fecal expression of Escherichia coli lysine decarboxylase (LdcC) is downregulated in E-cadherin negative lobular breast carcinoma.
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DOI: http://dx.doi.org/10.1016/j.bbabio.2018.04.002
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